Right Ventricular Outflow Limitation and Capacity for Exertion Associated with Age and Iron Status

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Dedicated to my family

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Abstract

This thesis is concerned with the role of iron in modulating right ventricular (RV) afterload during exercise in healthy people aged between 50 and 80 years. This is predicated on the requirement of the hypoxia-inducible factor (HIF) pathway for ferrous iron. A secondary objective is to examine the reactive oxygen species (ROS) hypothesis in human hypoxic pulmonary vasoconstriction (HPV) using exposure to hyperoxia.

Chapters 3 and 4 describe basal relationships that may affect the HIF pathway and exercise capacity during ageing. These were explored in 113 participants using blood tests and exercise tests. Age and inflammatory factors, C-reactive protein, and ferritin were associated with impaired exercise capacity. In addition, ageing did not significantly affect haematological variables or iron status indicators.

Chapters 5 and 6 describe the effect of a single intravenous iron infusion on the haematological variables in 32 participants in a randomised, placebo-controlled and double-blinded study. The effects of iron infusion on RV afterload during light exercise, and exercise capacity during heavy exercise, were examined in these participants. With iron infusion, erythropoietin production, and the increase in RV afterload during light exercise were blunted, potentially indicating involvement of the HIF pathway. However, blunting of RV afterload neither influenced the cardiac output during light exercise nor exercise capacity.

Chapter 7 describes a study of 11 healthy volunteers, which investigated the ROS hypothesis in HPV using acute isocapnic hypoxia following an 8-hour exposure to hyperoxia. This sustained hyperoxic exposure did not influence the hypoxic behaviour of the pulmonary vasculature.

This thesis demonstrates the complex relationship between iron status and exercise capacity in older adults. It shows that the decrease in RV afterload during exercise caused by intravenous iron supplementation does not lead to an augmented cardiac output or exercise capacity. Finally, it calls into question the role of ROS in HPV.

Abbreviations

CODD	C-terminal oxygen-dependent degradation domain
COPD	chronic obstructive pulmonary disease
CRP	C-reactive protein
DEF	dynamic end-tidal forcing
DFO	desferrioxamine
DMT1	divalent metal ion transporter 1
ELISA	enzyme-linked immunosorbent assays
EPO	erythropoietin
ETC	electron transport chain
FIH	factor inhibiting HIF
Hct	haematocrit
Hgb	haemoglobin
HIF	hypoxia-inducible factor
HPV	hypoxic pulmonary vasoconstriction
HRE	hypoxia response elements
IL-6	interleukin-6
IRE	iron responsive elements
IRP	iron responsive protein
MCV	mean cell volume
MPAP	mean pulmonary arterial pressure
NODD	N-terminal oxygen-dependent degradation domain
PAP	pulmonary arterial pressure
PASMC	pulmonary arterial smooth muscle cell
PHD	prolyl hydroxylase domain
P_{O_2}	partial pressure of oxygen
PVR	pulmonary vascular resistance
RES	reticuloendothelial system
ROS	reactive oxygen species
RV	right ventricular
$S_{a}O_2$	arterial oxygen haemoglobin saturation
SPAP	systolic pulmonary arterial pressure
sTfR	soluble transferrin receptor
Tf	transferrin
Tfsat	transferrin saturation
TPR	total pulmonary resistance
vHL	von-Hippel Lindau
ZIP14	ZRT/IRT-like protein 14
$\dot{V}_{O_2}$	oxygen consumption

Subscript

A	aleovlar
a	arterial
Δ	changes in or gradient
E	expired
ET	end-tidal
I	inspired
Max	maximum
Peak	peak
s	stimulus
$\bar{v}$	mixed venous

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Chapter 1

General Introduction

1.1 Background

Over the last three decades, right ventricular (RV) afterload, which is commonly referred to as pulmonary arterial pressure (PAP), has been considered to be a limiting factor in exercise at high altitudes (Groves et al., 1987; Naeije and Chesler, 2012; Naeije et al., 2010). Besides impairing gas exchange, lack of oxygen, known as hypoxia, causes pulmonary vasoconstriction, thus increasing PAP. This results in a reduction in cardiac output, oxygen delivery to exercising muscles and exercise performance.

Recently, there has been a growing interest in the increased RV afterload during exercise in people aged above 50 years. In comparison to people aged below 50 years, older people have a significantly higher mean pulmonary arterial pressure (MPAP) and lower cardiac output during exercise (Kovacs et al., 2009, 2012). However, little is known about the mechanism causing increased RV afterload in older people. Moreover, research has not fully considered the consequence of increased RV afterload on

exercise capacity in older people (e.g. does it actually affect exercise capacity in older people?). Nor has it addressed whether there is a possibility of attenuating RV afterload and thus improving exercise capacity in the group.

The aim of this chapter is to address these questions and raise several hypotheses to be examined in later chapters of this thesis. Section 1.2 summarises the current findings and characteristics of increased RV afterload during exercise in older people. In Section 1.3, a theoretical mechanism, hypoxic pulmonary vasoconstriction (HPV), for the increased RV afterload in older people is proposed, which is intended to be the central hypothesis of this thesis. Details of HPV and its regulators will then be described in Section 1.4. A key regulator for HPV, the hypoxia-inducible factor (HIF) family, and possible manipulation of HIF through iron are presented in the same section. Section 1.5 describes how iron status is changed by intravenous iron agents and how iron status is assessed in humans. Finally, Section 1.6 provides a roadmap of this thesis and illustrates key hypotheses.

1.2 Rise in RV afterload during exercise in older people

During exercise, the contractility of the heart increases, inducing a progressive increase in PAP. Compared with younger people, the increase in MPAP during exercise in older people is more substantial (Kovacs et al., 2009; Naeije and Chesler, 2012; Reeves et al., 2005). Findings of this greater increase in MPAP were summarised in a systematic review by Kovacs and colleagues (Kovacs et al., 2009). They found that the resting MPAP in the supine position did not differ greatly between different age groups, < 30, 30-50 and ≥ 50 years. However, during mild exercise, MPAP in participants aged $\geq$

50 years became almost twice the resting value, compared with an increase of 50% in participants aged < 50 years.

A concern of using MPAP as an indicator of RV afterload is that it does not show the impact of the flow, cardiac output, on MPAP. Another way to represent RV afterload is to use the total pulmonary resistance (TPR), which is defined as MPAP divided by cardiac output. Kovacs *et al.* extended an earlier study and used TPR in their results (Kovacs et al., 2012). They found that during both rest and moderate exercise in the supine position, participants aged above 50 years had significantly higher TPR than participants aged below 50 years. For example, in the oldest group ($\geq$ 70 years), TPR at rest and during moderate exercise were 223 ± 45 dyn·s/cm^5 and 212 ± 42 dyn·s/cm^5 respectively. The corresponding TPRs in the youngest group (< 24 years) were 165 ± 50 dyn·s/cm^5 and 103 ± 23 dyn·s/cm^5. More interestingly, changes in TPR from mild to moderate exercise were markedly different. The TPR in participants aged $\geq$ 50 years increased slightly during mild exercise from rest. Then it decreased to around the resting value during moderate exercise. This differed from for the participants aged < 50 years, whose TPR decreased continuously with increased exercise intensity.

The results of Kovacs *et al.* were in agreement with previous findings in other studies (Naeije and Chesler, 2012; Reeves et al., 2005), and suggested that older people have significantly higher RV afterload during exercise. Of further significance, their results indicate that the increased MPAP during exercise in older people is associated with a decrease in cardiac output. This may cause subsequent decrease in exercise performance and restriction in exercise capacity.

1.3 Possible mechanisms for the increased RV afterload in older people

The mechanism behind the increased RV afterload in older people is still unclear. Reeves *et al*. proposed that the main cause of this phenomenon is a decrease in the distensibility of pulmonary arteries with age (Reeves et al., 2005). One possibility explaining this is an increasing vascular calcification with ageing (Demer and Tintut, 2008). Another possibility is a fall with age in medial collagen content in pulmonary arteries (Mackay et al., 1978). However, it is also likely that the decreased distensibility and high RV afterload in older people is the result of HPV, since exercise consumes oxygen in the blood, thus inducing a hypoxia-related constriction in the pulmonary circulation.

Several pieces of evidence suggest that older people are vulnerable to low levels of oxygen in the blood during exercise and rest. For example, Proctor *et al*. demonstrated that, at the same workloads (70 W, 140 W and 210 W), older endurance athletes (63 ± 2 years) had significantly lower venous P_{O_2} from femoral venous samples in cycling exercise tests than younger endurance athletes (27 ± 1 years) (Proctor et al., 1998). In addition, Sorbini and colleagues demonstrated how P_{O_2} in systemic arterial blood decreases with age (Sorbini et al., 1968). On average there was a decrease of 0.42 mmHg per year in P_{O_2} in arterial blood in adults at rest. Together, these may contribute to the lower P_{O_2} in the mixed venous blood ($P_{\bar{v}O_2}$) during exercise.

It is possible that the low oxygen partial pressure in the mixed venous blood during exercise in older people causes HPV and increases their RV afterload. Marshall and

colleagues varied different levels of oxygen partial pressure in the alveoli and mixed venous blood in anesthetised dogs to study the stimulators for HPV (Marshall et al., 1994). They summarised the quantification of the P_{O_2} stimulus (P_{sO_2}) for HPV as a function of both alveolar and mixed venous P_{O_2} ($P_{sO_2} = {P_{AO_2}}^{0.62} \times {P_{\bar{v}O_2}}^{0.38}$). Although these experiments have not been performed on humans due to ethical difficulties, it is possible that a similar behaviour exists in human lungs and that a low venous P_{O_2} in older people during exercise leads to HPV, thereby increasing RV afterload.

1.4 HPV and iron status

In humans, there are two distinct phases of the pulmonary vascular response to hypoxia. Talbot *et al.* identified a rapid and a gradual phase of pulmonary vascular response to hypoxia, and suggested that there are at least two distinct underlying processes (Talbot et al., 2005). A few years later, several hypotheses have been proposed for the processes, but none of them are conclusive yet.

In this thesis, two hypotheses for the pulmonary vascular response to hypoxia were adapted and examined: 1) the mitochondria-derived reactive oxygen species (ROS) hypothesis (Waypa et al., 2001) and 2) the HIF family hypothesis (Smith et al., 2008b).

1.4.1 Mitochondria-derived ROS hypothesis

The mitochondria-derived ROS hypothesis proposes that lack of oxygen interrupts the electron transfer on the electron transport chain (ETC), which results in the production of intermediate substances such as superoxide radicals (Waypa et al., 2001). These subsequently generate hydrogen peroxide, which functions as a signalling messenger

that leads to an increase in the intracellular Ca^{+2} and muscle contraction in pulmonary arterial smooth muscle cells (PASMC).

There is a growing interest in the central messenger, ROS, of the mitochondrial hypothesis in the pulmonary circulation, since high concentrations of oxygen are commonly used in emergency conditions, home oxygen treatment and cabin pressurisation systems. Potentially, this excessive oxygen produces ROS and causes physiological responses or even damage. In this thesis, we have adapted the hypothesis to investigate the role of ROS in the pulmonary circulation with exposure of humans to hyperoxia.

1.4.2 HIF family

HIFs are a family of transcription factors, which regulate gene expression in energy metabolism and other functional genes that change vascular tone (Smith et al., 2008b). In addition, genetic variations in HIF (Petousi et al., 2014) and mutations in HIF regulatory proteins, such as the von-Hippel Lindau (vHL) protein (Formenti et al., 2011; Smith et al., 2006), influence pulmonary vascular response to hypoxia and exercise capacity significantly in humans. These suggest an important role of the HIF family in HPV in humans.

HIF is a heterodimer consisting of α and β subunits, which bind to hypoxia response elements (HRE) of target genes, activating their transcription (Pugh et al., 1991; Semenza et al., 1991; Wang et al., 1995). The regulation of HIF relies on the degradation of HIF-α subunits. In an oxygen-dependent mechanism, the degradation depends on the enzymatic hydroxylation of HIF-α domains by prolyl hydroxylase domain (PHD) enzymes and Factor inhibiting HIF (FIH) enzymes. PHD enzymes require oxy-

gen, 2-oxoglutarate as a co-substrate, as well as iron (Fe^{+2}) and ascorbate as co-factors to hydroxylate the N-terminal oxygen-dependent degradation domain (NODD) and the C-terminal oxygen-dependent degradation domain (CODD) on HIF-α (Jaakkola et al., 2001; Masson et al., 2001). The hydroxylation of these residues enhances the binding of the VHL protein to HIF-α, which leads to ubiquitylation and finally proteasomal destruction. Similarly, FIH requires oxygen, 2-oxoglutarate and iron (Fe^{+2}) to hydroxylate the asparagine residue on the trans-activation domain (TAD) of HIF (Lando et al., 2002a,b). This prevents HIF from binding to transcriptional co-activators (p300) and thus blocks the activation of transcription.

An interesting consideration is that both PHD and FIH enzymes in the regulation of HIF degradation need iron (Fe^{+2}) as a co-factor to function. In addition, HIFs regulate several genes and protein synthesis that are involved in the regulation of iron metabolism. These prompt the possibility of manipulating the HIF pathway through altering iron status. For example, Smith and co-workers investigated the impact of iron status on the pulmonary vascular response to 8 hours of sustained hypoxia in humans (Smith et al., 2008a). They found that participants receiving intravenous iron showed a significant decrease in pulmonary vascular response to acute hypoxia exposure after 8 hours of sustained hypoxia, compared with the placebo group. In addition, participants receiving 8 hours of deferoxamine (DFO; an iron chelator) infusion showed a significant increase in PVR to acute hypoxia, compared with the placebo group. The underlying mechanism, that iron status is associated with HIF regulation, is confirmed by other studies. For example, Ren *et al*. found that infusion of DFO increased levels of serum erythropoietin (EPO), a downstream HIF-regulated gene, in

humans (Ren et al., 2000b). Cotroneo *et al.* found that expression of HIF-α in the lungs was increased in rats with iron deficient diets (Cotroneo et al., 2015). These results support the possibility of manipulating the HIF system by altering iron status. The findings reviewed above have prompted us in this thesis to investigate the impact of iron status on the pulmonary circulation during exercise in older people.

1.5 Manipulation of iron status and iron indicators

In healthy humans, iron is maintained at a stable concentration in the plasma due to its regulatory mechanism (Ganz and Nemeth, 2012). There is no regulated mechanism for the excretion of iron in the body. Daily iron loss from the body is a mere 1-2 mg by sloughing intestinal mucosal cells (Nemeth and Ganz, 2006). As a result, iron absorption from diet and guts plays the main regulatory role in iron homeostasis in healthy conditions (Ganz and Nemeth, 2012). Another characteristic of iron homeostasis is that the daily movement of iron occurs mainly through the erythroid compartment (Winter et al., 2014). As the majority of iron in the body is found in red blood cells as haemoglobin iron, the major portion of iron supply in the body is from destroyed red blood cells, the iron from which is then reused for erythropoiesis in the bone marrow. This forms an iron circuit during the turnover of erythroid cells. The body's efficient iron recycling and the regulated absorption contribute to the difficulty of altering iron status through oral routes in iron-replete humans. Low absorption rate and adverse effects, such as gastrointestinal intolerance, often occur in iron-replete humans (Macdougall, 1999). Therefore, parenteral iron administration is used to change the iron status in our participants in this thesis.

Simple iron compounds cannot be administered parenterally because of the hazard to health. Iron exists in either ferrous (Fe^{+2}) or ferric (Fe^{+3}) states, but ferrous iron is reactive and readily oxidized to become ferric iron in the body (Geisser and Burckhardt, 2011). Ferric iron needs to be bound by ligands in the plasma to increase its solubility since ferric compounds, for example ferric oxide-hydroxide, precipitate in the plasma (Brissot et al., 2012; Geisser and Burckhardt, 2011). Therefore, parenteral iron needs to be formulated before administration. Early preparation, such as high-molecular-weight (HMW) iron dextran, has been used to achieve a higher dose of injectable iron by surrounding ferric iron with a carbohydrate shell (Danielson, 2004), which controls the release of free iron from the complex and avoids health hazards. However, the carbohydrate shell of HMW iron dextran has also induced incidences of serious anaphylactic reactions (Jahn et al., 2011), leading to its limited application in clinical use. The safety of parenteral iron formulas has been dramatically improved recently. The latest preparations, for example ferric carboxymaltose (Ferinject) used in this thesis, have permitted significantly fewer incidences of adverse reactions without test doses, and high administrative doses as much as 1,000 mg of iron over 15 minutes (Lyseng-Williamson and Keating, 2009). These new intravenous iron agents provide a safe and convenient way to research the effects of iron on animals and humans in clinical studies.

Cellular uptake of iron from intravenous iron agents is through several routes. A small proportion of labile iron is released directly from iron agents (Jahn et al., 2011). Labile iron in the circulation can directly interact with the iron transporter protein, transferrin (Tf), to deliver iron into the cells. Cells take up Tf by the binding of Tf to the

transferrin receptor 1 (TfR1), which is a receptor-mediated endocytosis (Winter et al., 2014). Some labile iron from intravenous iron agents that does not bind to Tf (non-Tf bound iron, NTBI) can interact with other proteins in the plasma, such as albumin, or form soluble ferric compounds, such as ferric citrate (Brissot et al., 2012). Uptake of NTBI can go through transmembrane metal ion transporters, such as divalent metal ion transporter 1 (DMT1) and ZRT/IRT-like protein 14 (ZIP14), to enter cells (Brissot et al., 2012). However, it is also possible that NTBI molecules pass iron back to Tf and through the aforementioned route since Tf has a much higher affinity to iron (Aisen et al., 1978).

The majority of iron agents is taken up by macrophages of the reticuloendothelial system (RES) in the liver and spleen. Through the acidification of endocytosis, these iron-contained particles release iron into the cytoplasm of macrophages (Geisser and Banke-Bochita, 2010). Whether this iron is stored in the macrophage or exported into the circulation for demands via Tf primarily depends on the level of circulating hepcidin. Hepcidin is a liver-derived peptide that regulates a transmembrane iron exporter, ferropotin (Ganz and Nemeth, 2012). Once the level of circulating hepcidin is high, it prevents iron being exported out of the macrophages. At the same time, this increases the cellular iron in the macrophage and alters the post-transcriptional regulation in the iron responsive elements (IRE)/iron responsive protein (IRP) system (Muckenthaler et al., 2008). IREs are sequences of mRNA containing codes for the TfR and for ferritin. IRP is a bifunctional protein, of which confirmation changes by binding iron. When the cellular iron condition is abundant, it results in IRP binding to the 5' untranslated region on IREs, which promotes the production of iron storage protein, ferritin,

to store excessive iron. In a scenario where there is a lack of iron, it results in IRP binding to the 3' prime untranslated region to promote the production of TfR and DMT1 transporter allowing more iron uptake.

Changes induced by intravenous iron agents can be determined by several indicators permitting assessment of iron status in humans. For example, soluble forms of ferritin and TfR in the plasma can be used to interpret the size of iron stores and iron demands respectively in humans (WHO, 2007). The amount of Tf occupied by iron in the plasma (Tf saturation, Tfsat) can be used to estimate the iron concentration in the blood since the majority of iron in the blood is bound by Tf (Wish, 2006). The concentration of hepcidin, as a primary iron regulator, also correlates to iron status (Zaritsky et al., 2009). However, no single, or combination of iron indicators, has been agreed upon to be the best means for assessing the iron status of a normal person (WHO, 2007). In addition, the interpretation of iron status indicators requires caution as both hepcidin and ferritin levels can be influenced by inflammation (Feelders et al., 1998; Gabay and Kushner, 1999; Ganz, 2003), due to the complex interaction between iron homeostasis and other signalling pathways. Therefore, an acute phase inflammatory protein, C-reactive protein (CRP), and the aforementioned iron indicators will be included to assess the iron status of participants in the studies.

1.6 Roadmap of this thesis

We hypothesised that the increased RV afterload during exercise in older people is one of the factors limiting their exercise capacity. The increased RV afterload is caused by a hypoxia-related mechanism, namely activation of HIF family, which is similar to the

slow component of HPV. As there is a tight connection between HIF regulation and iron status, we hypothesised that altering iron status can attenuate RV afterload during exercise in older people and improve their exercise capacity.

Several studies are designed to examine these hypotheses. These include a cross-sessional study is designed to observe the impacts of ageing on the HIF pathway and iron status (Chapter 3). Furthermore, the impact of ageing on exercise capacity and a discussion about the impact of natural iron status on exercise capacity are included (Chapter 4). A double-blind, randomised, placebo-controlled study aiming to investigate the effect of iron infusion on RV afterload, measured by systolic pulmonary arterial pressure (SPAP), and cardiac output, during light exercise, and also on peak exercise capacity, in healthy older people is later described (Chapter 6). The effects of iron infusion on iron homeostasis and haematological variables in older people are reported in Chapter 5. In a hyperoxia study (Chapter 7), we revisit the mitochondrial ROS hypothesis and investigate the effects of hyperoxia on acute hypoxia exposures in healthy people. Finally, other miscellaneous chapters contain participant information (Appendices) and a description of the general methods and techniques used in this thesis (Chapter 2).

Chapter 2

Experimental apparatus and methods

2.1 Introduction

This chapter describes the experimental apparatus and methods relevant to the studies discussed in later chapters. Participants served as sources of data. Echocardiography was used to observe responses in the pulmonary circulation to interventions. An incremental exercise test was used to measure participants' exercise capacity. Enzyme-linked immunosorbent assays (ELISA) were used to measure chemokine and protein levels and their changes after intervention. Finally, gas control was used to induce sustained hyperoxia and hypoxia in participants for physiological observations. These will be described in the following sections.

2.2 Participants

All human studies presented in this thesis received ethical approval from the National Research Ethics Service (NRES). The relevant ethical approval reference numbers for

each study are mentioned in the appropriate chapter. The research protocols and participant information sheets are included in the Appendix. Studies were performed in accordance with the Declaration of Helsinki and Good Clinical Practice (GCP). Participants were informed of the protocols in each study, the potential risks involved and the recruitment criteria. In addition, participants were given opportunities and time to ask questions regarding any aspect of their participation in the study. All participants were aware that they were free to withdraw from the study at any time. Written consent was obtained from each participant before each study began.

2.2.1 Blinding of experiments

In studies with interventions, participants were blinded to the intervention to avoid bias in the results and the placebo effect. Two types of blinded experiments were applied, one was a single-blind experiment (Chapter 7) and the other one was a double-blind experiment (Chapter 5 and 6). In the single-blind experiment, only the participants were ignorant of the intervention, i.e. the type of gas exposure received (room air or hyperoxia), until the end of the study. Investigators were not allowed to discuss the intervention with or reveal the details to blinded participants during the study.

In the double-blind study, participants remained blinded until the end of the study in which they participated. Some investigators remained blinded until full completion of the study. The completion of the study was defined as the finish of the last data acquisition from the last participant in the study. The intervention, i.e. the type of infusion (iron or placebo), was operated by unblinded investigators. Participants wore eye masks during the infusion so that they could not watch the intervention. Blinded

investigators were not allowed to access the blood results, be involved in the infusion operation or communicate with unblinded physicians with regards to relevant information until full completion of the study.

After completion of the study, individual participants were allowed to contact the unblinded physicians about the intervention or to ask for relevant information. However, after being unblinded, these participants were not allowed to contact blinded investigators regarding the details of intervention until the completion of the study.

2.2.2 Randomisation

Participants were randomly assigned to interventions in order to avoid a selection bias. In the cross-over study (Chapter 7), the intervention sequence (room air or hyperoxia exposure) was decided by flipping through pages in a book. Those that ended upon an odd page number were exposed to room air exposure first; those with an even page number were exposed to hyperoxia first.

In the double-blind study (Chapter 5 and 6), the intervention type (iron infusion or placebo) was decided by block randomisation. Male and female participants were randomised separately. Since the total number of participants was a multiple of 4, blocks of size 4 were chosen. Each block allowed half of the participants to be allocated to receiving an iron infusion, and the other half to a placebo. However, in each block, the order of intervention was random.

In such a block of 4 equally divided to undergo 2 different interventions, iron infusion (I) and placebo (P), there are six different possible permutations that may describe the treatment of the 4 participants:

1. IIPP 2. IPIP 3. IPPI 4. PIIP 5. PIPI 6. PPII

A random number table was then used to select a permutation to allocate to each block of participants. For example, if 1532 were selected from the table, then the first 16 participants would be allocated to treatments as follows:

1	5	3	2
↓	↓	↓	↓
IIPP	PIPI	IPPI	IPIP

2.2.3 Recruitment

The relevant detailed inclusion criteria are listed for each study in the associated chapters. Current smokers or participants with a history of moderate or severe respiratory or cardiovascular disease were excluded. Participants should have had no intravenous iron supplements or medication affecting the pulmonary circulation during the previous three months. Participants with specific health concerns and medication were encouraged to consult a physician before their participation. Females were asked to participate in only the first 14 days of their menstrual cycle. All recruitment was carried out through online advertising, posters and internal emails distributed throughout the departments and colleges in the University of Oxford.

2.3 Echocardiography

The first physician to use ultrasound to examine the heart was Wolfe Dieter Keidel but his results were not fruitful (Edler and Lindstrom, 2004). Credit is hence usually given to Inge Edler and Carl Hellmuth Hertz, since the diagnostic techniques and equipment

that they used were similar to those of the modern settings (Singh and Goyal, 2007). In addition, they refined the equipment to make echocardiography more popular and approachable. Since then, echocardiography has been widely used for diagnosis and management in medicine. It is able to give a wealth of information, such as images of heart structures and vascular tissues, assessment of the blood flow through cardiac valves and blood vessels, as well as measurement of the ejection fraction of ventricles. Recent developments in echocardiography have been able to form three dimensional structures for more detailed anatomical assessment. In addition, this technique provides the advantages of non-invasiveness, high reproducibility and low-cost when compared with other methods in cardiovascular studies, such as cardiac catheterisation, magnetic resonance imaging and computed tomography scanning. These contributed to the choice of echocardiography over other methods in this thesis. In this section, basic principles of echocardiography and the methods in studies used to estimate SPAP and cardiac output will be described.

2.3.1 Basic principles of echocardiography

Ultrasound images are constructed using sound waves and the data from reflected sound waves such as their transmission time, phases and strength. By using a piezoelectric transducer to produce sound waves, ultrasound machines are able to display desired objectives through different modes of ultrasound. The two-dimensional (2D) Doppler modes were heavily used in the studies. The 2D mode was used to construct a two-dimensional image as a plane by exciting sound waves from linear-array transducers. Examples of 2D images, such as four or five-chamber views of hearts, are shown

in the upper panels of Figures 2.1 A and 2.1 B.

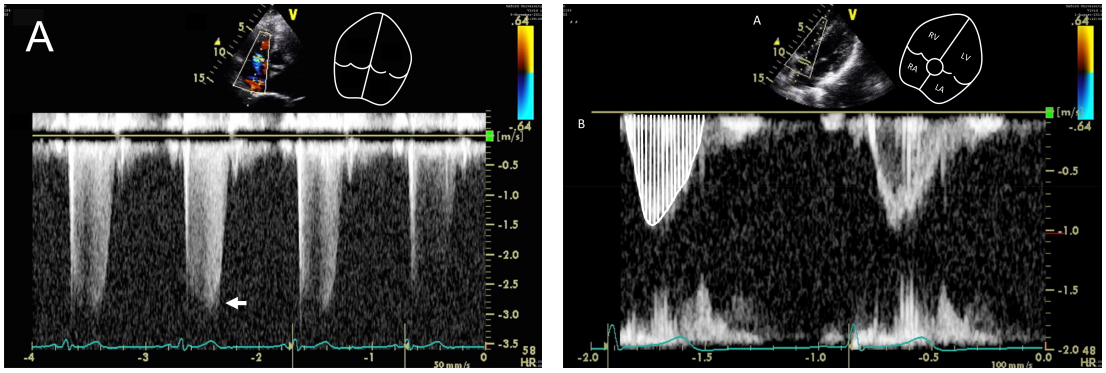

Figure. 2.1 **Images of echocardiography measurement on a representative participant. A.** Systolic pulmonary arterial pressure (SPAP) measurement. A four-chambered heart view in coloured Doppler wave mode is in the upper panel. A schematic heart next to it shows the anatomical structure. The lower panel shows an image of the velocity of tricuspid jets. The arrow shows where the maximum velocity of the tricuspid jet is; **B.** Cardiac output measurement. A five-chambered heart view is shown in the upper panel in pulsed-wave Doppler mode. The lower panel shows image of the pulsed Doppler echocardiography trace. The striped area shows the area with respect to the integration of the velocity with time.

The Doppler mode was used to visualise and then measure blood flow in the heart. The principle of the measurement involves the Doppler effect. The Doppler effect was first described by Christian Doppler in 1842 after observing a light colour shift in stars in the sky. Later on, it was found to be applicable to waves such as sound. The effect, in short, explains the change in frequency of a wave as an observer moves relative to a wave source. A classic example is the way the frequency of a police siren heard by a standing pedestrian changes as the police car goes past them. Accordingly, blood velocity is measured by pulsing ultrasound waves at red blood cells. The frequency of the returned ultrasound changes (Δf) according to the movement of the cells as in the following equation:

$$\Delta f = \frac{V}{c} \times f_0 \tag{2.1}$$

where Δf is the change in frequency between the observed and the emitted frequency; V is the velocity of the blood cell; c is the velocity of the ultrasound; f_0 is the emitted frequency.

Three types of Doppler echocardiography have been developed and used in studies to help with blood flow measurements: colour Doppler, pulsed-wave Doppler and continuous Doppler. Colour Doppler is able to visualise the velocity information of the blood flow and overlays it on top of 2D images as shown in Figure 2.1 A. It shows the direction of the blood flow by the colour (blue: away from the probe; red: toward the probe) and the velocity by the brightness (dark: slow; light: fast). This is extremely useful for detecting a tricuspid jet (regurgitation) for SPAP measurement, which shows up as bright blue on tricuspid valves in Figure 2.1 A.

Pulsed-wave Doppler sends batches of ultrasound waves and waits for the returned signals. Hence it gives information about the depth of the blood flow being identified. It is particularly useful for detecting the amount of blood crossing the aortic root, i.e. in the measurement of stroke volume, in the cardiac output calculation shown in Figure 2.1 B. The limit of pulsed-wave Doppler is its failure to function under 'aliasing' when the blood velocity is too high (velocity > 2 m/s). This results from interference between the transit time in waves and the distance to the heart. Therefore, it is not suitable for measuring high speed jets, such as tricuspid regurgitation. Continuous Doppler overcomes aliasing by using two different crystals to constantly and simultaneously send a single-frequency sound and receive the reflected signal. However, it cannot give information about the depth as pulsed-wave Doppler can do.

2.3.2 Systolic pulmonary arterial pressure

In this thesis, changes in pulmonary vascular tone were described by changes in SPAP. Since SPAP has a high correlation with pulmonary vascular resistance (PVR), it suggests that SPAP can be used as an index of pulmonary vascular tone (Dorrington et al., 1997). The measurement requires 2D echocardiography, colour Doppler and continuous Doppler techniques using echocardiography machines (Vivid i with 3S-RS transducer, GE Healthcare; Vivid q with M4S-RS transducer, GE Healthcare). Measurement started when the participant lay in the left lateral recumbent position (Figure 2.2 A). His/her heart was first examined by 2D echocardiography. A clear four-chambered heart view was generated to locate the tricuspid valve and anatomical heart images (Figure 2.1 A). Colour Doppler was used to assess and locate the tricuspid regurgitant jet. By using continuous Doppler, the velocity of tricuspid jet can be obtained (Figure 2.1 A) and used to estimate systolic right ventricular pressure from a simplified version of Bernoulli's relationship in the following equation:

$$\mathrm{RVP} - \mathrm{RAP} = \frac{1}{2}\rho {\mathrm{V_{TR}}}^2 \tag{2.2}$$

where RVP is the right ventricular systolic pressure; RAP is the right atrial pressure; ρ is the density of blood; $\mathrm{V_{TR}}$ is the velocity of tricuspid jet.

The standard international (SI) derived unit of pressure [Pa, kg/(m·s^2)] can be converted into the conventional unit (mmHg) by the conversion:

$$1\ \mathrm{Pa} = \frac{1}{133.3}\ \mathrm{mmHg} \tag{2.3}$$

If the density of blood (1051 kg/m^3 at body temperature) is known (Trudnowski and Rico, 1974), the equation can be further simplified as follows:

$$\text{RVP} - \text{RAP} = \frac{1 \times 1051}{133.3 \times 2} \, {\text{V}_{\text{TR}}}^2 \tag{2.4}$$

$$\text{RVP} - \text{RAP} = 3.9 \, {\text{V}_{\text{TR}}}^2 \tag{2.5}$$

$$\text{SPAP} = \text{RVP} \approx 4 \, {\text{V}_{\text{TR}}}^2 + \text{RAP} \tag{2.6}$$

As the right ventricle pumps blood into the pulmonary artery, it is assumed that SPAP equals RVP. In addition, 5 mmHg of RAP was assumed (Rudski et al., 2010) in this thesis.

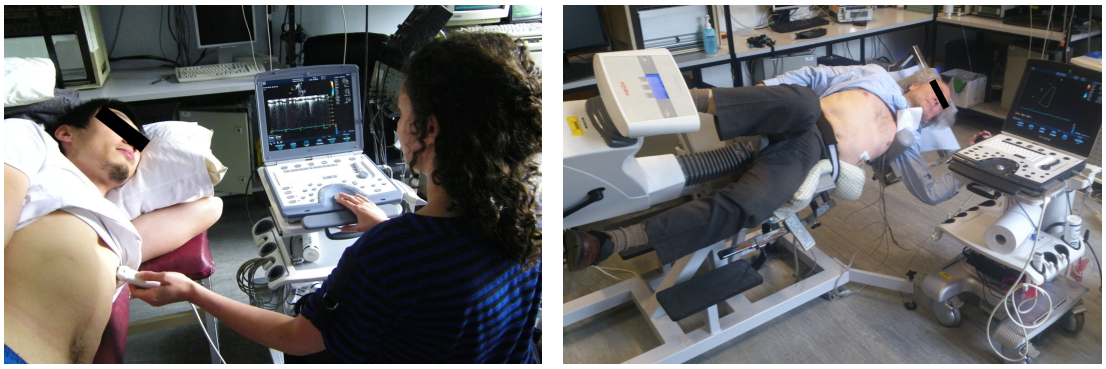

Figure. 2.2 **Echocardiography measurement of an participant. A.** Echocardiography measurement. The participant remained in a left lateral recumbent position. The echocardiography operator sat next to the participant to acquire data; **B.** Stress echocardiography measurement. The participant remained in a left lateral recumbent position on a stress echocardiography ergometer. The participant's left hand, wrist, hip and head were fixed to stabilise the body during exercise.

During light exercise, as described in Chapter 6, the procedure for SPAP measurement was similar to that described above. However, challenges to the measurement were present during exercise in terms of the limb movement, fast breathing and elevated heart rate, making the localization of tricuspid jets difficult and technically de-

manding. Therefore, to counter the challenge, participants were restrained on a stress echocardiography ergometer (Ergoselect 1200EL, Ergoline, Ferraris Respiratory Europe Ltd). Participants' left hands, wrists, hips and heads were fixed to stabilise the body during exercise (Figure 2.2 B). In addition, participants were encouraged to pedal with low cadence (40–50 rpm) to minimise the limb movement and smooth the measurement.

2.3.3 Cardiac output and stroke volume

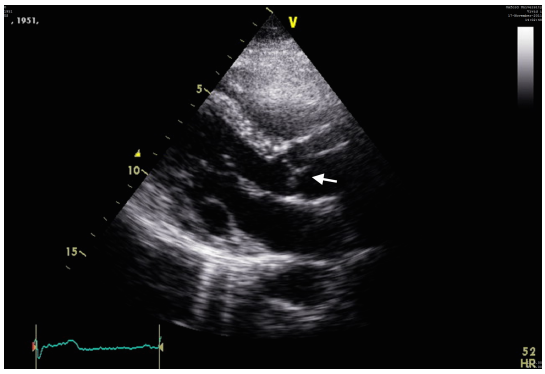

Figure. 2.3 **An image of measurement of the diameter of the aortic root.** The two-dimensional Doppler image shows a parasternal long-axis view of heart. The location of the aortic root is indicated by an arrow.

Cardiac output was measured using Doppler echocardiography to measure blood velocity through the ventricular outflow tract. Strictly speaking, echocardiography only measures stroke volume, and cardiac output is obtained via heart rate multiplied by stroke volume. Instead of using continuous Doppler, stroke volume was measured by the pulsed-wave Doppler mode. Participants stayed in a left lateral recumbent position. The heart was first examined by 2D echocardiography. A clear five-chambered heart view was obtained to give pictures of aortic valves and relevant locations (Figure 2.1 B). Colour Doppler was used to assess and locate aortic valves. By integrating the velocity with time (Figure 2.1 B) and measuring the diameter of the aortic root on a parasternal long-axis view (Figure 2.3), stroke volume can

be estimated by the following equation:

$$\text{Stroke volume} = \text{VTI} \times \pi \times (\frac{\text{r}}{2})^2 \tag{2.7}$$

where VTI is the integration of the velocity with time; r is the diameter of the aortic root. If the heart rate is known, cardiac output can be calculated via the equation:

$$\text{Cardiac output} = \text{Stroke volume} \times \text{Heart rate} \tag{2.8}$$

2.4 The incremental exercise test

The incremental exercise test was used to determine the peak oxygen uptake ($\dot{\text{V}}_{\text{O}_2\text{peak}}$) for each participant. As the ability to perform exercise is related to the cardiovascular system's ability to deliver oxygen to exercising muscle and clear carbon dioxide from the blood, $\dot{\text{V}}_{\text{O}_2\text{Peak}}$ has been used in the assessment of athletes' performances and for research purposes to evaluate a participant's physical fitness. This is expressed in equation 2.9 as the product of cardiac output and arteriovenous oxygen concentration difference [$\text{C}_{(\text{a-v})\text{O}_2}$] at peak exercise:

$$\dot{\text{V}}_{\text{O}_2\text{peak}} = (\text{Heart rate} \times \text{Stroke volume}) \times [\text{C}_{(\text{a-v})\text{O}_2}] \tag{2.9}$$

All three components of $\dot{\text{V}}_{\text{O}_2\text{peak}}$ are known to increase from values at rest and therefore contribute to the increase in oxygen flux from rest to peak exercise (Lumb and Nunn, 2010).

2.4.1 Exercise protocols

The exercise protocol used in this thesis is adapted from Rossiter and colleagues (Rossiter et al., 2006). Each participant performed a standard incremental exercise test on an electrically-braked cycle ergometer (Cariokinetic, Mijnhardt KEM-3, UK) (Figure 2.4 A). The participant sat stationary on the cycle ergometer for the first 3 minutes (Figure 2.4 C). Then he/she began to pedal with the work rate rising incrementally from 0 W by 15 W per minute up to the point of volitional exhaustion. Following exhaustion, the participant pedalled at 0 W for 5 minutes before cycling against 105% of his/her maximum work rate as determined from the immediately previous standard incremental exercise test conducted up to the point of exhaustion. Exhaustion was determined by participants stopping or by the cadence going below 60 rpm.

2.4.2 Measurement of oxygen consumption

Oxygen consumption ($\dot{V}_{O_2}$) was measured by the breath-by-breath method in this thesis. In principle, in unit time, oxygen consumption can be calculated by the difference in the oxygen content between inhalation and exhalation:

$$\dot{V}_{O_2} = (\dot{V}_I \cdot F_{IO_2}) - (\dot{V}_E \cdot F_{EO_2}) \tag{2.10}$$

where $\dot{V}_I$ is the inspired volume in unit time; F_{IO_2} is the fraction of O_2 in the inspired gas; $\dot{V}_E$ is the expired volume in unit time; F_{EO_2} is the fraction of O_2 in the expired gas.

In Chapter 4, $\dot{V}_{O_2}$ was measured by a customised mass spectrometer system. The

participant breathed through a mouthpiece with his/her nose occluded. The concentration of O_2 in the participant's breath was constantly detected by a mass spectrometer (Airspec MGA 3000, UK) via a thin capillary tube connecting to the mouthpiece drawing the sample gas at a rate of 20 ml/min. The output of the mass spectrometer was updated every 20 milliseconds (ms). Ventilation data consisted of inputs from a turbine volume device (Cardiokinetics Ltd., UK) and pneumotachograph (Fleisch, Switzerland) attached to the mouthpiece. The turbine volume device was optimised for accurate measurement of inspiratory and expiratory volumes (1 revolution = 2 ml). The revolution was measured by a photodetector (Ventilation Measurement Module, SensorMedics, USA). However, the turbine generated delay due to impeller momentum. Therefore, the change in respiratory phase was detected by the pneumotachograph.

The pneumotach is a cylinder filled with smaller tubes with the structure of a honeycomb. This allows laminar flow through these small tubes, and results in a nearly linear relationship between the flow and the pressure drop across the tubes. This allows calculation of flow by measuring pressure changes (Poiseuille's law). The pressure change was detected using a pressure transducer (P.K. Morgan Ltd., UK). Ventilation data (flow) from the pneumotach were corrected by the turbine volume device. These data were then used for the oxygen consumption calculation. All data were recorded using customised *BreathM* software (written by T. Pragnell).

A metabolic measurement system, Oxycon^{TM} (Carefusion®, Germany), was used in Chapter 6 (Figure 2.4 B). The system works similarly to the aforementioned mass spectrometer system. The gas composition from the participant's breath was measured by a fast-response O_2 analyser and a fast-response CO_2 analyser. The O_2 analyser uses

the paramagnetic property of O_2 to detect the O_2 concentration. The CO_2 analyser uses the absorption of infrared light by CO_2 to measure its concentration. The ventilation was measured by a volume sensor (TripleV-Volume Sensor, Carefusion®, Germany).

2.4.3 Determination of oxygen consumption

The $\dot{V}_{O_2peak}$ was determined as the average of the oxygen consumption throughout the exercise test over a 30-second interval incorporating the maximum oxygen consumption by the customised mass spectrometer system in Chapter 4 or a 15-second interval by Oxycon™ in Chapter 6 (Figure 2.4 C). If the maximum $\dot{V}_{O_2}$ values between the two exhaustion values were different, the higher $\dot{V}_{O_2}$ value was chosen as the participant's $\dot{V}_{O_2peak}$.

Baseline $\dot{V}_{O_2}$ and CO_2 elimination were taken as averages from measurements over the second minute on the ergometer during incremental exercise tests before pedalling.

2.4.4 Other relevant measurements

The oxy-haemoglobin saturation (S_{aO_2}) was measured using a pulse oximeter (Ohmeda Biox 3740 Pulse Oximeter, BOC Healthcare) with a finger probe (OXY-F4-H finger sensor, GE Healthcare). Heart rate was measured using a three-lead electrocardiogram (Micromon 7142 B ECG, Kontron Medical).

2.4.5 Calibration

The mass spectrometer was tuned, zeroed and calibrated before each experiment. Tuning involves scanning the specific mass spectrometry peaks for O_2, CO_2, N_2 and Ar

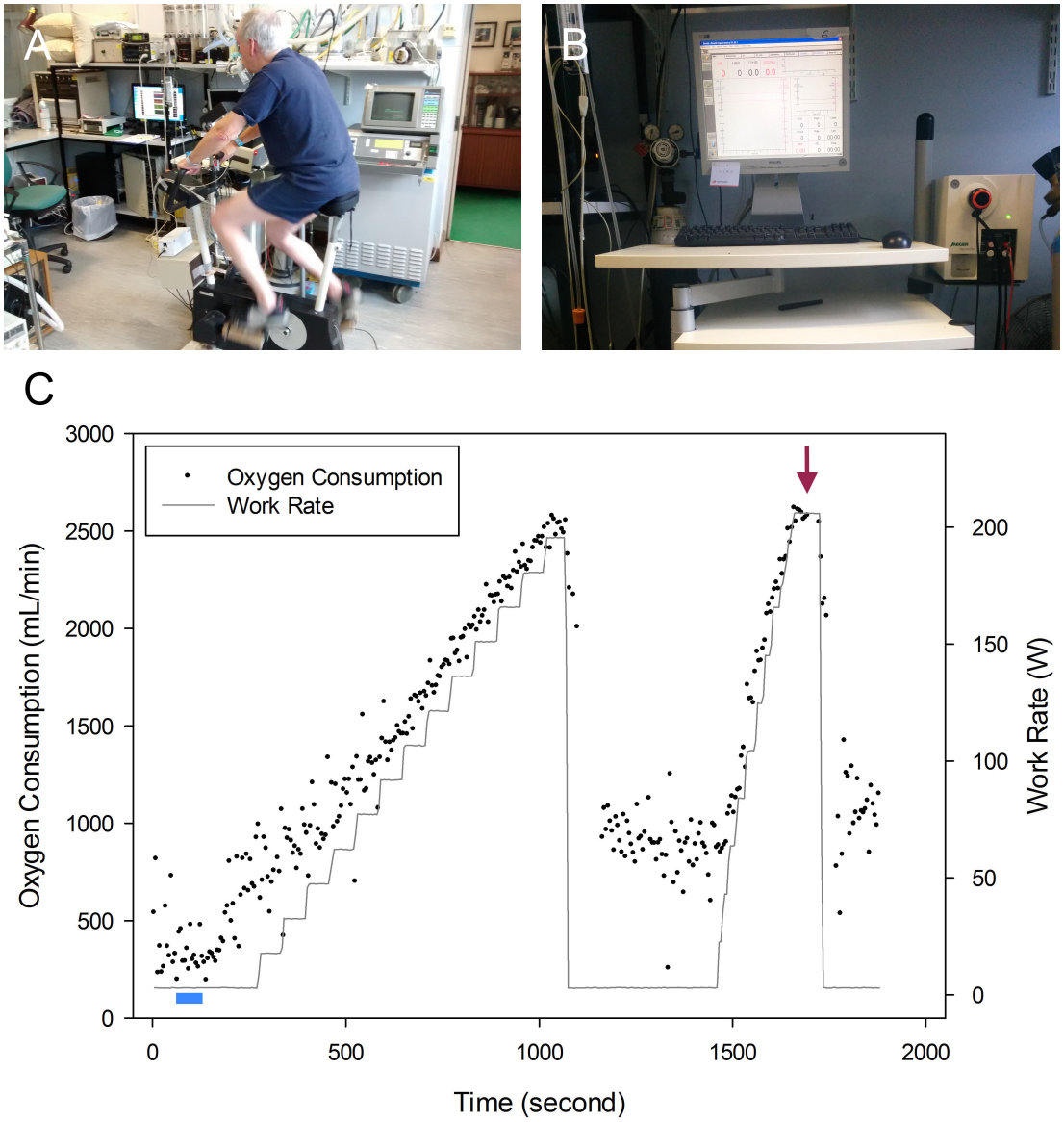

Figure. 2.4 **The incremental exercise test. A.** A participant performing the incremental exercise test. In the centre of the picture, a participant can be seen performing the test on a cycle ergometer. His nose was occluded and he breathed through a mouthpiece during the exercise test. On his right hand side, the mass spectrometer can be seen. The computer, heart rate monitor and oxy-haemoglobin monitor can be seen in the background of the picture; **B.** The Oxycon^{TM} machine and its screen; **C.** The exercise protocol and measurement from a representative participant. Peak oxygen consumption was determined as the average of oxygen consumption values over a 30-second interval during the period of exhaustion in the exercise test. The higher value between the two exhaustion periods was chosen as indicated in this case in the second period of exercise by *red arrow* in the graph. Baseline oxygen consumption and carbon dioxide elimination were determined by the average of the 2nd minute period (*blue bar*).

to optimise the signals. Zeroing involves a gas cylinder containing 100% helium to detect and suppress the background noise. The calibration used room air and a cali-

bration gas cylinder containing $\sim$7% of O_2 and $\sim$6% of CO_2 balanced with nitrogen. The calibration method was a two-point calibration.

Ventilation calibration was using an artificial pump with known volume. The signal delay between equipment, such as between the pneumotach and mass spectrometer, was measured by switching a solenoid valve from room air to the calibration gas. An electronic signal from the solenoid valve was sent to a computer using the same signalling system as the pneumotach and the turbine. After switching the valve, the delay time was treated as the gap in time of the computer receiving the change in gas composition (from room air to the calibration gas) from the mass spectrometer. Typically the delay was between 100 ms and 300 ms, depending on the length of the sampling catheter.

2.5 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISA) use antibodies and colour change in the solution to identify a protein or peptide. By using known standard concentrations for calibration, the concentration of substance can be quantified through Beer's law. Assuming the molar absorptivity is constant in the solution, the attenuation of light across the same depth is then related to the concentration of the substance in the solution. An ELISA allows for the measurement of a large number of samples simultaneously. In addition, an ELISA requires only small amounts of a substance and has the characteristics of high sensitivity and high reproducibility. Therefore, it has been used widely in medicine and biomedical research. The methods used to quantify substances in this thesis are sandwich ELISA and competitive ELISA.

2.5.1 Sample preparation for ELISA

Venous blood samples were drawn from antecubital veins using a needle and a syringe under standard aseptic conditions. For participants whose withdrawal of blood samples from antecubital veins proved difficult, blood samples were taken from a vein in the forearm via an indwelling cannula. Details of sampling volume and frequency are given in relevant chapters, but in general participants gave 10 ml of venous blood for an ELISA use. Half of the blood was transferred immediately into tubes containing EDTA (EDTA BD Vacutainer®, BD) for plasma samples. The other half was transferred into tubes containing micronised silica particles (SST BD Vacutainer®, BD) for serum samples. Tubes were spun for 20 minutes at 3000 rpm to obtain $\sim$3 ml plasma and serum respectively for subsequent analysis. All samples were stored at –80°C.

2.5.2 Sandwich ELISA

Sandwich ELISA uses two antibodies to measure a substance. One is pre-coated at the bottom of the plate to capture the substance. The other binds to the substance, and part of the antibody binds another coloured, fluorescent or electrochemically signalled chemical to identify the substance. The detailed protocols, dependent on the substance and manufacturers, differ but in principle involve the following procedures: 1) pipetting the standard or sample into antibody pre-coated microplates; 2) incubation for substance binding by the antibody; 3) washing off excessive substance; 4) conjugation; 5) washing off excessive conjugate reagents; 6) pipetting substrate for colouring; 7) stopping the reaction for absorbance detection.

Measurements of EPO (Quantikine® IVD® Erythropoietin ELISA, R&D system),

CRP (Quantikine® Human C-Reactive Protein ELISA, R&D system), interleukin-6 (IL-6; Quantikine® HS Human IL-6 ELISA, R&D system), and soluble transferrin receptor (sTfR; Quantikine® IVD® Soluble Transferrin Receptor ELISA, R&D system) are based on this principle. All controls, standards and samples were assayed at least in duplicate and performed at room temperature.

2.5.3 Competitive ELISA

Competitive ELISA plates have coated plates with the antigen at the bottom. The substance and antibody then compete with each other for the antigen in the bottom. After the reaction, unbounded substance and antibody are washed out. If there is any more substance left in the well, there would be fewer antibody-antigen complexes left for the identification by fluorescent or electrochemically signalled chemical. Therefore, the more substance there is in the well, the fewer the number of colour/fluorescent responses.

The hepcidin assay (Human Hepcidin-25, Peninsula Laboratories International, Inc) used in this thesis is an example. The protocol for the assay was in accordance with protocol V in the manufacturer's instructions. In short, the antiserum was added and incubated in the microplate for an hour at room temperature. The standard and diluted samples (15 to 1) were then added and incubated for two hours at room temperature. A biotinylated tracer was finally added into the microplate and competed to bind to the antiserum with the standard and the samples. The microplate with antiserum, samples, standards and tracers was incubated at 4°C for 16 hours. After the long incubation, a washing off process for excessive tracer, standard and samples was performed. Then

conjugation was started after adding streptavidin-conjugated horseradish peroxidase (SA-HRP) for an hour at room temperature. Following another washing off process, a substrate, tetramethylbenzidine (TMB), was added and incubated for an hour. Then the reaction was terminated for absorbance detection. All controls, standards and samples were assayed at least in duplicate and the assay was performed at room temperature.

2.5.4 Technical details

The ELISA technical information is summarised in Table 2.1.

	Sample	Assay	Sensitivity	Calibration
EPO	Serum	Sandwich	0.6 IU/l	The 2nd International Reference Preparation 67/343
CRP	Plasma	Sandwich	0.022 mg/l	The NIBSC/WHO 1st International Standard 85/506
IL-6	Serum	Sandwich	0.11 ng/l	The NIBSC/WHO 1st International Standard 89/548
sTfR	Plasma	Sandwich	0.5 nmol/l	NA
Hepcidin	Plasma	Competitive	1.5 µg/l	NA

Table 2.1 **Summary of ELISA technical information.** EPO: erythropoietin; CRP: C-reactive protein; IL-6: Interleukin-6; sTfR: soluble transferrin receptor; NIBSC: The National Institute for Biological Standards and Control; WHO, The World Health Organization.

2.6 Gas Control

A chapter of this thesis discusses the effects of sustained hyperoxia on the pulmonary circulation in humans. It is difficult to achieve stable hyperoxic and hypoxic conditions at sea level. In addition, without inspired carbon dioxide being controlled, both hyperoxia and hypoxia cause hypocapnia, which has been found to alter pulmonary vascular

tone, observed as a reduction in SPAP and cardiac output (Balanos et al., 2003). The laboratory has developed two systems for the purpose of controlling oxygen levels in the body under isocapnic conditions. One is a dynamic end-tidal forcing (DEF) system for short exposures (< 1 hr). Another is a gas-control chamber for longer exposures (> 1 hr). Both systems control the end-tidal partial pressures of oxygen and carbon dioxide. We assume that these are the same as those in alveolar gas. The gas control is achieved by clamping end-tidal partial pressure of gases with computer algorithms.

2.6.1 Dynamic end-tidal forcing system

The DEF system was designed for short exposures with accurate control of end-tidal gas composition by breath-by-breath adjustments of inhaled gases. The composition of the DEF system is similar to the one used in the incremental exercise test. However, the system includes gas inputs as shown in Figure 2.5 A and schematised in Figure 2.5 B. Therefore, the ventilation and gas sampling methods are the same as described in Section 2.4.2. Similarly, the calibration procedure for the DEF system is the same as described in Section 2.4.5.

In experiments, the participant lay on a couch and breathed through a mouthpiece with the nose occluded. The participant's inhaled and exhaled gases were sampled close to the mouth continuously at a rate of 20 ml/min via a catheter and analysed by a mass spectrometer (Airspec MGA 3000, UK). Ventilatory volumes and timings were measured by ventilation sensors, a turbine volume device (Cardiokinetics Ltd., UK) and a pneumotachograph (Fleisch, Switzerland). The ventilation sensors sent signals

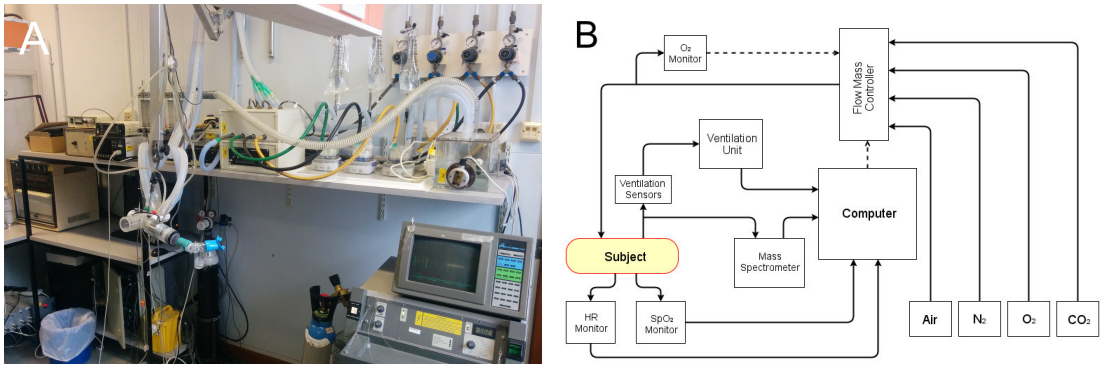

Figure. 2.5 **The dynamic end-tidal forcing system. A.** The DEF system. On the top right corner of the picture, valves for gas flow can be seen. On the bottom right corner of the picture, the mass spectrometer can be seen. In the centre region of the picture, a flow mass controller and a mouthpiece connecting to the ventilation sensors is present. The ventilation unit, heart rate monitor, oxy-haemoglobin monitor and computer, are on the left side of the picture; **B.** The schematic graph of DEF system. The solid arrows show the direction of the gas and signal flows. The dashed arrows show the control output, i.e. the computer controls the flow mass controller to deliver the specific inspired gas to the participant in the experiment.

to ventilation units, a photodetector (Ventilation Measurement Module, SensorMedics, USA) and a pressure transducer indicator (P.K. Morgan Ltd., UK), to be processed and amplified before ventilatory data were recorded by a computer.

Ventilatory data were recorded constantly by the computer and used for the gas control. The gas control was achieved by a prediction-correction scheme developed by Robbins *et al.* (Robbins et al., 1982). In the prediction phase, inhaled gas was predicted and controlled by the algorithm below:

$$P_I = \frac{\dot{V}_A P_A + V_L \cdot \frac{dP_A}{dt} + \lambda \dot{Q}(C_a - C_{\bar{v}})}{\dot{V}_A} \tag{2.11}$$

where $\dot{V}_A$ is the alveolar ventilation; V_L is the mean lung volume; λ is a particular coefficient dependents on units; $\dot{Q}$ is the pulmonary blood flow; C_a is the concentration of the gas in the arterial blood; $C_{\bar{v}}$ is the concentration of the gas in the mixed venous blood.

The correction phase reduces the inadequacies of the model and errors to minimise the effect of drift and steady-state error by considering the variation in each breath (proportional feedback) and the sum of all previous differences in breaths (integral feedback). The algorithm for the correction is in the equation as breath (*n*th):

$$P_{I(n)} = P_{I_c(n)} + g_p \cdot (P_{ET_d(n-1)} - P_{ET_m(n-1)}) + g_i \cdot [\sum_{j=1}^{n-1} (P_{ET_d(j)} - P_{ET_m(j)})] \qquad (2.12)$$

where P_{I_c} is the current predicted inspired partial gas pressure from equation 2.11; P_{ET_d} is the desired end-tidal partial pressure; P_{ET_m} is the measured end-tidal partial pressure; g_p is the proportional feedback gain; g_i is the integral feedback gain.

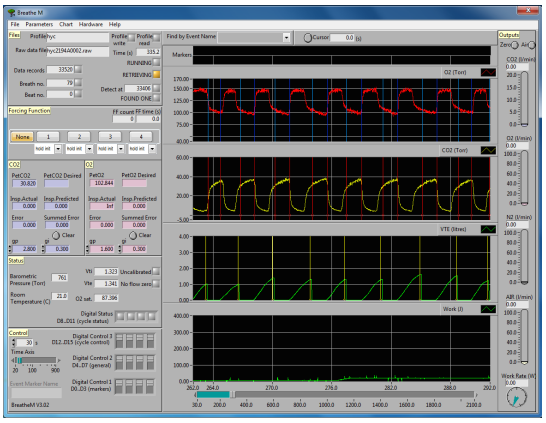

Figure. 2.6 **The operation window in *BreathM* software** The status of the system is on the left side. The right side shows participant's P_{O_2} trace, P_{CO_2} trace, exhalation and work rate (*from top to bottom*) in a 30-second period.

In practice, the correction was adjusted by changing gains, g_p and g_i, to improve the accuracy of gas control. The gas control and ventilatory volumes were implemented by computer software, *Breath M* (written by T. Pragnell).

2.6.2 Gas-control chamber

A customised chamber of volume 8.6 m^3 [2.3 m (L) x 1.7 m (W) x 2.2 m (H)] was used for long exposures. The gas-control chamber system is shown in Figure 2.7 A and schematised in Figure 2.7 B. The gas control in the chamber was achieved by sampling each breath (Howard and Robbins, 1995). Participants' inspired and expired

gases were monitored through nasal catheters continuously by a gas analyser, Datex (Datex Normocap 200, GE Healthcare). Since the ventilation was not measured in the chamber, the detection of breaths was achieved by sudden changes in the partial pressure of gases. Automated alterations of chamber gas composition were made every five minutes, based on the difference between measured and desired gas values via the equations:

$$P_{I_{(new)}} = P_{A_{(new)}} + \frac{(P_{I_{(old)}} - P_{A_{(old)}}) \cdot \dot{V}_{E_{(old)}}}{\dot{V}_{E_{(new)}}} \tag{2.13}$$

where P_I is the predicted inspired partial gas pressure as mentioned by equation 2.12; P_A is the partial gas pressure in the alveoli; $\dot{V}_E$ is the expiratory ventilation; the subscripts indicate the predicted value (*new*) from the previous 5 minutes of averaged data (*old*).

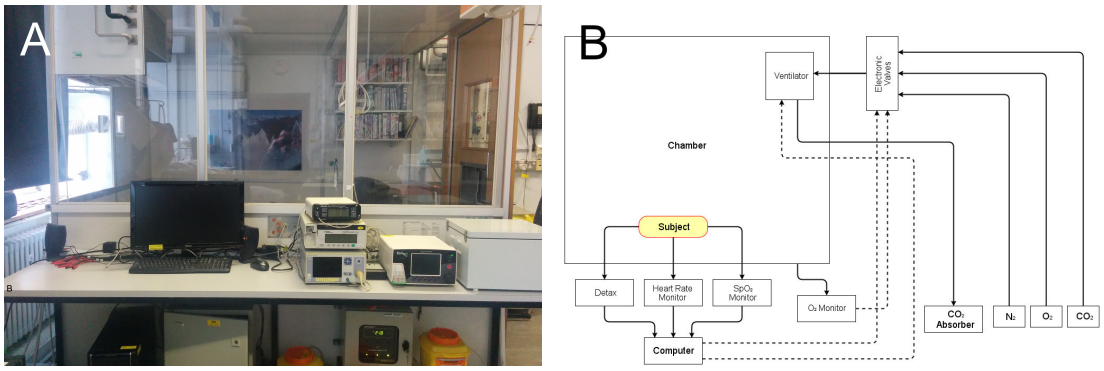

Figure. 2.7 **The gas-control chamber. A.** The chamber can be seen in the centre of the picture. Two sides of the chamber consist of glass. Inside the chamber, some video equipment, such as a TV and video tapes, are available for participants' entertainment. An air conditioner is installed inside of the chamber for temperature regulation. The computer, heart rate monitor and oxy-haemoglobin monitor can be seen in front of the long glass facade. A separate oxygen monitor with an alarm for low oxygen for safety is under the desk; **B.** The schematic graph of gas-control chamber. The solid arrows show the gas or signal flow direction. The dashed arrows show the control output, i.e. the computer controls electronic valves to deliver gases into the chamber in the experiment.

Measurement of ventilation is not available in the chamber, so the ventilation is estimated based on the Lloyd-Cunningham equation (Lloyd et al., 1958).

Once newly inspired gas partial pressures were obtained from the equations 2.13, the adjustment of gas compositions in the chamber was achieved by equation 2.14 below. The flow rates of gases were known (105 l/min for O_2; 85 l/min for CO_2; 882 l/min for N_2) and designed to change the concentration of the gases at a rate of 1% per minute.

$$V_{gas} = V_{chamber} \times \log_e \frac{1 - F_{I_{(old)}}}{1 - F_{I_{(new)}}} \tag{2.14}$$

where F_I is the fraction in inspired gas.

Excessive CO_2 was removed by re-circulating chamber air into a soda-lime CO_2 absorber with a flow rate of 1.2 l/min and the new CO_2 fraction was calculated using the equation below:

$$V_{absorber} = \frac{V_{chamber}}{0.9997} \times \log_e \frac{F_{CO_{2(old)}}}{F_{CO_{2(new)}}} \tag{2.15}$$

where 0.9997 is equal to 1 minus the fraction of CO_2 in air.

The gas control was implemented by a computer software, *Chamber* (written by T. Pragnell) (Figure 2.8).

2.6.3 Calibration for chamber system

The Datex monitor was calibrated with a calibration gas cylinder containing 55% of O_2, 5% of CO_2 and 40% of N_2O. The Datex monitor was first flushed with room air

and then with calibration gas according to the specified calibration procedure. The calibration method was a two-point calibration. All gas valves and flow meters were checked before each experiment.

2.6.4 Safety for gas-control experiments

Physicians were present during all experiments in case of emergency. The laboratory was equipped with a resuscitation trolley, as well as first aid equipment and an automated external defibrillator (AED).

Figure. 2.8 **The operation window in *Chamber* software** The status of the system is on the left side. The right side shows participant's P_{O_2} trace, P_{CO_2} trace and oxygen saturation (*from top to bottom*) in a 1 minute period.

Both DEF and gas-control chamber systems had separate oxygen monitors (Oxygen analyser Model 571 & 1175, Servomex®, UK) within the breathing system or chamber (Figure 2.5 B and 2.7 B). They measured oxygen concentration in the inspired gas in the DEF system and gas composition in the chamber constantly. Once the oxygen level in the inspired gas and chamber was below 5% for more than 20 seconds, the alarm would sound. In addition, the alarm would trigger the flow mass controller to deliver air (DEF system) or electronic valves to deliver oxygen (gas-control chamber). The computer would then stop recording. The experiment would be terminated.

Chapter 3

Age, iron homeostasis and erythropoiesis

3.1 Introduction

Erythropoiesis, a process that involves haemoglobin (Hgb) production, is important for maintaining the oxygen delivery in the body. This process is regulated by EPO, which is secreted within the kidneys in adults. When renal oxygen tension decreases under conditions of, for example, decreased S_{aO_2} (Knaupp et al., 1992), decreased Hgb concentration (Adamson, 1968) and decreased renal blood flow rate (Fehr et al., 2004), EPO production is up-regulated to accelerate erythropoiesis in order to overcome a shortage in oxygen delivery capacity. On average Hgb concentration was estimated to decline at a rate of 0.06 g/dl per year with age in adults without anaemia or chronic kidney disease (Ershler et al., 2005). The decrease in Hgb concentration with age might be expected to correspond with an increase in EPO level with age. Indeed several

studies have demonstrated that EPO level increases with age. Kario and co-workers surveyed 156 subjects aged 60 to 98 and found an increase in EPO level with age (Kario et al., 1991). In addition, in a longitudinal study of 143 adults over 8 years, Ershler and co-workers found a modest but steady increase in EPO level with age (Ershler et al., 2005).

The increase in EPO level with age leads to the question of whether iron homeostasis is altered with age. Iron is an essential element for oxygen binding in Hgb. More than two-thirds of iron present in the body is contained in Hgb and utilised for erythropoiesis (Finch, 1982; Goodnough et al., 2000). Furthermore, a vast iron turnover from Tf (Cazzola et al., 1985; Cook et al., 1970) associated with circulating Tf receptors mostly originates from erythroblasts (Feelders et al., 1999). This suggests that the prodcution and destruction of Hgb should affect iron homeostasis. It is not clear to what extent erythropoiesis and iron homeostasis are related to each other and whether the correlation is affected by age. Therefore, the main aim of this study is to assess the relationship between iron homeostasis and erythropoiesis in healthy humans aged between 50 and 80 years. In addition, the secondary aim is to measure the impact of ageing on iron homeostasis and erythropoiesis in this group.

3.2 Methods

3.2.1 Participants

In total, 113 men and women volunteered to participate in this study. Experimental details were given to each volunteer, with consent obtained from each before partic-

ipation. This study was approved by the NRES Committee South Central-Oxford B (Reference: 11/SC/0221).

Participants were recruited through online advertising, posters and internal emails distributed throughout the departments and colleges of Oxford University. The inclusion criteria were as follows: not a current smoker; not overweight; no history of severe cardiac diseases, respiratory diseases or epilepsy; have not undergone iron therapy in the preceding three months and capable of performing the required cycling exercise.

Participants were advised against vigorous exercise and heavy drinking the day before the study. Participants were requested to refrain from alcohol and caffeinated drinks on the day of the study.

3.2.2 Experimental protocol

Each subject gave details of his/her medical history and lifestyle before the following procedures were carried out in sequence: 1) blood samples, 2) echocardiographic measurements and 3) incremental exercise tests. The details and results of echocardiographic measurements and incremental exercise are included in Chapter 4.

3.2.3 Blood samples

Blood samples were obtained from a vein in the forearm and distributed into tubes containing EDTA (EDTA BD Vacutainer®, BD) or micronised silica particles (SST BD Vacutainer®, BD). Tubes were sent to the haematology department at the Oxford University Hospital and analysed for Hgb, haematocrit (Hct), mean cell volume (MCV), ferritin, serum iron, Tf, Tfsat and CRP.

Tubes for plasma sTfR, EPO and hepcidin were centrifuged at 3,000 g for 20 minutes. Plasma was immediately frozen at –80ºC. The same procedure was followed for tubes for serum, which were immediately frozen at –80ºC. Plasma sTfR (Quantikine® ELISA, R&D system), EPO (Quantikine® ELISA, R&D system) and hepcidin (Hepcidin-25 (human)-EIA Kit, Bachem) were measured by ELISA kits. All analytical procedures followed the guidelines of the manufacturers.

3.2.4 Definition of blood disorders

Anaemia is defined as an Hgb concentration below 12 g/dl for females and 13 g/dl for males according to the World Health Organization (WHO) guideline (WHO, 1968). Iron deficiency follows the definition by Johnson *et al*. as falling into at least one of two criteria: 1) ferritin < 12 µg/l; 2) Tfsat < 16% (Johnson et al., 1994). Iron overload follows the definition by Dooley *et al*. as falling into at least one of two criteria: 1) ferritin > 300 µg/l; 2) Tfsat > 50% (Dooley and Worwood, 2000). Iron deficiency anaemia is defined as when anaemia and iron deficiency co-exist.

3.2.5 Statistical analysis

Data are presented as the mean ± SD for normally distributed data or as the median (interquartile range) for non-normally distributed data. The distribution of data were tested by the Shapiro-Wilk test. Non-normally distributed data were compared by using the Mann-Whitney U test. Normally distributed data were compared using a two-tailed Student's *t*-test. The correlation between variables was analysed using Spearman's rank correlation coefficients (β) and linear regression. Data were analysed using

computer software (SigmaPLOT, Version 12.0, Systat Software).

3.3 Results

3.3.1 Participant characteristics

After excluding participants with blood disorders and diseases that might affect iron homeostasis, the basic and haematological characteristics of 83 enrolled participants are listed in Table 3.1. Of the 113 participating volunteers, 7 were excluded due to acute inflammatory response (CRP > 8 mg/l), 6 were excluded due to anaemia, 3 were excluded due to iron deficiency anaemia, 7 were excluded due to iron deficiency, and 7 were excluded due to iron overload (Figure 3.1).

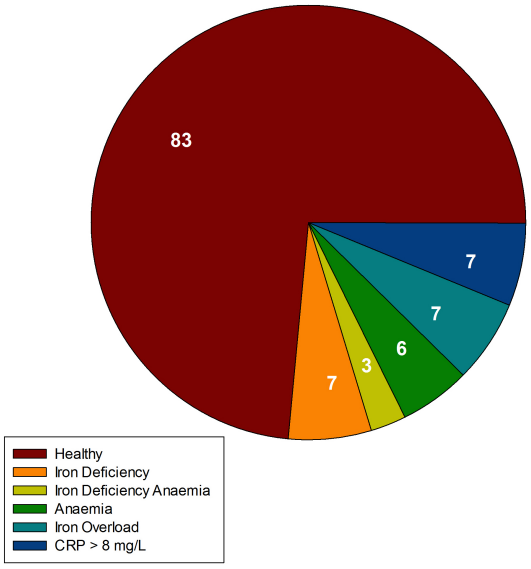

Figure. 3.1 **Distribution of participants according to iron status and haematological results.** The pie chart shows the number of participants by blood disorders.

Despite male participants having higher Hgb concentration (14.3 [13.7-14.7] g/dl) and Hct (0.429 ± 0.023 l/l) than female participants' Hgb concentration (13.2 [12.7-13.7] g/dl) and Hct (0.404 ± 0.025 l/l) respectively in the studied age group ($p < 0.001$), both sexes of participants had similar MCV and EPO concentrations (Table 3.1). The only significant differences between male and female participants in iron status indicators were serum iron concentration (male: 19.5 ± 4.5 µmol/l; female: 17.2 ± 4.7

µmol/l, p = 0.025) and Tf concentration (male: 2.70 ± 0.36 µmol/l; female: 2.51 ± 0.32 µmol/l, p = 0.015) (Table 3.1).

	Male	**Female**	**p Value**
Number, n	42	41	
Age, yr	61.9 ± 6.8	62.3 ± 7.1	0.794
Height, cm	177.2 ± 5.0	166.2 ± 6.5	< 0.001
Weight, kg	76.3 ± 8.8	65.8 ± 10.7	< 0.001
BMI, kg/m^2	24.3 ± 2.8	23.8 ± 3.4	0.466
Hgb, g/dl	14.3 (13.7-14.7)	13.2 (12.7-13.7)	< 0.001
Hct, l/l	0.429 ± 0.023	0.404 ± 0.025	< 0.001
MCV, fl	92.7 ± 4.3	93.1 ± 4.3	0.673
EPO, UI/l	8.73 (7.41-11.4)	7.91 (6.71-9.16)	0.058
Serum iron, µmol/l	19.5 ± 4.5	17.2 ± 4.7	0.025
Serum ferritin, µg/l	73.2 (29.0-113.6)	69.1 (42.0-108.3)	0.870
Tf, g/l	2.70 ± 0.36	2.51 ± 0.32	0.015
Tfsat, %	28.0 (23.5-36.6)	25.7 (21.6-32.6)	0.266
sTfR, nmol/l	14.2 (12.6-16.0)	12.9 (11.1-17.5)	0.068
Hepcidin, µg/l	28.8 (14.1-48.5)	35.1 (20.9-51.5)	0.196
CRP, mg/l	0.80 (0.2-1.74)	0.80 (0.43-1.19)	0.837

Table 3.1 **Participant characteristics.** Hgb: haemglobin; Hct: haematocrit; MCV: mean cell volume; EPO: erythropoietin; Tf: transferrin; Tfsat: transferrin saturation; sTfR: soluble transferrin receptor; CRP: C-reactive protein. Values are shown as the mean ± SD or the median (interquartile range).

3.3.2 Effects of age on haematological variables

Hgb concentration, Hct, MCV, EPO concentration and CRP concentration in both sexes, and overall, showed no changes with age (Figure 3.2 A to E). All correlations were not significant.

3.3.3 Effects of age on iron status indicators

Serum iron, ferritin, and Tf concentrations, Tfsat, sTfR concentration and hepcidin concentration in both sexes and overall showed no changes with age (Figure 3.3 A to F). All correlations were not significant.

3.3.4 Relationship between erythropoietin and selective variables

Hgb, ferritin, Tf and hepcidin concentrations showed no correlations with EPO concentration in male participants, female participants or overall (Figure 3.4 A, B, C and F).

Tfsat had a significant negative correlation with EPO concentration in male participants (β = –0.325, p = 0.036, Spearman's correlation) (Figure 3.4 D). However, there was no correlation between Tfsat and EPO concentration in female participants (p = 0.142, Spearman's correlation). sTfR concentration had a significant positive correlation with EPO concentration in male participants (β = 0.316, p = 0.042, Spearman's correlation), but the correlation was not seen in female participants (p = 0.534, Spearman's correlation) (Figure 3.4 E).

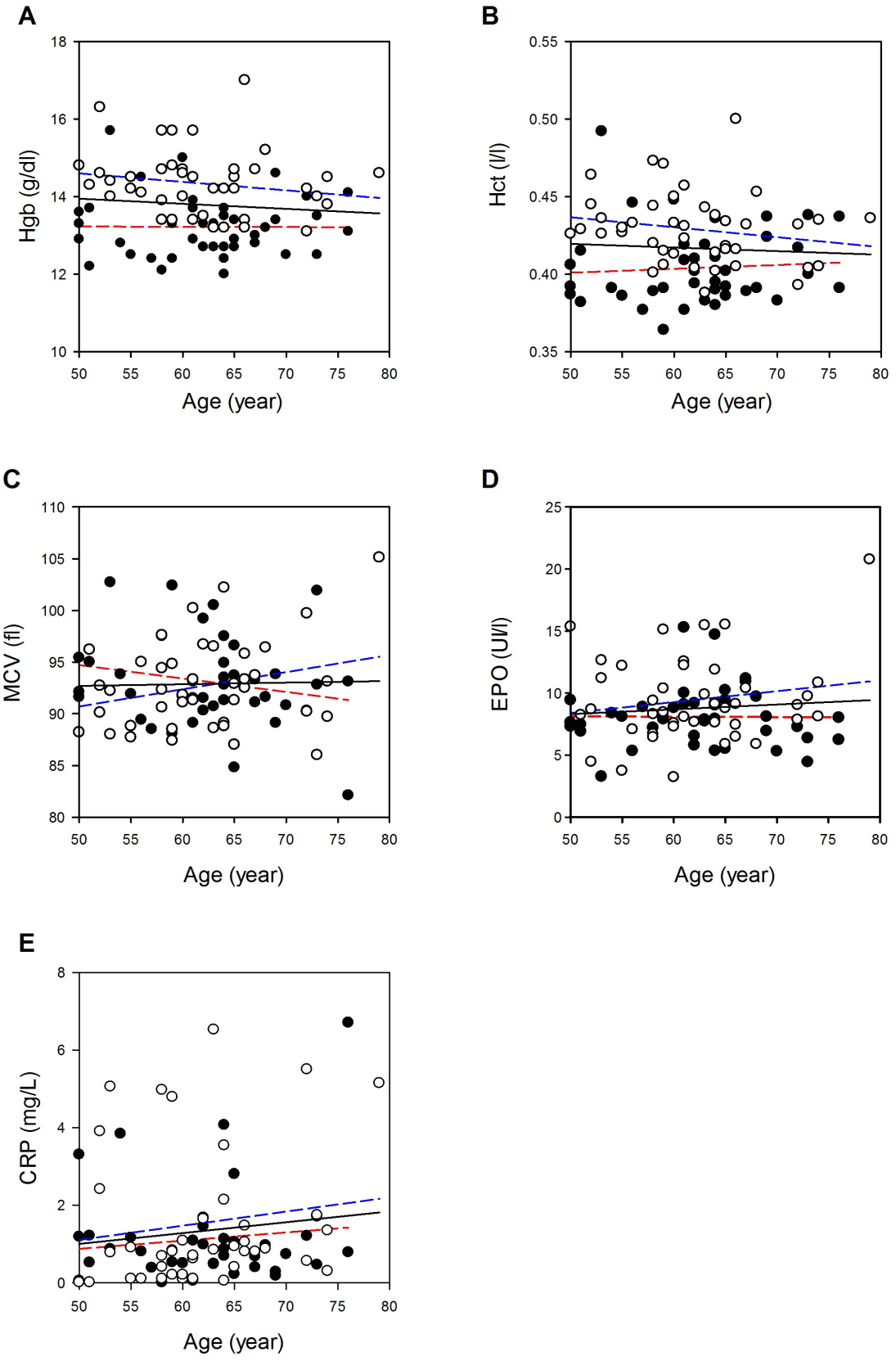

Figure. 3.2 **Relationships between haematological variables and age.** Scatter graphs show the correlations between age (horizontal axis) and haematological variables (vertical axes). **A**. Haemoglobin (Hgb) concentration; **B**. Haematocrit (Hct); **C**. Mean cell volume (MCV); **D**. Erythropoietin (EPO) concentration; **E**. C-reactive protein (CRP) concentration. Data from females (*filled circles*) and males (*empty circles*) are shown together. Trends with age are shown as regression lines, female (*red dashed*), male (*blue dashed*) and overall (*black solid*).

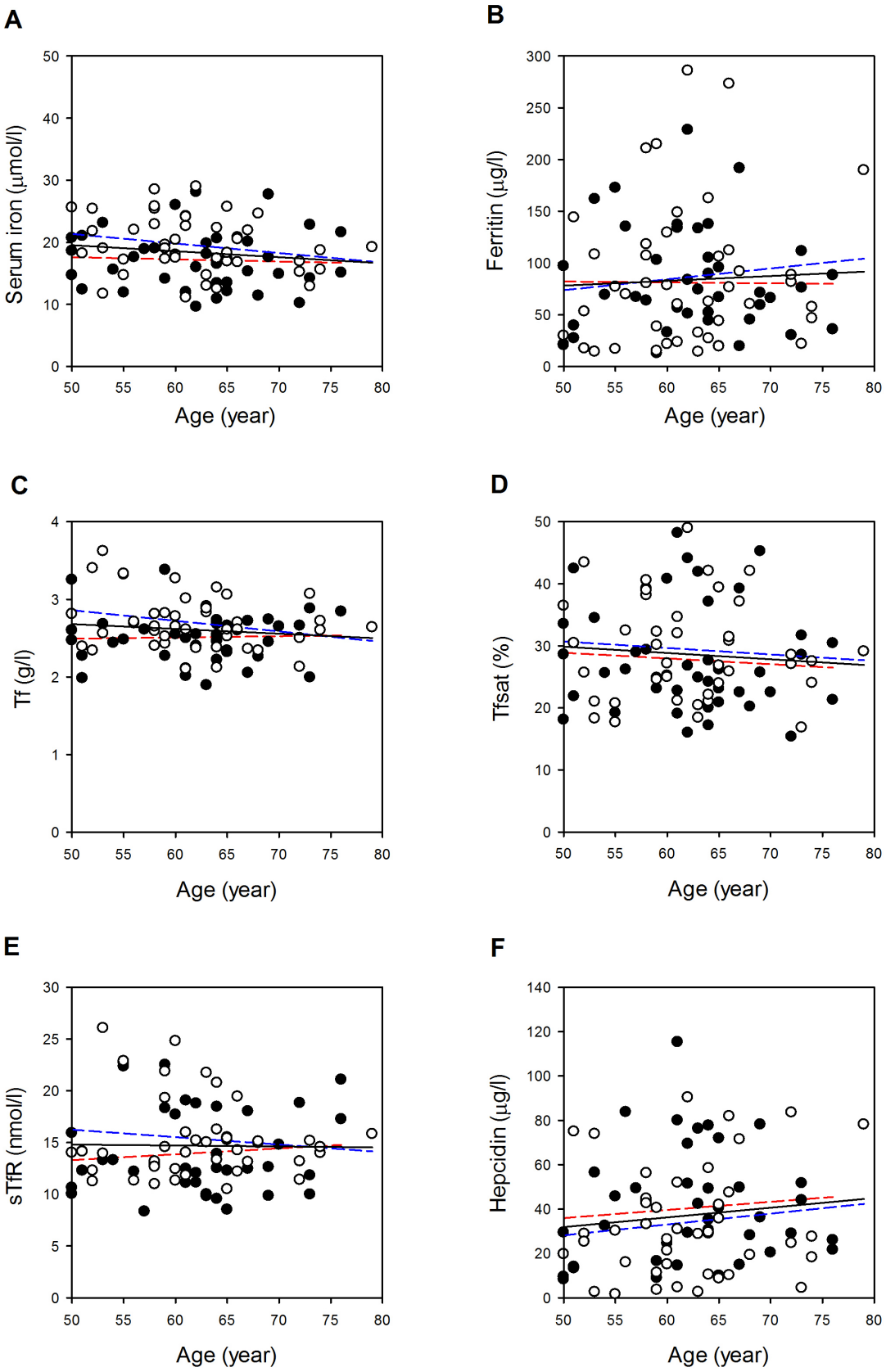

Figure. 3.3 **Relationships between iron status indicators and age.** Scatter graphs show the correlations between age (horizontal axis) and iron status indicators (vertical axes). **A**. Serum iron concentration; **B**. Ferritin concentration; **C**. Transferrin (Tf) concentration; **D**. Transferrin saturation (Tfsat); **E**. Soluble transferrin receptor (sTfR) concentration; **F**. Hepcidin concentration. Data from females (*filled circles*) and males (*empty circles*) are shown together. Trends with age are shown as regression lines, female (*red dashed*), male (*blue dashed*) and overall (*black solid*).

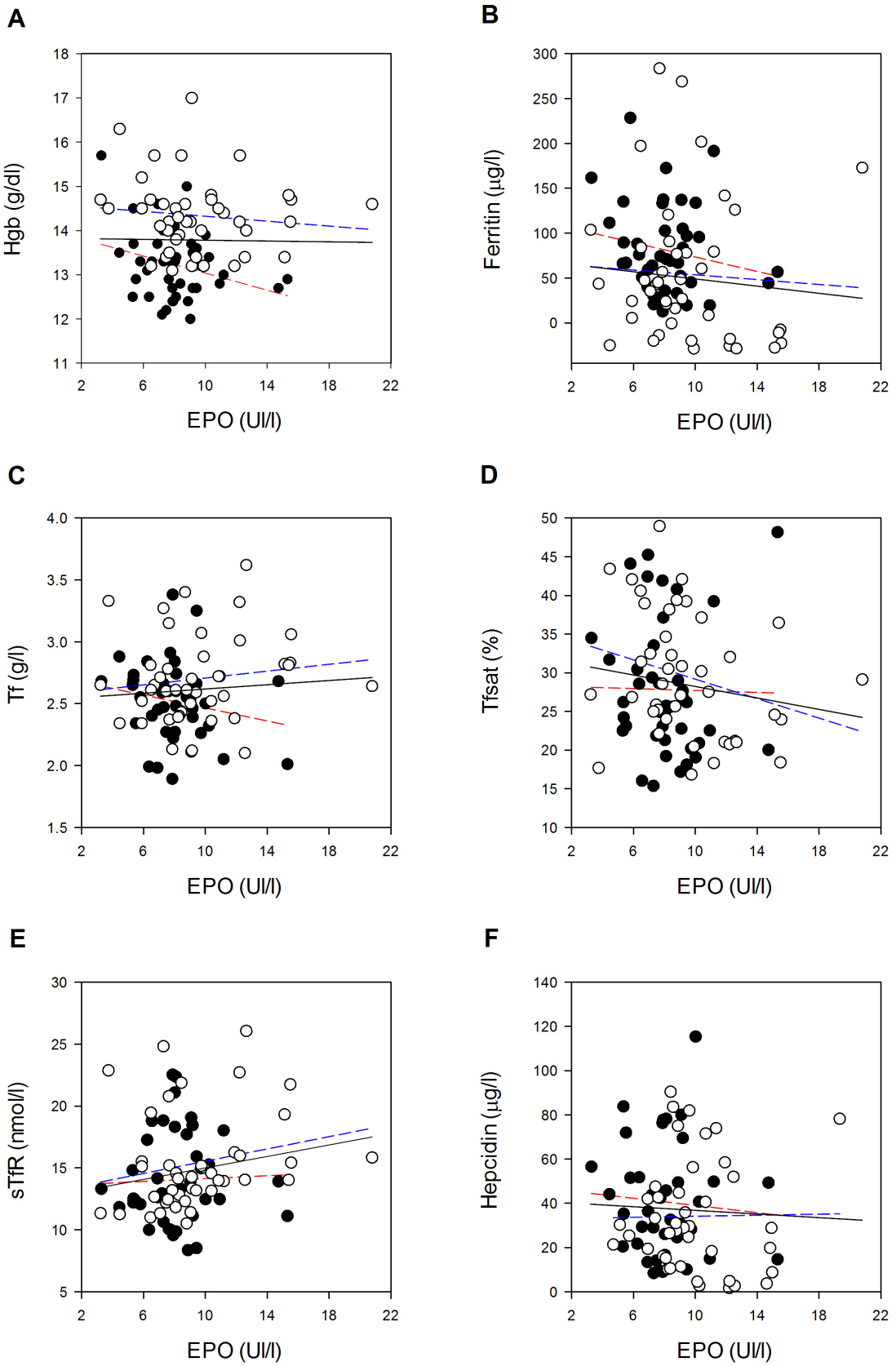

Figure. 3.4 **Relationships between erythropoietin (EPO) and selected variables.** Scatter graphs show the correlations between erythropoietin (horizontal axis) and selected variables (vertical axes). **A**. Haemoglobin (Hgb) concentration; **B**. Ferritin concentration; **C**. Transferrin (Tf) concentration; **D**. Transferrin saturation (Tfsat); **E**. Soluble transferrin receptor (sTfR) concentration; **F**. Hepcidin concentration. Data from females (*filled circles*) and males (*empty circles*) are shown together. Trends with erythropoietin are shown as regression lines, female (*red dashed*), male (*blue dashed*) and overall (*black solid*).

3.4 Discussion

In this study, we observed that healthy male and female participants aged between 50 and 80 years only showed significant differences in Hgb concentrations, Hct, serum iron and Tf concentrations. In addition, ageing did not alter haematological variables or iron status indicators significantly in the studied group. Finally, EPO concentration was negatively correlated to Tf saturation and positively correlated to sTfR concentration in male participants, but no correlation was found between EPO concentration and other variables in this group or in female participants.

The study results showed no significant correlations between the age and studied variables, such as Hgb, EPO and hepcidin. The results of Hgb in relation to age in this study contradicted other previous studies (Ershler et al., 2005; Zakai et al., 2013). For example, regression analysis showed insignificant rates of Hgb decline with age in our patients. The figures for female and male participants in our study were 0.0014 ($p = 0.936$) and 0.0159 ($p = 0.400$) g/dl per year respectively. Compared with the rate of 0.06 g/dl per year seen by Ershler *et al.* (Ershler et al., 2005), age showed no influence on the Hgb concentration in our participants. The contradiction could result from the small sample size of the participants in the study, which contributed to the low statistical power of the analysis. For example, the powers of the regression analysis were 3% and 13% for female and male participants respectively, compared with the desired power of 80% in most studies (based on two-tail; alpha level = 0.05). These suggest the need for a greater number of participants to detect significance.

The absence of trend was also observed when the EPO concentrations were compared with a younger healthy group (mean age = 36.7 ± 13.2 years; internal commu-

nication with Dr Frise). This could be result of the profound individual variations in EPO concentrations or small changes in EPO concentration with age. However, another concern is measurement variation in the ELISA kits (Bechensteen et al., 1993; Marsden, 2006) since the concentrations of EPO measured in this study were lower than other studies, despite the fact that the kit was calibrated against the Second International Reference Preparation (67/343). Another unexpected result was the observation of a non-significant change in the hepcidin concentration with age. Our results are contrast with Galesloot *et al.*, who demonstrated that hepcidin increases with age from onset of adulthood (Galesloot et al., 2011). The cause for this contrast with some previous studies is not clear, but it could the result of a small sample size, relatively good fitness and health among enrolled participants or measurement variations, since hepcidin measurement has yet to be fully established and standardised (Kroot et al., 2012; Macdougall et al., 2010).

Interestingly, our study results showed similar ferritin levels in male and female participants in contast to other studies. Zacharski and co-workers observed that ferritin levels in males are higher than those of females in an age group corresponding to this study from the National Health and Nutrition Examination Survey (NHNES III) (Zacharski et al., 2000). Findings from Custer and colleagues also showed a difference in ferritin levels between males and females (Custer et al., 1995). Since inflammation (Birgegard et al., 1978; Elin et al., 1977), anthropometry (Lecube et al., 2008), genetic factors (Beutler et al., 2000; Burt et al., 1998; Datz et al., 1998) and race (Popkin et al., 1996; Zacharski et al., 2000) have been shown to impact variations in ferritin levels in the population, the similar ferritin levels found in males and females

in this study may be the result of selective participant enrollment criteria.

EPO concentration was significantly correlated to Tfsat and sTfR in male participants, but not in female participants. The significant positive correlation between sTfR and EPO has been found in animal (R'zik et al., 2001) and human studies (Soininen et al., 2010). Since erythropoiesis demands iron, it may explain why Tfsat is negatively correlated with EPO concentration. However, it is unclear why there was a gender difference in the results. This could have been the result of the intrinsic difference in EPO potency or microvasculature regulation between the sexes. For example, Ifudu and colleagues found that female haemodialysis patients require a higher dose of recombinant EPO to reach the equivalent Hct of males regardless of the disease status or intravenous iron dose (Ifudu et al., 2001). This suggests that in females, erythropoiesis may be less sensitive to the level of EPO. It would be interesting to measure EPO receptor concentrations in both groups to further identify the underlying mechanism. On the other hand, Murphy *et al.* suggested that EPO stimulation in females is affected by oestrogen, which dilates the renal microvasculature and increases tissue oxygenation in spite of a lower Hgb level (Murphy, 2014). Although our studied group consists of postmenopausal women and similarly aged men, it is hard to rule out the involvement of hormone in erythropoiesis without actual hormone measurements. Therefore, it requires further studies to clarify the gender differences in erythropoiesis and iron homeostatsis.

Surprisingly, we did not find any correlation between EPO and Hgb concentrations in the studied group. The correlation between EPO and Hgb concentrations has been demonstrated in anaemic patients (Adamson, 1968) and renal failure patients (Fehr

et al., 2004). However, no significant correlation was found in this study. The reason for this may be the result of only including healthy volunteers in this study, since plasma EPO concentration has been shown to remain relatively stable in the normal range of Hgb concentration. Furthermore, EPO concentration has shown to increase logarithmically only when the Hgb concentration falls below 12 g/dl (Hillman et al., 2011).

In conclusion, we have demonstrated that EPO concentration and iron status indicators remain steady in healthy humans aged between 50 and 80 years. The observed association between EPO concentration and sTfR concentrations in healthy male participants implies a tight link between erythropoiesis and iron status in this group. However, these correlations were not seen in healthy female participants, implying an underlying difference between the sexes in erythropoiesis and iron homeostasis regulation, even at postmenopausal age. The extent of gender modulation in erythropoiesis and iron homeostasis regulation needs to be investigated and addressed in future studies.

Chapter 4

Iron status and exercise capacity in healthy aged individuals

4.1 Introduction

The 'Iron Hypothesis' proposed by Sullivan and colleagues (Sullivan, 1981) put forward the idea that mild iron depletion in humans protects against cardiovascular diseases, such as ischemic heart disease (Salonen et al., 1998), myocardial infarction (Holay et al., 2012; Klipstein-Grobusch et al., 1999; MacDonald, 1993; Magnusson et al., 1994; Tuomainen et al., 1998), atherosclerosis (Kiechl et al., 1994, 1997; Syrovatka et al., 2011; Wolff et al., 2004) and peripheral arterial disease (Depalma et al., 2010; Zacharski et al., 2007, 2011). While the underlying mechanism is under investigation, it may be linked to the role of iron in redox-mediated injury in myocyte and vascular walls (Sullivan, 2005; Zheng et al., 2005). However, a confounding factor in this hypothesis is that physical activity, such as exercise, also protects against cardiovascular

diseases and impacts iron status. Depletion of iron stores is commonly seen in athletes involved in intensive training (Ehn et al., 1980). Furthermore, temporary increases in hepcidin and ferritin levels have been observed immediately after running exercise in young runners and triathletes (Schumacher et al., 2002). In elderly males after resistance training altered iron status, such as decreased total iron binding capacity and increased Tfsat, was observed (Murray-Kolb et al., 2001). As physical activity can affect indices of iron status, this raises the possibility of a correlation between iron status and physical fitness in older individuals. Therefore, the main aim of this study is to investigate the relationship between iron status and physical fitness, measured by $\dot{V}_{O_2}$, in healthy individuals aged between 50 and 80 years.

4.2 Methods

4.2.1 Participants and experimental protocol

The participants were the same as described in Chapter 3. The experimental protocol was the same as described in Chapter 3. Each subject gave details of his/her brief medical history and lifestyle before the following procedures were carried out in sequence: 1) blood samples, 2) echocardiographic measurements and 3) incremental exercise tests.

4.2.2 Incremental exercise test

The exercise was performed on a bicycle ergometer (KEM-3, CardioKinetics Limited, UK). A customised metabolic measurement system was used to determine $\dot{V}_{O_2Peak}$.

Further details of the metabolic measurement system can be found in Chapter 2. In short, participants breathed through mouthpieces with noses occluded. The mouthpieces were connected via a sampling catheter to an air mass spectrometer (3000, Airspec Ltd., UK) to constantly measure the composition of the gas exchanging with participants. Ventilation was measured by a turbine volume device (Cardiokinetics, UK) and a pneumotach (Fleisch, Switzerland). The values of gas composition and ventilation were recorded by a customised software, *BreathM* and used to calculate $\dot{V}_{O_2}$ offline by a customized software, *Exhale*.

The exercise protocol was adapted from Rossiter *et al.* (Rossiter et al., 2006). Each participant performed a standard incremental exercise test until volitional exhaustion, with the work rate rising incrementally from 0 W by 15 W per minute. Then after 5 minutes of pedalling at 0 W, participants cycled against 105% of their maximum work rate to the point of exhaustion. The work rate was determined from the preceding standard incremental exercise test. Exhaustion was determined by participants stopping or by the cadence going below 60 rpm. The $\dot{V}_{O_2Peak}$ was determined as the average of the maximum $\dot{V}_{O_2}$ throughout the exercise test over a 30-second interval. If the maximum $\dot{V}_{O_2}$ values between the two exhaustion values were different, the higher $\dot{V}_{O_2}$ value was chosen as the participant's $\dot{V}_{O_2Peak}$. Baseline O_2 consumption and CO_2 elimination were taken as averages from measurements over the second minute on the ergometer before pedalling. $S_{a}O_2$ was measured using a pulse oximeter (Ohmeda Biox 3740 Pulse Oximeter, BOC Healthcare) with a finger probe (OXY-F4-H finger sensor, GE Healthcare). Heart rate was measured using a three-way electrocardiogram (Micromon 7142B ECG, Kontron Medical). All equipment was calibrated before each

experiment.

4.2.3 The evaluation of exercise performance

The evaluation of exercise performance was based on the difference ($D_{\dot{V}_{O_2Peak}}$) between measured $\dot{V}_{O_2Peak}$ and predicted $\dot{V}_{O_2Peak}$ values ($D_{\dot{V}_{O_2Peak}}$ = measured $\dot{V}_{O_2Peak}$ − predicted $\dot{V}_{O_2Peak}$). The predicted $\dot{V}_{O_2Peak}$ values were calculated according to regression formulas proposed in meta-analyses by Fitzgerald *et al*. (for women; Table 4.1) (Fitzgerald et al., 1997) and by Wilson *et al*. (for men; Table 4.2) (Wilson and Tanaka, 2000). The formulae used participants' ages to estimate the $\dot{V}_{O_2Peak}$ in the following three arbitrary groups in both sexes: 1) endurance-trained; 2) active; 3) sedentary. The endurance-trained group refers to participants who performed vigorous endurance exercise (e.g. running and cycling) $\geq$ 3 times/week for > 1 year. The active group refers to participants who did occasional or irregular aerobic exercise (e.g. walking and dancing) $\leq$ 2 times/week. The sedentary group refers to participants who did no aerobic exercise. Group assignment was carried out according to participants' self-reports and lifestyles given on their arrival at the laboratory. Participants were asked about activities (e.g. walking, cycling, running, gym, etc) undertaken in a typical week, as well as their duration, frequency and intensity.

Female	
Endurance-trained	$\dot{V}_{O_2Peak} = 72.41 - 0.62 \times \text{Age}$
Active	$\dot{V}_{O_2Peak} = 54.02 - 0.44 \times \text{Age}$
Sedentary	$\dot{V}_{O_2Peak} = 43.82 - 0.35 \times \text{Age}$

Table 4.1 **Formulae used for the estimation of $\dot{V}_{O_2Peak}$ (ml/min/kg) in female participants by age (year) in different activity groups.**

Male	
Endurance-trained	$\dot{V}_{O_2Peak} = 77.2 - 0.46 \times \text{Age}$
Active	$\dot{V}_{O_2Peak} = 61.4 - 0.39 \times \text{Age}$
Sedentary	$\dot{V}_{O_2Peak} = 54.2 - 0.40 \times \text{Age}$

Table 4.2 **Formulae used for the estimation of $\dot{V}_{O_2Peak}$ (ml/min/kg) in male participants by age (year) in different activity groups.**

4.2.4 Statistical analysis

Participant characteristics are presented as the mean ± SD for normally distributed data or as the median (interquartile range) for non-normally distributed data. Distribution of data were examined using the Shapiro-Wilk test. Non-normally distributed data were compared by using the Mann-Whitney U test. Normally distributed data were compared using a two-tailed Student's *t*-test. Different iron indicators, including ferritin concentration, Tf concentration, Tfsat and sTfR concentration, were chosen to represent a participant's iron status to outweigh the disadvantages of using a single iron indicator, which may be affected by other signalling pathways. In this case, multiple linear regression was chosen since it allows one to evaluate a relationship between the interested variable $\dot{V}_{O_2Peak}$ (dependent) and a cluster of variables, iron status in-

dicators (independents), as well as other possible confounding variables, such as age, height and Hgb concentration (forced entry of independents) to minimise the bias that would occur if one performed simple regression or correlation analysis. The same analysis was performed to explore the relationships between $D_{\dot{V}_{O_2Peak}}$ (dependent) and iron status indicators (independents). Ferritin concentration was logarithmically transformed before performing multiple regression to prevent violating the assumption of multiple regression (normal distribution of errors) in the small simple size. Data were analysed using computer software (SPSS, Version 20, IBM).

4.3 Results

4.3.1 Basic characteristics on exercise performance

The participants were either sedentary, or recreational joggers, or cyclists. None were ex-elite or competitive athletes.

Participants with blood disorders as described in Chapter 3, and participants who did not reach peak exercise levels (n = 3) were excluded. These participants (three females; aged 65, 67 and 67 years respectively) had a low maximum heart rate ($\leq$ 130 bpm) during both exhaustion periods in the exercise test.

Multiple regression was carried out on the basic characteristics of the 80 remaining participants to assess the performance in their exercise tests (Table 4.3). For the group as a whole, sex ($\beta = 0.517$, $p < 0.001$) was found to be the main factor affecting exercise performance. Age also had a significant effect (β = –0.242, p = 0.008). Therefore, results are presented by sex.

	B	**Std. Error**	β	**p Value**
Sex	0.882	0.241	0.517	< 0.001
Age	−0.030	0.011	−0.242	0.008
Height	0.012	0.014	0.110	0.419
Weight	−0.009	0.009	−0.112	0.318
Hgb	0.092	0.092	0.109	0.318
Ferritin	−0.091	0.104	−0.082	0.387
Tf	−0.117	0.274	−0.048	0.672
Tfsat	0.017	0.011	0.168	0.109
sTfR	0.028	0.021	0.129	0.188

Table 4.3 **Results of multiple linear regression analysis with $\dot{V}_{O_2Peak}$ (l/min) as the dependent in the studied group.** B: unstandardised coefficients; Std. Error: standard mean of error; β: standardised coefficients

4.3.2 Decline in $\dot{V}_{O_2Peak}$

In male participants, $\dot{V}_{O_2Peak}$ showed a significant decline with age (β = –0.052, p = 0.005) (Figure 4.1 A). However, the decline was not significant in female participants (p = 0.090). Similar results were shown when $\dot{V}_{O_2Peak}$ values were adjusted by body weight. In male participants, $\dot{V}_{O_2Peak}$ showed a significant decline with age (β = –0.608, p = 0.024) (Figure 4.1 B) but not in female participants (p = 0.272).

4.3.3 Basic characteristics

The basic characteristics and haematological results did not show many differences between male and female participants, besides height (p < 0.001), weight (p < 0.001), Hgb concentration (p < 0.001) and Tf concentration (p = 0.017) (Table 4.4). Significant differences were also found in the performance of exercise tests. For example, male participants had a higher baseline $\dot{V}_{O_2}$ (p = 0.002) and ventilation (p < 0.001) than female participants. In addition, male participants performed significantly better than female participants in $\dot{V}_{O_2Peak}$ (p < 0.001), peak ventilation (p < 0.001) and peak work

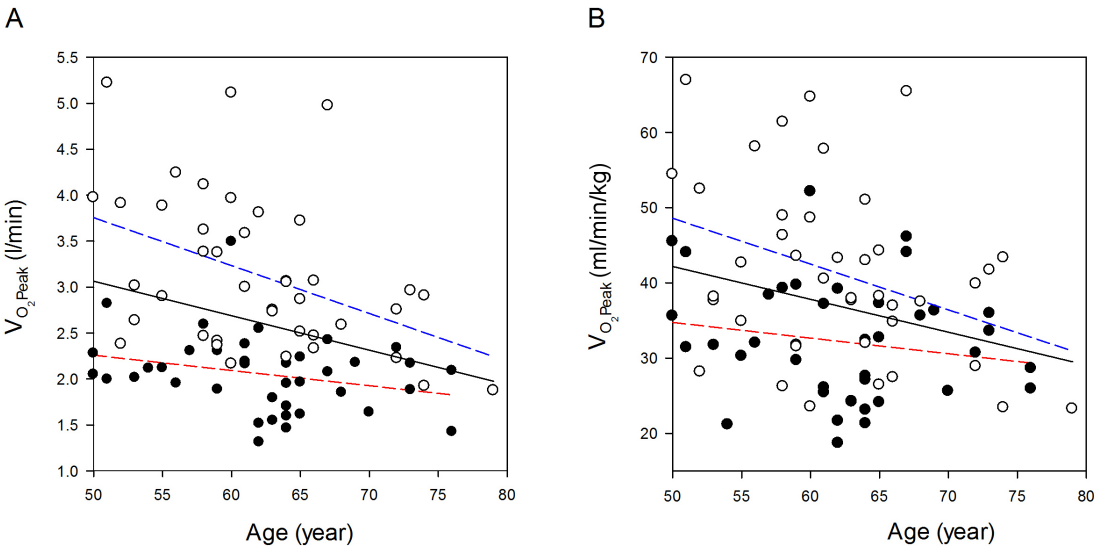

Figure. 4.1 **Relationship between $\dot{V}_{O_2Peak}$ and age by sex.** The scatter graph shows the correlation between age (*horizontal axis*) and $\dot{V}_{O_2Peak}$ (*vertical axis*) in l/min (*graph **A***) and ml/min/kg (*graph **B***). Data from females (*filled circles*) and males (*empty circles*) are presented in the same graph. Trends with age are shown as regression lines, female (*red dashed*) and male (*blue dashed*).

Regression lines:

Graph A

Female $\dot{V}_{O_2Peak}$ (l/min) = 3.088 – 0.017 × Age (year), R^2 = 0.076, p = 0.090

Male $\dot{V}_{O_2Peak}$ (l/min) = 6.364 – 0.052 × Age (year), R^2 = 0.184, p = 0.005

Graph B

Female $\dot{V}_{O_2Peak}$ (ml/min/kg) = 45.157 – 0.208 × Age (year), R^2 = 0.033, p = 0.272

Male $\dot{V}_{O_2Peak}$ (ml/min/kg) = 79.002 – 0.608 × Age (year), R^2 = 0.125, p = 0.024

rate ($p < 0.001$).

4.3.4 Relation between iron status indicators and exercise performance by sex

The $\dot{V}_{O_2Peak}$ of female participants was correlated with height ($\beta = –0.467$, $p = 0.004$), CRP ($\beta = –0.336$, $p = 0.020$), and ferritin ($\beta = –0.388$, $p = 0.008$) (Table 4.5). In male participants, age ($\beta = –0.394$, $p = 0.021$) and CRP ($\beta = –0.425$, $p = 0.015$) were the factors correlated with $\dot{V}_{O_2Peak}$ (Table 4.6).

In order to show the influence of other independents in the regression model, the relationships between $\dot{V}_{O_2Peak}$ and significant independents are shown in Figure 4.2 by using partial regression plots.

4.3.5 Relation between iron status indicators and $D_{\dot{V}_{O_2Peak}}$ by sex

The $D_{\dot{V}_{O_2Peak}}$ of female participants was correlated with height ($\beta = –0.440$, $p = 0.007$), CRP ($\beta = –0.395$, $p = 0.007$), and ferritin ($\beta = –0.440$, $p = 0.003$) (Table 4.7). In male participants, CRP ($\beta = –0.507$, $p = 0.006$) was the only factor correlated with $D_{\dot{V}_{O_2Peak}}$ (Table 4.8). The partial regression plots for the significant independent variables in the anaylsis are shown in Figure 4.3.

	Male	**Female**	**p Value**
Number, n	41	39	
Age, yr	62.0 ± 6.9	62.4 ± 6.0	0.767
Height, cm	177.2 ± 5.1	166.3 ± 6.7	< 0.001
Weight, kg	76.8 ± 8.4	65.3 ± 10.5	< 0.001
BMI, kg/m^2	24.5 ± 2.6	23.6 ± 3.2	0.158
FEV_1, predicted %	114.0 ± 16.3	111.4 ± 18.8	0.519
FVC, predicted %	113.4 ± 17.3	113.8 ± 19.1	0.933
FEV_1/FVC	75.0 (69.0-81.5)	79.0 (74.5-82.0)	0.096
SBP, mmHg	132.0 (120.0-143.5)	122.0 (115.0-136.0)	0.066
DBP, mmHg	82.0 (78.0-87.5)	80.0 (69.0-86.0)	0.122
Hgb, g/dl	14.3 (13.9-14.7)	13.2 (12.7-13.7)	< 0.001
CRP, mg/l	0.8 (0.3-1.9)	0.8 (0.5-1.2)	0.197
Serum ferritin, µg/l	69.8 (28.4-110.2)	69.1 (44.3-111.5)	0.641
Tf, g/l	2.64 (2.47-2.86)	2.50 (2.27-2.68)	0.017
Tfsat, %	28.5 (24.0-36.8)	25.6 (21.3-33.5)	0.225
sTfR, nmol/l	14.3 (12.5-16.1)	13.3 (11.8-17.7)	0.138
Baseline $\dot{V}_{O_2}$, ml/min	335 (275-391)	289 (248-325)	0.002
Baseline ventilation, l/min	11.9 (11.1-14.8)	9.6 (8.6-11.6)	< 0.001
Baseline heart rate, bpm	64 (56-72)	64 (60-71)	0.359
Peak $\dot{V}_{O_2}$, l/min	2.96 (2.47-3.76)	2.07 (1.79-2.28)	< 0.001
Peak $\dot{V}_{O_2}$, ml/min/kg	39.9 (31.8-48.8)	31.7 (25.6-37.3)	< 0.001
Predicted $\dot{V}_{O_2Peak}$, ml/min/kg	35.7 (33.3-40.7)	27.2 (26.1-30.8)	< 0.001
$D_{\dot{V}_{O_2Peak}}$, ml/min/kg	3.56 ± 14.66	4.09 ± 7.18	0.655
Peak ventilation, l/min	110.9 ± 31.6	70.0 ± 16.3	< 0.001
Peak heart rate, bpm	158.8 ± 12.6	153.2 ± 13.7	0.062
Peak work rate, W	207.7 ± 51.0	136.3 ± 25.5	< 0.001

Table 4.4 **Participant characteristics.** Values are shown as the mean ± SD or median (interquartile). BMI: body mass index; FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; SBP: systolic blood pressure; DBP: diastolic blood pressure; Hgb: haemoglobin; CRP: C-reactive protein; Tf: transferrin; Tfsat: transferrin saturation; sTfR: soluble transferrin receptor; $D_{\dot{V}_{O_2Peak}}$: the difference between measure $\dot{V}_{O_2Peak}$ and predicted $\dot{V}_{O_2Peak}$ values.

	B	**Std. Error**	β	**p Value**
Age	−0.308	0.166	−0.267	0.073
Height	−0.555	0.181	−0.467	0.004
Hgb	1.162	1.407	0.118	0.415
CRP	−2.078	0.848	−0.336	0.020
Ferritin	−4.456	1.573	−0.388	0.008
Tf	−5.548	3.844	−0.227	0.159
Tfsat	0.240	0.142	0.226	0.101
sTfR	0.309	0.304	−0.388	0.080

Table 4.5 **Results of multiple linear regression analysis with $\dot{V}_{O_2Peak}$ (ml/min/kg) as the dependent in female participants.** B: unstandardised coefficients; Std. Error: standard mean of error; β: standardised coefficients

	B	**Std. Error**	β	**p Value**
Age	−0.017	0.007	−0.394	0.021
Height	−0.003	0.009	−0.047	0.769
Hgb	−0.002	0.055	−0.005	0.974
CRP	−0.068	0.026	−0.425	0.015
Ferritin	−0.068	0.060	−0.202	0.263
Tf	−0.308	0.195	−0.368	0.124
Tfsat	0.003	0.007	−0.079	0.706
sTfR	−0.003	0.014	−0.037	0.850

Table 4.6 **Results of multiple linear regression analysis with $\dot{V}_{O_2Peak}$ (ml/min/kg) as the dependent in male participants.** B: unstandardised coefficients; Std. Error: standard mean of error; β: standardised coefficients

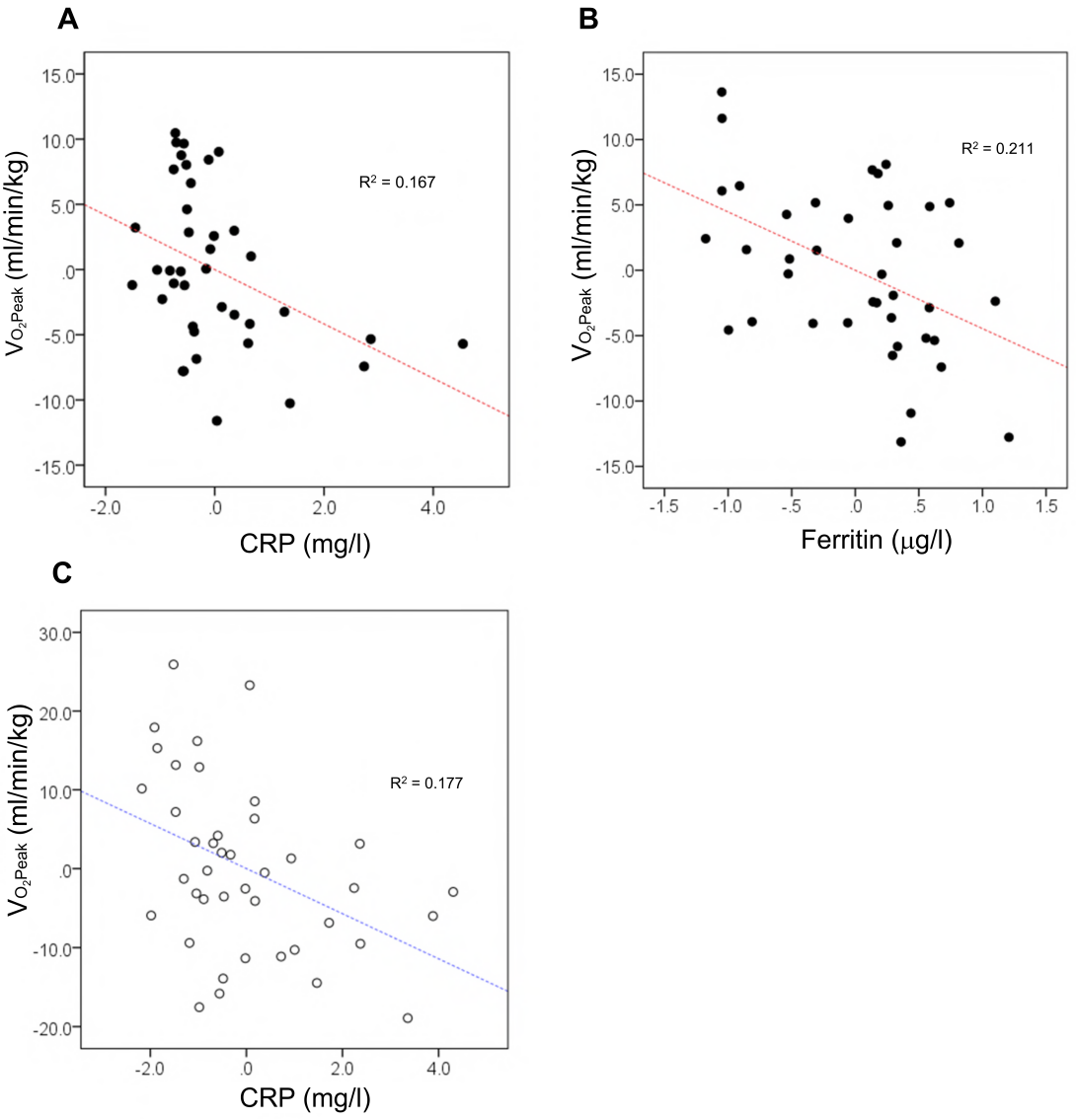

Figure. 4.2 **Partial regression plots of $\dot{V}_{O_2Peak}$ in relation to signficant independents in studied participants.** The partial regression plots show the correlation between $\dot{V}_{O_2Peak}$ (*vertical axis*) and significant independents (*horizontal axis*) found in the multiple regression analysis in the study. **A.** CRP in female participant (*filled circles*); **B.** ferritin in female participants (*filled circles*); **C.** CRP in male participants (*empty circles*). Trends with $\dot{V}_{O_2Peak}$ and independents are shown as regression lines, female (*red dashed*) and male (*blue dashed*). Numbers on axes are not absolute values of variables since the vertical axis represents the residuals from regressing the dependent on all independents, except the specific significant independent. The horizontal axis represents the residuals from regressing the specific significant dependent on all other independents.

	B	**Std. Error**	β	**p Value**
Age	−0.036	0.150	−0.035	0.811
Height	−0.473	0.163	−0.440	0.007
Hgb	1.220	1.272	0.137	0.345
CRP	−2.206	0.767	−0.395	0.007
Ferritin	−4.570	1.422	−0.440	0.003
Tf	−4.527	3.475	−0.205	0.203
Tfsat	0.149	0.128	0.183	0.256
sTfR	0.171	0.275	−0.090	0.537

Table 4.7 **Results of multiple linear regression analysis with $\mathbf{D_{\dot{V}_{O_2Peak}}}$ (ml/min/kg) as the dependent in female participants.** B: unstandardised coefficients; Std. Error: standard mean of error; β: standardised coefficients

	B	**Std. Error**	β	**p Value**
Age	−0.271	0.361	−0.128	0.458
Height	−0.318	0.483	−0.110	0.515
Hgb	0.591	2.884	0.035	0.839
CRP	−4.110	1.396	−0.507	0.006
Ferritin	−3.020	3.169	−0.177	0.348
Tf	−14.130	10.299	−0.334	0.180
Tfsat	−0.120	0.391	−0.066	0.761
sTfR	−0.223	0.731	−0.061	0.762

Table 4.8 **Results of multiple linear regression analysis with $\mathbf{D_{\dot{V}_{O_2Peak}}}$ (ml/min/kg) as the dependent in male participants.** B: unstandardised coefficients; Std. Error: standard mean of error; β: standardised coefficients

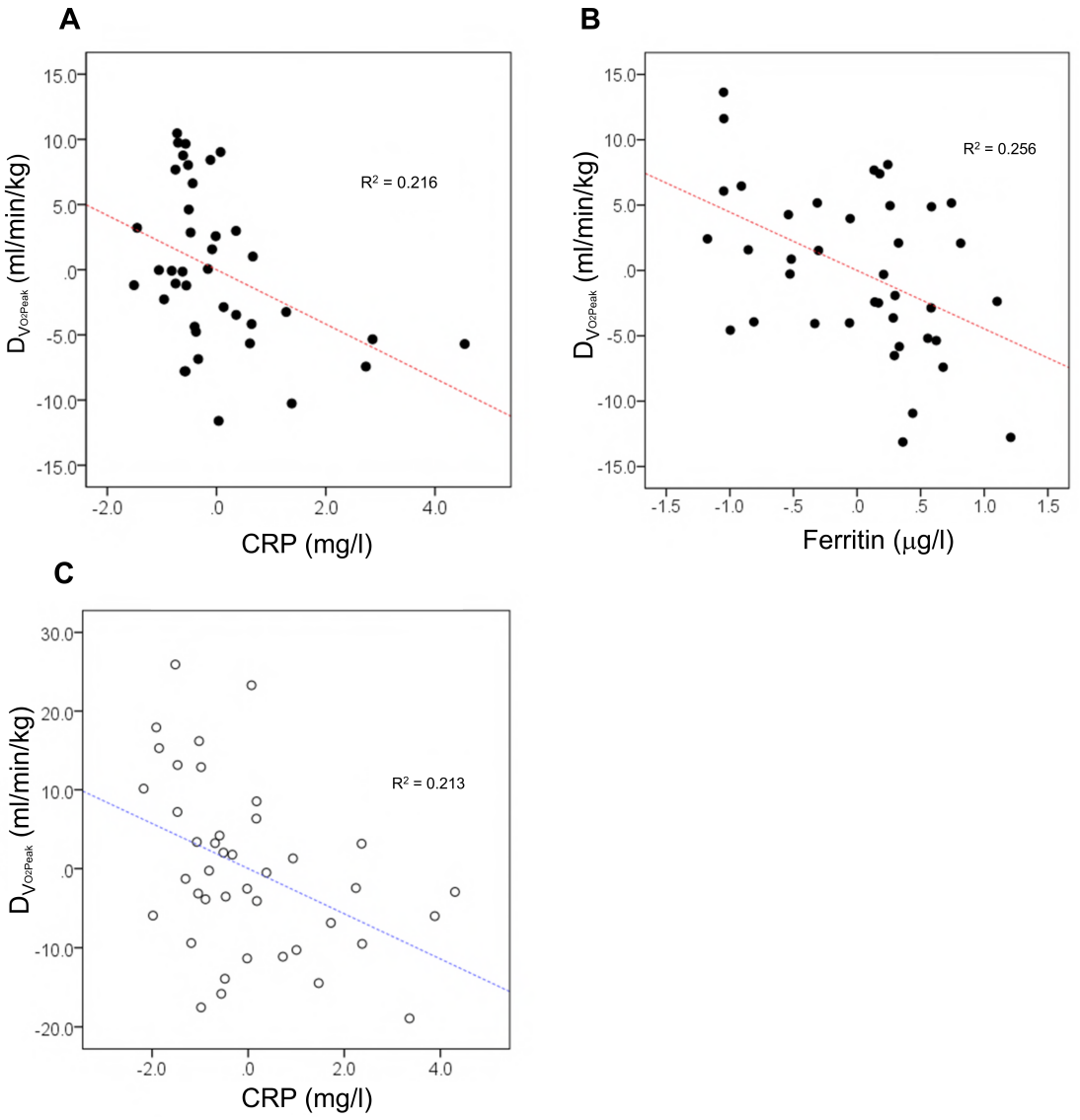

Figure. 4.3 **Partial regression $D_{\dot{V}_{O_2Peak}}$ in relation to signficant independents in studied participants.** The partial regression plots show the correlation between $D_{\dot{V}_{O_2Peak}}$ (*vertical axis*) and significant independents (*horizontal axis*) found in the multiple regression analysis in studied participants. **A.** CRP in female participant (*filled circles*); **B.** ferritin in female participants (*filled circles*); **C.** CRP in male participants (*empty circles*). Trends with $D_{\dot{V}_{O_2Peak}}$ and indpendents are shown as regression lines, female (*red dashed*) and male (*blue dashed*). Numbers on axes are not absolute values of variables since the vertical axis represents the residuals from regressing the dependent on all independents, except the specific significant independent. The horizontal axis represents the residuals from regressing the specific significant dependent on all other independents.

4.4 Discussion

In this study we observed that, in healthy participants aged between 50 and 80 years, sex and age are the main factors that determine performance in incremental exercise tests. Male participants had a significantly higher exercise performance, measured by work rate and $\dot{V}_{O_2}$, than female participants. However, the decline in the $\dot{V}_{O_2Peak}$ with age was more significant and profound in male participants, compared with female participants. Finally, results from multiple regression showed that $\dot{V}_{O_2Peak}$ correlated inversely with ferritin and CRP in female participants. In male participants, CRP was the only factor correlated inversely with $\dot{V}_{O_2Peak}$. These results were confirmed by treating $D_{\dot{V}_{O_2Peak}}$ as the dependent variable in the same regression analyses.

Together with having higher performance in exercise tests than female participants, male participants showed a substantially greater rate of decline in $\dot{V}_{O_2Peak}$ with age. These results are consistent with other studies. For example, Fleg and Lakatta found the rate of decline in $\dot{V}_{O_2Max}$ was –0.39 ml/min/kg per year in men and –0.25 ml/min/kg per year in women using treadmill exercise tests in 184 participants aged between 22 and 87 years (Fleg and Lakatta, 1988). Similarly, results from Posner *et al.* supported these findings with –0.44 ml/min/kg per year in men and –0.36 ml/min/kg per year in women using cycling exercise in healthy participants aged between 20 and 89 years (Posner et al., 1987). However, it is not clear why the difference in the rate of decline between males (–0.608 ml/min/kg per year) and females (–0.208 ml/min/kg per year) in our results would be more profound, compared with other studies (Paterson et al., 1999; Toth et al., 1994). It could result from selection bias as there were some active men in their early 50s in this study or it could be due to the small sample

size as the decline with age in our female participants was not found to be significant.

This study included multiple linear regression analyses to investigate the correlation between iron status parameters and $\dot{V}_{O_2Peak}$. Ferritin was found to be negatively correlated to $\dot{V}_{O_2Peak}$ in female participants but not in male participants. The underlying reason for this is not clear. In addition, from our understanding, this is the first report showing the correlation between iron status and exercise performance in iron-replete subjects. This finding supports the idea of the 'Iron Hypothesis' and other studies showing the association between iron status and risk of cardiovascular diseases. The underlying mechanism of such has been suggested to be linked to vascular function. Zheng and colleagues demonstrated that low ferritin levels are correlated with better vascular function, measured by flow-mediated dilation in the brachial artery (Zheng et al., 2005). They examined voluntary blood donors aged from 50 to 75 years with the same Hcts but different frequencies of donation. High-frequency blood donors with lower serum ferritin concentrations were found to have increased flow-mediated dilation, compared to low-frequency blood donors. Furthermore, they suggested that the underlying mechanism lies with reduced oxidase stress by measurement of a marker, 3-nitrotyrosine.

CRP was found to be negatively correlated with $\dot{V}_{O_2Peak}$ (ml/min/kg) in both male and female participants. This significant finding is consistent with other studies. For example, Taaffe and colleagues found that people aged 70 to 79 years with active lifestyle had lower CRP level and IL-6 than sedentary people (Taaffe et al., 2000). A large cohort study investigating 5,888 men and women above 65 years also found that participants in the highest physical activity group had significantly lower CRP levels

than those in the lowest physical activity group (Geffken et al., 2001). Elevated CRP levels were also found to be tightly linked with decreased insulin sensitivity and decreased endothelin function (Buffiere et al., 2015; Yudkin et al., 1999). Conversely, exercise training and physical activities have been found to reduce CRP levels through attenuated IL-6 and IL-1β expression (Gielen et al., 2003). Interestingly, both ferritin and CRP productions are affected by similar mechanisms and cytokines, such as IL-6 (Northrop-Clewes, 2008). In addition, higher physical activity in leisure time was also associated with lower iron stores, measured by ferritin (Lakka et al., 1994). Therefore, an interesting question is whether they are confounding results caused by the same mechanism or whether there is a gender specific modification in the cytokine stimulation through physical activity.

Besides applying direct regression analyses on the relation between iron status indicators and V_{O_2Peak}, this study also performed the same regression analysis using $D_{\dot{V}_{O_2Peak}}$ to examine the relation. As $D_{\dot{V}_{O_2Peak}}$ was calculated by finding the difference between the measured $\dot{V}_{O_2Peak}$ with the predicted $\dot{V}_{O_2Peak}$, the higher values in $D_{\dot{V}_{O_2Peak}}$ suggested that participants had better exercise performance than the predicted values. In the study, the factors that were found to be correlated with $D_{\dot{V}_{O_2Peak}}$ were the same as those found in the regression analysis using V_{O_2Peak} as the dependent. These reinforce the finding that these factors, ferritin (female participants) and CRP (both male and female participants), indeed affect $\dot{V}_{O_2Peak}$ in our participants.

Unexpectedly, Hgb levels were not found to affect $\dot{V}_{O_2Peak}$ in the studied group. Hgb is the main oxygen carrier in the blood, given the low solubility of oxygen in blood plasma. Each gram of Hgb has a capacity of 1.34 ml of oxygen, compared with

0.3 mL for every 100 ml plasma (Mairbaurl, 2013). In addition, in the normal range of Hgb levels, $\dot{V}_{O_2peak}$ increases by approximately 1% for every 0.3 g/dl Hgb (Otto et al., 2013), suggesting the high correlation between Hgb levels and $\dot{V}_{O_2Peak}$. Therefore, Hgb levels should be able to predict $\dot{V}_{O_2Peak}$. The reason why a weak correlation was found in this study could be a result of changes in participant blood volumes. This could be overcome using the measurement of total Hgb mass (Otto et al., 2013).

One concern of the $\dot{V}_{O_2Peak}$ results is that our values seem to be higher than predicted $\dot{V}_{O_2Peak}$ values and those of other studies. In addition, the reference values for maximum oxygen consumption for active men and women aged between 55 and 64 years are 35.3 ± 6.2 (SD) ml/min/kg and 28.6 ± 6.1 (SD) ml/min/kg respectively (Herdy and Uhlendorf, 2011), compared to our results, 41.3 ± 11.9 (SD) ml/min/kg for men and 32.2 ± 7.9 (SD) ml/min/kg for women. The higher $\dot{V}_{O_2Peak}$ among male and female participants can result from the selection criteria since smokers, overweight participants and those with cardiac and respiratory illness were precluded. Furthermore, the advertisement used in this study could attract relatively healthy participants to join in the study. Last, the evaluation of predicted $\dot{V}_{O_2Peak}$ relied on participants' self-reports, which could draw another bias from the perception of exercise among individuals. However, these errors should not affect our results since they appear to be systematic.

In conclusion, we have demonstrated gender and age are significant factors in the determination of $\dot{V}_{O_2Peak}$ in healthy people aged between 50 and 80 years. Furthermore, ferritin and CRP were the factors found to be negatively correlated with the $\dot{V}_{O_2Peak}$ in female participants and CRP in male participants. Reduction of inflammatory markers by active physical activity may contribute to these results. However, to

what extent $\dot{V}_{O_2Peak}$ is affected by iron status in female participants and how ferritin contributes to oxidative stress and reduced $\dot{V}_{O_2Peak}$ warrants further investigation.

Chapter 5

Iron homeostasis in healthy humans aged 50-80 years after a single intravenous dose of ferric carboxymaltose

5.1 Introduction

Iron supplements are widely administered intravenously to treat iron deficiency and iron deficiency associated anaemia (Polin et al., 2013), and in patients with kidney disease (Kowalczyk et al., 2011; Macdougall and Geisser, 2013), heart failure (Anker et al., 2009; Cohen-Solal et al., 2014), and inflammatory bowel disease (Gomollon and Gisbert, 2013; Khalil et al., 2011; Koutroubakis et al., 2010). Given tight links between iron metabolism and oxygen sensing, iron supplementation has been explored

to modify hypoxia-related conditions (Smith et al., 2009; Talbot et al., 2011) and diseases (Portillo et al., 2013; Silverberg et al., 2014; Viethen et al., 2014). The improvement in iron preparations, such as the introduction of carbohydrate-iron complexes, has reduced serious adverse events (Auerbach et al., 2007; Larson and Coyne, 2014) and administration frequency (Keating, 2015; Macdougall et al., 2014; Munoz and Martin-Montanez, 2012) and made possible the use of higher doses given by a single administration (Auerbach et al., 2013; Keating, 2015; Munoz and Martin-Montanez, 2012).

This chapter is the preceding part of a study, which investigates the effects of a single dosage of iron as ferric carboxymaltose (Ferinject®) on the pulmonary circulation during exercise in healthy humans aged between 50 and 80 years (Chapter 6). Elsewhere this iron agent has been reported for cases of iron-deficiency anaemia and other diseases (Funk et al., 2010; Keating, 2015; Lyseng-Williamson and Keating, 2009). With a single dose of 15 mg/kg of iron (maximum dosage: 1,000 mg), we aimed to describe the time-course of haematological changes over 8 weeks in 16 healthy iron-replete humans. Sixteen acted as control by being administered placebo.

5.2 Methods

5.2.1 Participants

Thirty-two healthy men and women aged 50-80 years volunteered to participate in this study. All participants were informed about the potential risks and discomfort involved before written consent to participate was obtained. This study was approved by NRES

Committee South Central-Oxford B (Reference: 11/SC/0221).

Participants with detectable tricuspid regurgitation were recruited through the study described in Chapter 3. Inclusion criteria were as follows: not a current smoker; not overweight; no history of severe cardiac diseases, respiratory diseases or epilepsy; having no iron supplements or medications affecting the pulmonary circulation during the previous three months; able to perform cycling exercise.

5.2.2 Experimental protocol

Before enrollment in the study, a medical history was obtained from each participant for screening pulmonary and cardiovascular abnormalities and other conditions that could influence the blood test results. Participants arrived in the morning around 9 a.m. for the first blood sample and exercise tests, echocardiography during light bicycle exercise and $\dot{V}_{O_2Peak}$ exercise. After a short break (20-30 minutes), participants received the infusion. The same tests were repeated at 3 hours, 23 hours, 7 days, 4 weeks and 8 weeks after the infusion (Figure 5.1).

Details of echocardiography during light bicycle exercise and $\dot{V}_{O_2Peak}$ exercise are described in Chapter 6.

Figure. 5.1 **Experimental design.** Blood: blood samples; EC: echocardiography during light bicycle exercise; EX: $\dot{V}_{O_2Peak}$ exercise test.

5.2.3 Infusion

The participants were randomized into two groups relating to the type of infusions to be administered: iron and placebo, according to a block randomization using groups of 4. The infusion procedure was double-blind. Volunteers wore eye masks during infusions. Infusions were administered by physicians. Blood pressure was monitored during the infusion and 30 minutes after the infusion using an automated device (Omron M7 Digital Blood Pressure Moniter, Omron, UK). Each participant in the iron group received 15 mg/kg of iron (maximum dosage: 1,000 mg) as ferric carboxymaltose (Ferinject®, Vifor Pharma) diluted to 50 ml in total with 0.9% NaCl. Placebo infusion consisted of 50 ml 0.9% NaCl. The infusion time was 15 minutes and controlled by a syringe infusion pump (Graseby™ 3100 Syringe Pump, Smith Medical International Ltd, UK).

5.2.4 Blood samples and analytical procedures

The blood sampling was followed the same procedures as described in Chapter 3. In short, the blood samples were obtained from a vein of the forearm and distributed into tubes containing EDTA (EDTA BD Vacutainer®, BD) or micronized silica particles (SST BD Vacutainer®, BD) for the instant haematology and biochemistry analysis. Tubes for plasma and serum preparation were centrifuged at 3,000 g and immediately frozen at –80ºC for later use in ELISA kits. Plasma sTfR (Quantikine IVD® ELISA, R&D system), EPO (Quantikine® ELISA, R&D system), hepcidin (Hepcidin-25 human - EIA Kit, Bachem) and serum IL-6 (high-sensitivity Quantikine® ELISA, R&D system) were measured by ELISA kits. The sample preparation for ELISA kits is

described in Chapter 2. All analytical procedures followed the guidelines of the manufacturers.

5.2.5 Statistical analysis

Participant characteristics between groups were compared using the two-tailed Student's *t*-test. To assess changes after infusion, two-way repeated measures analysis of variance (ANOVA), using time and group as factors, was performed. The Bonferroni procedure was used in post hoc analysis to perform the mulitple comparisons. Results are given as mean ± SEM if not otherwise stated. All statistical analyses were performed using computer software (SigmaPLOT, Version 12.0, Systat Software).

5.3 Results

5.3.1 Participant characteristics

The 32 participants tolerated the infusion well. There were no severe adverse reactions or withdrawals. Two participants (one in each group) experienced headache after the infusion and one participant from the iron group reported a fever the day after the infusion. The basic characteristics of enrolled participants are listed by group in Table 5.1. There was no significant difference between the iron and placebo groups with regard to these characteristics.

When participants had a sign of acute infection (CRP > 8 mg/l) in the blood test, their results on the visit were excluded to avoid the effects of inflammatory responses on iron status (Feelders et al., 1998; Gabay and Kushner, 1999; Ganz, 2003).

	Iron Group	Placebo Group	p Value
Number, n	16	16	
Sex, %male	50%	50%	
Age, yr	65.4 ± 8.6	64.6 ± 4.9	0.749
Height, cm	167.5 ± 10.1	168.0 ± 8.5	0.881
Weight, kg	67.3 ± 14.8	67.9 ± 11.0	0.898

Table 5.1 **Participant Characteristics.** Values are shown in mean ± SD.

5.3.2 Iron status indicators

Before receiving the infusion, the iron and the placebo groups had similar levels of iron status indicators (Figure 5.2). After the infusion, a rapid increase in serum iron was seen in the iron group. The highest serum iron concentration (129.0 ± 3.4 μmol/l, n = 15), was reached at 4 hours and dropped to a normal range 4 weeks after the infusion (Figure 5.2 A). In contrast, serum iron levels in the placebo group remained the same as pre-infusion level during the study. Tfsat followed a similar trend to that of serum iron concentration in both groups, with the highest level of 212.5 ± 7.7% (Figure 5.2 B).

Hepcidin concentrations increased at 4 hours after the infusion in both groups (Figure 5.2 F). In the placebo group, the level of hepcidin at 4 hours (38.6 ± 5.8 μg/l, n = 15) did not reach significance ($p = 0.526$ for time), compared to its own pre-infusion level (25.5 ± 3.7 μg/l, n = 15). The hepcidin concentration remained close to its pre-infusion level over the remaining of the study period. In the iron group, the hepcidin concentration increased substantially and significantly at 4 hours after the infusion, reaching 62.3 ± 7.1 μg/l (n = 15; $p < 0.001$ for time). Then the hepcidin concentration

plateaued from 23 hours to 7 days, with the concentration of 141.0 ± 12.9 µg/l (n = 14). The concentration then dropped to a similar level to that at 4 hours after the infusion at the end of the study.

Compared to the iron status indicators mentioned above, ferritin, Tf and sTfR concentrations responded more slowly in the iron group after infusion (Figure 5.2 C, D, and E). For example, ferritin and Tf concentrations did not start to change until 23 hours after the infusion. For sTfR concentration, a significant difference between groups was first observed at 7 days ($p < 0.05$) and this persisted at 8 weeks ($p < 0.05$). In the placebo group, ferritin, Tf and sTfR concentrations showed no significant changes during the study (Figure 5.2 C, D, and E).

5.3.3 Haematological variables

Although there was a slight difference in the Hgb levels between the iron (14.0 ± 0.3 g/dl, n = 15) and the placebo groups (14.5 ± 0.2 g/dl, n = 14) at pre-infusion, it did not reach significance ($p = 0.458$) (Figure 5.3 A). Hgb levels dropped marginally in both groups at 23 hours after the infusion. The levels of Hgb were significantly lower than pre-infusion at 7 days after the infusion in both the iron group (13.7 ± 0.3 g/dl, n = 14, $p < 0.05$ for time), and the placebo group (13.9 ± 0.3 g/dl, n = 16, $p < 0.05$ for time). After 7 days, Hgb levels recovered slightly in both groups (Figure 5.3 A).

Hcts in both groups showed similar trends to the respective Hgb concentrations. Hcts dropped slightly by 23 hours after the infusion, but in neither group did the Hct differ significantly from pre-infusion levels, except at 7 days in the iron group ($p < 0.05$) (Figure 5.3 B).

The EPO concentrations of both groups were similar at the pre-infusion time point (iron group: 7.23 ± 0.81 Ul/l, n = 15; placebo group: 7.41 ± 0.57 Ul/l, n = 15), and increased slightly at 4 hours after the infusion (iron group: 8.93 ± 0.67 Ul/l, n = 15; placebo group: 8.56 ± 0.59 Ul/l, n = 15) (Figure 5.3 D). EPO concentration was significantly increased from the pre-infusion concentration in the iron group ($p = 0.017$ for time) but not in the placebo group ($p = 0.228$ for time). There was a marked decrease in the EPO concentration at 23 hours after the infusion in the iron group (5.70 ± 0.55 Ul/l, n = 15), compared to the placebo group ($p < 0.01$). EPO concentration in the iron group then plateaued from 7 days after the infusion (6.76 ± 0.62 Ul/l, n = 14), to the end of the study. In contrast, EPO levels in the placebo group peaked at 7 days after the infusion (9.12 ± 0.93 Ul/l, n = 14), and returned to pre-infusion levels at 4 weeks after the infusion (Figure 5.3 D).

MCV, CRP and IL-6 concentrations in both groups remained at pre-infusion levels throughout the study (Figure 5.3 C, E, and F).

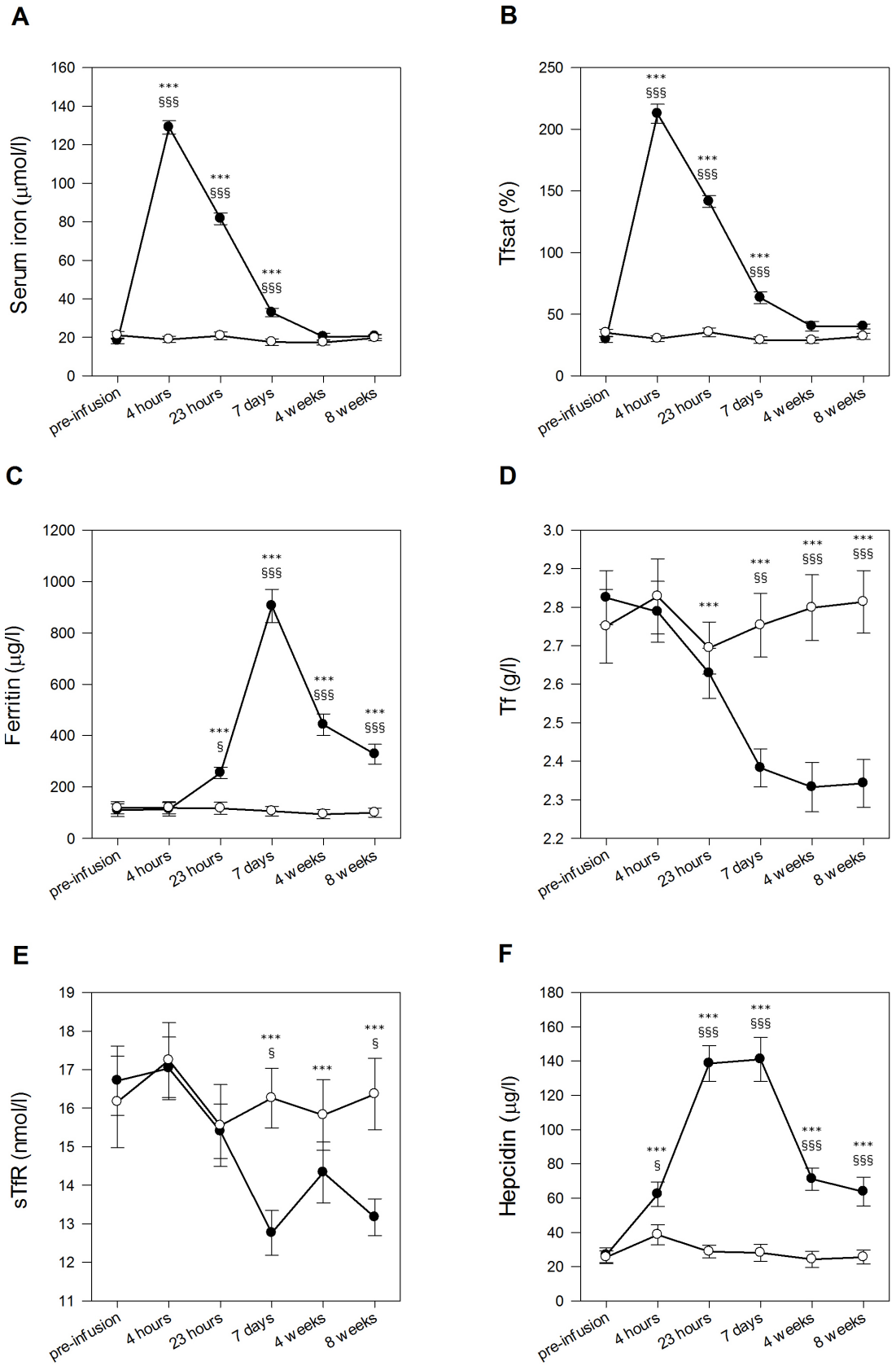

Figure. 5.2 **Iron-status indicators.** **A**. Serum iron concentration; **B**. Transferrin Saturation (Tfsat); **C**. Ferritin concentration; **D**. Transferrin (Tf) concentration; **E**. Soluble transferrin receptor (sTfR) concentration; **F**. Hepcidin concentration. *Filled circles* represent responses in the iron group. *Empty circles* represent responses in the placebo group. Values are mean ± SEM.

* Different from the pre-infusion in the iron group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

§ Different between groups; § $p < 0.05$, §§ $p < 0.01$, §§§ $p < 0.001$

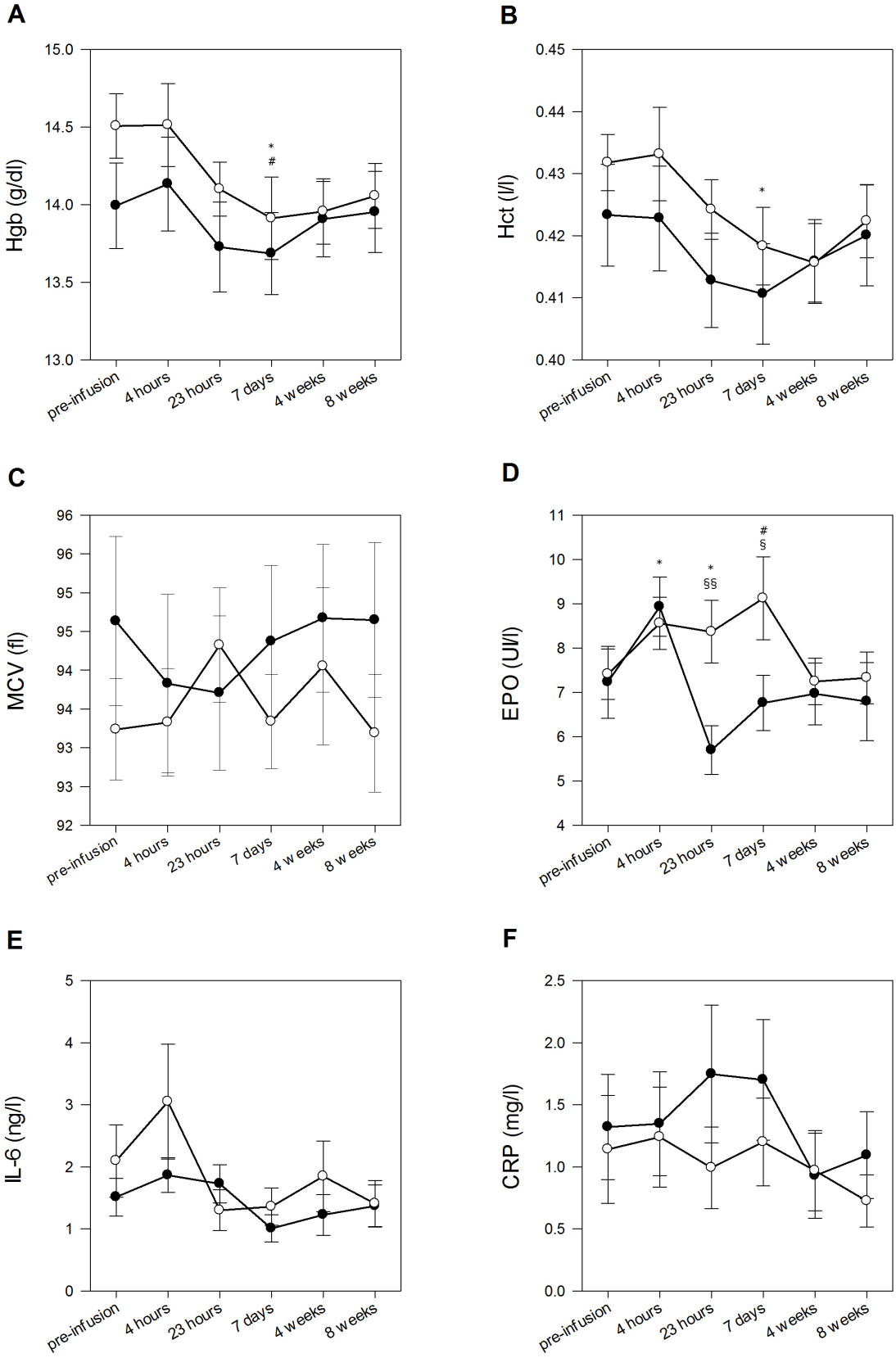

Figure. 5.3 **Haematological variables. A**. Haemoglobin (Hgb) concentration; **B**. Haematocrit (Hct); **C**. Mean cell volume (MCV); **D**. Erythropoietin (EPO) concentration; **E**. Interleukin-6 (IL-6) concentration; **F**. C-reactive protein (CRP) concentration. *Filled circles* represent responses in the iron group. *Empty circles* represent responses in the placebo group. Values are mean ± SEM.

* Different from the pre-infusion in the iron group; * $p < 0.05$

Different from the pre-infusion in the placebo group; # $p < 0.05$

§ Different between groups; § $p < 0.05$, §§ $p < 0.01$

5.4 Discussion

In this study, we observed that a single dose of ferric carboxymaltose induced rapid changes in serum iron concentration, hepcidin concentration and Tfsat. Tf and ferritin concentrations started to reach a trough and a peak respectively from 23 hours to 7 days after the infusion of iron. Despite the fact that the serum iron concentration and Tfsat returned to their pre-infusion levels at 4 weeks after the infusion, the levels of ferritin, Tf, sTfR and hepcidin remained significantly different, both compared with their pre-infusion levels and those in the placebo group. Furthermore, these changes in iron status did not affect Hgb and Hct levels significantly over 8 weeks, despite the fact that the concentration of EPO in the iron group dropped by $\sim$20% at 23 hours after the infusion compared with the pre-infusion level. Finally, we observed that the large dose of iron as ferric carboxymaltose did not cause any inflammatory response (based on CRP and IL-6) nor severe adverse effects in our participants.

The present study showed that a single dose of intravenous ferric carboxymaltose caused long-lasting effects in iron-replete participants. Despite similar peak concentrations and time to peak concentration in the serum iron level and Tfsat, it took a longer time for iron status indicators to return to normal ranges in our participants than iron deficient anaemia patients with the same dose of iron infusion (Geisser and Banke-Bochita, 2010). The serum iron levels in the present study fell to pre-infusion levels after 4 weeks, compared to 72 hours in the study on iron deficient anaemia patients (Geisser and Banke-Bochita, 2010). It is clear that the pharmacokinetics of ferric carboxymaltose is altered according to the starting iron status and Hgb level in our participants. As iron elimination from the body is by the gradual loss of cells (Ganz and

Nemeth, 2012; Nemeth and Ganz, 2006), a slower clearance of ferric carboxymaltose in healthy iron replete participants is not surprising. Furthermore, the same dose of intravenous ferric carboxymaltose caused a higher peak ferritin level in our participants than in iron deficient anaemia patients (Geisser and Banke-Bochita, 2010), suggesting a stronger replenishment of iron stores in healthy iron replete participants than in iron depleted participants.

Interestingly, an equivalent dose of ferric carboxymaltose caused a significant decline in sTfR and Tf concentrations between 7 days and 8 weeks after the infusion in our iron replete participants. However, this was not seen at iron deficient anaemia patients (Geisser and Banke-Bochita, 2010). Declines in sTfR and Tf concentrations were observed in our participants, suggesting that the amount of circulating iron is beyond the capacity of utilization and storage. This may have activated iron regulatory mechanisms, including the IRE/IRP, to reduce sTfR and Tf productions as a feedback mechansim. Moreover, this raises a concern of iron toxicity by excess iron (Bishu and Agarwal, 2006) and specifically non-Tf-bound iron (Brissot et al., 2012) since the elimination of iron in the body is limited (Ganz and Nemeth, 2012). One limitation of this study is the lack of identification of haemosiderin and haemosiderosis, which are signatures of iron toxicity (Batts, 2007). However, no significant acute adverse effect was observed after the infusion in this study. In addition, the infusion of ferric carboxymaltose caused no inflammatory response (measured by CRP and IL-6), which is reassuring in terms of the safety of large doses of ferric carboxymaltose.

After infusion, the EPO concentration increased slightly in both groups at 4 hours. This elevation lasted until 7 days in the placebo group after the infusion but had disap-

peared 4 and 8 weeks after the infusion, suggesting that it might be the result of exercise (Roberts et al., 2000), repeated venipuncture (Maeda et al., 1992) or the infusion itself. In contrast, the EPO concentration in the iron group declined significantly 23 hours after the infusion and recovered to pre-infusion level 7 days after the infusion. To our knowledge, iron has not been reported to down-regulate EPO production, though DFO (an iron chelator) up-regulated EPO production in humans was seen (Ren et al., 2000b). One possible mechanism could be the activation of the HIF hydroxylase by the iron infusion (Nagel et al., 2010) since EPO gene expression is controlled primarily by HIF-2α (Morita et al., 2003; Rosenberger et al., 2002). As iron has been shown to activate the proteasomal degradation of the HIF (Jaakkola et al., 2001; Knowles et al., 2003; Nandal et al., 2011) and causes inhibition of hypoxic responses in pulmonary vasculature (Smith et al., 2008a, 2009), iron loading in our subjects might improve the degradation of HIF and subsequently reduce the production of EPO in our healthy aged individuals. However, the detailed mechanism requires further studies.

Besides the measurement of routine clinical iron status indicators, this study included hepcidin concentration to evaluate the temporal effects of iron infusion on the regulation of iron homeostasis. Hepcidin is a short peptide with antimicrobial properties that has been identified as an important iron regulator. It regulates the internalization of ferroportin, which is the only putative iron exporter from cells, such as those lining the gut (Ganz and Nemeth, 2012; Nemeth and Ganz, 2006). In this study, a fast response in the increase of hepcidin concentration was observed at 4 hours after the infusion in the iron group faster than some other iron status indicators. The increase in hepcidin concentration blunts the absorption of iron from the diet and iron release from

macrophages to reduce the amount of circulating iron via a feedback mechanism. This response of hepcidin to iron administration and its importance to iron metabolism confirm the possibility of hepcidin being an important iron regulator. Given that hepcidin concentration increases with age (Galesloot et al., 2011), it would have been interesting to include young controls to determine the changes in hepcidin levels in a similar experiment.

In conclusion, we demonstrate that a single infusion of ferric carboxymaltose induces significant changes in iron status indicators over 8 weeks in healthy iron replete participants safely without significant changes in Hgb, IL-6, CRP concentrations and Hct. Overall, it appears that, in healthy iron replete participants, hepcidin could be a useful indicator of iron status because of its responsiveness and importance in iron homeostasis. Finally, intravenous iron infusion might interrupt HIF signalling in the body, thus it could become a novel strategy for the treatment of hypoxia-related symptoms or diseases.

Chapter 6

The effect of a single intravenous dose of iron on systolic pulmonary arterial pressure and exercise capacity in healthy humans aged 50-80 years

6.1 Introduction

In 1985, Groves and colleagues recruited 8 healthy volunteers and studied their responses in the acclimatization during a 40-day simulated ascent of Mt. Everest (8,800 m) in a hypobaric chamber (Operation Everest II) (Groves et al., 1987). After two years, they published that healthy subjects had progressively increasing slopes on plots of MPAP against cardiac output as the simulated high altitude intensified. Their findings suggested that the right ventricle may serve as a limiting factor for exercise capac-

ity at high altitude. Subsequent studies have expanded on these findings and demonstrated that PAP lowered by sildenafil improved exercise performance along with increased cardiac output at high altitude (Ghofrani et al., 2004; Hsu et al., 2006) and in heart failure patients (Guazzi et al., 2011; Hamdan et al., 2014; Hirata et al., 2005; Lewis et al., 2007). However, some studies have reported contradictory views as to the effects of sildenafil on the recovery of exercise performance (Pavelescu and Naeije, 2012; Redfield et al., 2013; Sperandio et al., 2012). This has led to uncertainty as to whether exercise capacity in hypoxia might be limited by RV function as it works to discharge cardiac output into relatively high pressure of the hypoxic pulmonary circulation.

A recent finding has suggested that a similar mechanism might restrict the exercise capacity in aged individuals. Kovacs and colleagues observed a significantly higher MPAP during light exercise in healthy individuals aged over 50 years compared with that of younger individuals (age < 30 years) (Kovacs et al., 2009). In addition, Reeves and colleagues found a steeper slope of PAP against cardiac output during exercise for aged individuals than for young individuals (Reeves et al., 2005). While the underlying mechanism is unknown, a lower distensibility in the pulmonary vasculature in aged individuals has been suggested to be the cause (Naeije and Chesler, 2012; Reeves et al., 2005).

In addition to the stiffer vasculature in aged individuals, there is also the possibility of a more vigorous hypoxia-related pulmonary vasoconstriction in older people. Sorbini and colleagues demonstrated a negative correlation between the systemic P_{aO_2} and age (Sorbini et al., 1968). In addition, elderly athletes had a lower systemic

P_{aO_2} than young athletes at the same workload during an incremental exercise test (Prefaut et al., 1994). These data suggest that older people may be generally more hypoxic and consequently have an increased PAP during exercise. As HPV can be depressed by iron infusion in healthy participants (Smith et al., 2008a, 2009), the main aim of this study was to measure the impact of iron infusion on SPAP during light exercise in healthy individuals aged between 50 and 80 years. In addition, a secondary aim was to assess the effect of the changed SPAP on exercise capacity during heavy exercise, measured in terms of $\dot{V}_{O_2Peak}$ and work rate.

6.2 Methods

6.2.1 Participants, infusion and experimental protocol

The participants, infusion and experimental protocol are the same as described in Chapter 5. In short, thirty-two healthy men and women aged 50-80 years volunteered to participate in this study. Participants arrived in the morning around 9 a.m. for the first blood sample and exercise tests, echocardiography during light bicycle exercise, and $\dot{V}_{O_2Peak}$ exercise. After a short break (20-30 minutes), participants received the infusion. The infusion procedure was double-blind, placebo-control and randomised. Sixteen acted as subjects receiving a single dose of iron (15 mg/kg; maximum dosage: 1,000 mg) as ferric carboxymaltose (Ferinject®, Vifor Pharma) diluted in 50 ml in total with 0.9% NaCl. Another 16 acted as placebo receiving the same volume of 0.9% NaCl. The blood test and the two exercise tests were then repeated at 3 hours, 23 hours, 7 days, 4 weeks and 8 weeks after the infusion.

6.2.2 Echocardiography during light bicycle exercise

The light exercise was performed on a bicycle ergometer (Ergoline, Ergoline Company, Germany) in the semi-supine position with a 30-40 degree of rotation to the left. The exercise protocol included 5 minutes of resting, 20 minutes of exercise and 5 minutes of a recovery phase (Figure 6.1). SPAP was estimated using measurements of peak systolic tricuspid jet velocity as $4V_{TR}{}^2$ + 5 mmHg (estimated right atrial pressure (Rudski et al., 2010)) at rest and during a level of exercise achieved by increasing the heart rate to 30 bpm above participants' resting heart rate.

The baseline values of echocardiographic measurement were determined as the averaged echocardiographic data over the resting period. The exercise values of echocardiographic measurement were determined as the averaged echocardiographic data over the latter half of the exercise period (Figure 6.1). ΔSPAP, Δcardiac output, Δstroke volume and Δheart rate were obtained by subtracting baseline values from exercise values.

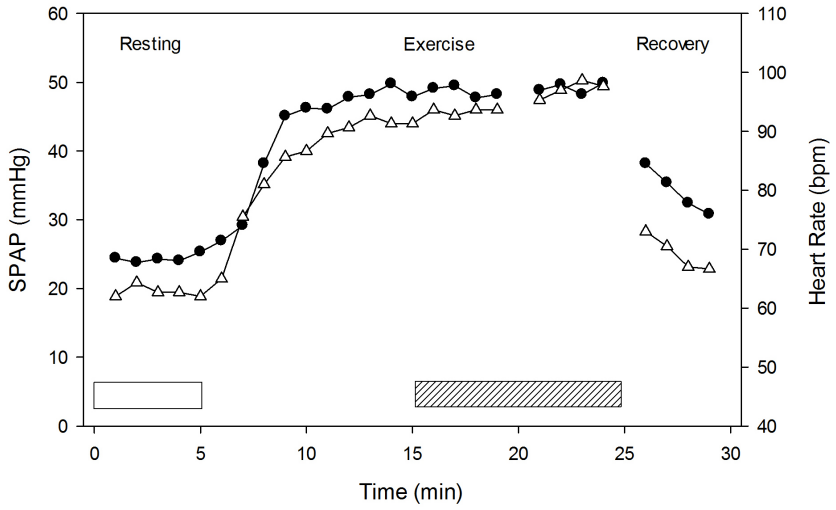

Figure. 6.1 **Data acquisition during the echocardiography bicycle exercise from a representative participant.** In the light exercise protocol, the participant rested on the ergometer for 5 minutes and started to reach the targeted heart rate (*empty triangles*) (30 bpm above the participant's resting heart rate). The exercise was sustained for 20 minutes and the participant allowed to recover. SPAP (*black circles*) was measured for the whole duration. Data obtained from first 5 minutes were averaged and taken as the baseline value (*blank bar*). Data between 16th and 25th minute were averaged and taken as the exercise value (*striped bar*).

6.2.3 Measurement of $\dot{V}_{O_2Peak}$ during heavy exercise

The heavy exercise was performed on a bicycle ergometer (KEM-3, CardioKinetics Limited) with a face mask (Metro Seal^{TM} 7900 Adult Face mask, Hans Rudolph Inc., USA) to determine the $\dot{V}_{O_2Peak}$ and work rate using a metabolic measurement system (Jaeger^{TM} Oxycon®, Carefusion, UK). Details are described in Chapter 2. In short, the exercise protocol was adapted from Rossiter *et al.* (Rossiter et al., 2006). Each participant performed a standard incremental exercise test up to volitional exhaustion with the work rate rising incrementally at 15 W per minute, from 0 W. After 5 minutes of pedalling at 0 W, the participant cycled at 105% of the participant's maximum work rate measured previously in the standard incremental exercise test to exhaustion. Exhaustion was determined by participants stopping or by the cadence going below 60 rpm.

The $\dot{V}_{O_2Peak}$ was determined as the average of $\dot{V}_{O_2}$ values over a 15-second period during the period of maximum $\dot{V}_{O_2}$ during the exercise test. If highest $\dot{V}_{O_2}$ values were different between two exhausion phases, the higher $\dot{V}_{O_2}$ value was chosen to represent the participant's $\dot{V}_{O_2Peak}$. Baseline $\dot{V}_{O_2}$ and CO_2 elimination ($\dot{V}_{CO_2}$) were taken as the averaged of measurements from the 2nd minute on the ergometer before pedalling. S_{aO_2} was measured using a pulse oximeter (Ohmeda Biox 3740 Pulse Oximeter, BOC Healthcare) with a finger probe (OXY-F4-H finger sensor, GE Healthcare). Heart rate was measured through three-lead electrocardiogram (Micromon 7142 B ECG, Kontron Medical). All equipment was calibrated before each experiment.

6.2.4 Statistical analysis

Participant characteristics were compared between groups using a two-tailed Student's *t*-test. The correlation at pre-infusion analysis between variables was analysed using Spearman's rank correlation (β) and linear regression. To assess the changes after infusion, two-way repeated ANOVA was performed using time and group as factors. The Bonferroni procedure was used in post hoc analysis to perform the multiple comparisons. Results are given as mean ± SEM unless otherwise stated. All statistics were calculated using computer software (SigmaPLOT, Version 12.0, Systat Software).

6.3 Results

6.3.1 Pre-infusion anaylsis

Pre-infusion data from 32 enrolled participants were anaylsed to assess the correlations between SPAP and clinical/echocardiographic variables (Table 6.1). Correlations between age and SPAP at rest, during light exercise and the rise between rest and light exercise are shown in Figure 6.2.

Resting SPAP was correlated to resting heart rate (β = –0.379, p = 0.032, Spearman's correlation), cardiac output during light exercise (β = –0.360, p = 0.043, Spearman's correlation), and Δcardiac output (β = –0.500, p = 0.004, Spearman's correlation).

SPAP during light exercise was correlated to resting SPAP (β = 0.418, p = 0.018, Spearman's correlation) and ΔSPAP (β = 0.691, p = < 0.001, Spearman's correlation).

ΔSPAP was correlated to age (β = 0.352, p = 0.048, Spearman's correlation), cardiac output during light exercise (β = 0.367, p = 0.039, Spearman's correlation) and Δcardiac output (β = 0.419, p = 0.017, Spearman's correlation).

6.3.2 Participant characteristics

The basic characteristics of the 32 enrolled participants are listed in Table 6.2 by group. The participants were either of sedentary lifestyle or recreational joggers/cyclists. None were former or current elite/competitive athletes.

	Iron Group	**Placebo Group**	**p Value**
Number, n	16	16	
Sex, %male	50%	50%	
Age, yr	65.4 ± 8.6	64.6 ± 4.9	0.749
Height, cm	167.5 ± 10.1	168.0 ± 8.5	0.881
Weight, kg	67.3 ± 14.8	67.9 ± 11.0	0.898
FEV_1, % predicted	108.1 ± 17.1	107.1 ± 16.9	0.867
FVC, % predicted	117.6 ± 16.5	111.9 ± 14.0	0.301
FEV_1/FVC	74.2 ± 8.8	76.2 ± 6.0	0.458
Systolic blood pressure, mmHg	124.4 ± 13.7	121.6 ± 15.4	0.591
Diastolic blood pressure, mmHg	80.0 ± 8.3	81.9 ± 10.9	0.583
Baseline $\dot{V}_{O_2}$, ml/min	299.0 ± 18.4	308.9 ± 17.5	0.129
Baseline $\dot{V}_{CO_2}$, ml/min	239.4 ± 17.3	239.2 ± 12.6	0.970

Table 6.2 **Participant characteristics at pre-infusion visit.** FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity. Values are shown as the mean ± SD.

6.3.3 Echocardiography measurement during light exercise

Throughout the study, heart rate was controlled as intended at a difference of ∼30 bpm between values in the baseline and exercise period (Figure 6.4 D). The heart rate in both groups followed a similar trend (p = 0.243 for interaction), despite differences in resting heart rate at pre-infusion (iron group: 59.4 ± 2.2 bpm; placebo group: 65.2 ± 1.7 bpm, p = 0.069 for group). The heart rate at rest between groups became statistically significant at 3 hours (p = 0.037), 23 hours (p = 0.024) and 4 weeks (p = 0.009) after the infusion (Figure 6.3 D).

There were no significant differences observed between the two groups in terms of SPAP, cardiac output and stroke volume during resting or exercise phases at any of the time points (Figure 6.3 A, B and C). However, SPAP during exercise in the iron group decreased significantly by ∼6% at 23 hours (37.2 ± 0.9 mmHg) (p < 0.001, for

time) and remained low until 8 weeks, compared to pre-infusion levels (39.42 ± 1.0 mmHg) (Figure 6.3 A). These caused significant drops in ΔSPAP from 23 hours to 8 weeks after the infusion in the iron group ($p < 0.001$), compared to pre-infusion levels (Figure 6.4 A). In contrast, ΔSPAP in the placebo group remained the same over the study, which showed a significant group and time interaction ($p < 0.001$).

Despite the significant changes in the resting cardiac output in both groups, compared to their own pre-infusion levels (Figure 6.3 B), the Δcardiac output in both groups showed no significant change from pre-infusion values (Figure 6.4 B). Stroke volume (Figure 6.3 C) and Δstroke volume (Figure 6.4 C) stayed comparatively stable over the study in both groups.

6.3.4 Exercise capacity

Both groups maintained similar heart rates during peak exercise, S_{aO_2} during rest and peak exercise, $\dot{V}_{O_2Peak}$ and peak work rate over time as shown in Figure 6.5.

There was a significant increase in the baseline heart rate for the placebo group at 4 hours after the infusion (75.3 ± 2.4 bpm) ($p < 0.001$ for time), compared with its pre-infusion levels (66.0 ± 2.4 bpm).

	SPAP at rest		SPAP at light exercise		ΔSPAP	
Variables	β	p Value	β	p Value	β	p Value
Age, year	0.112	0.539	0.319	0.074	0.352	0.048
Weight, kg	0.102	0.575	0.031	0.862	−0.116	0.526
Height, cm	0.018	0.919	0.006	0.974	−0.037	0.839
BMI, kg/m^2	0.140	0.443	0.117	0.522	−0.094	0.605
SBP at rest, mmHg	0.022	0.905	−0.134	0.460	−0.138	0.448
DBP at rest, mmHg	0.069	0.704	−0.010	0.954	−0.037	0.840
Heart rate at rest, bpm	−0.379	0.032	−0.008	0.966	0.194	0.285
Heart rate at peak exercise, bpm	−0.104	0.569	−0.344	0.053	−0.255	0.157
Cardiac output at rest, ml/min	−0.094	0.605	−0.005	0.978	0.071	0.698
Cardiac output at light exercise, ml/min	−0.360	0.043	0.049	0.789	0.367	0.039
Δcardiac output, ml/min	−0.500	0.004	< 0.001	0.999	0.419	0.017
$\dot{V}_{O_2peak}$, ml/min	−0.007	0.969	−0.140	0.440	−0.085	0.639
$\dot{V}_{O_2peak}$, ml/min/kg	−0.033	0.858	−0.231	0.202	−0.123	0.499
Peak work rate, W	−0.067	0.714	−0.238	0.189	−0.107	0.557
SPAP at rest, mmHg	–	–	0.418	0.018	−0.137	0.453
SPAP at light exercise, mmHg	–	–	–	–	0.691	< 0.001

Table 6.1 **Correlation between systolic pulmonary arterial pressure (SPAP) at rest, during light exercise, and the rise between rest & light exercise and clinical/echocardiographic variables.** BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure.

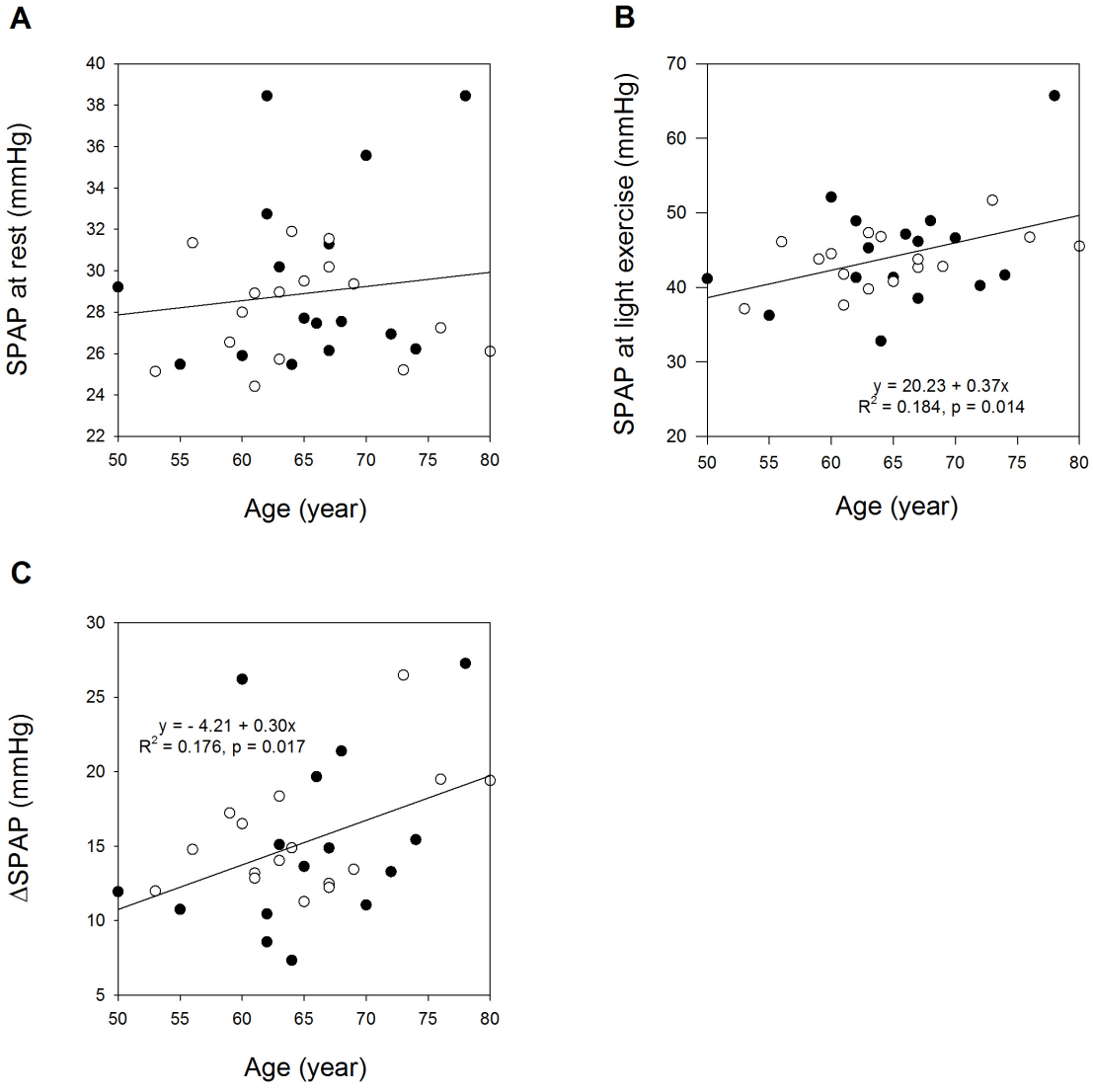

Figure. 6.2 **Relationships between age (horizontal axis) and systolic pulmonary arterial pressure (SPAP) at rest, during light exercise and the rise between rest & light exercise (vertical axes). A**. SPAP at rest; **B**. SPAP during light exercise; **C**. ΔSPAP. Data from females (*filled circles*) and males (*empty circles*) are shown together. Trends with age are shown as linear regression lines overall.

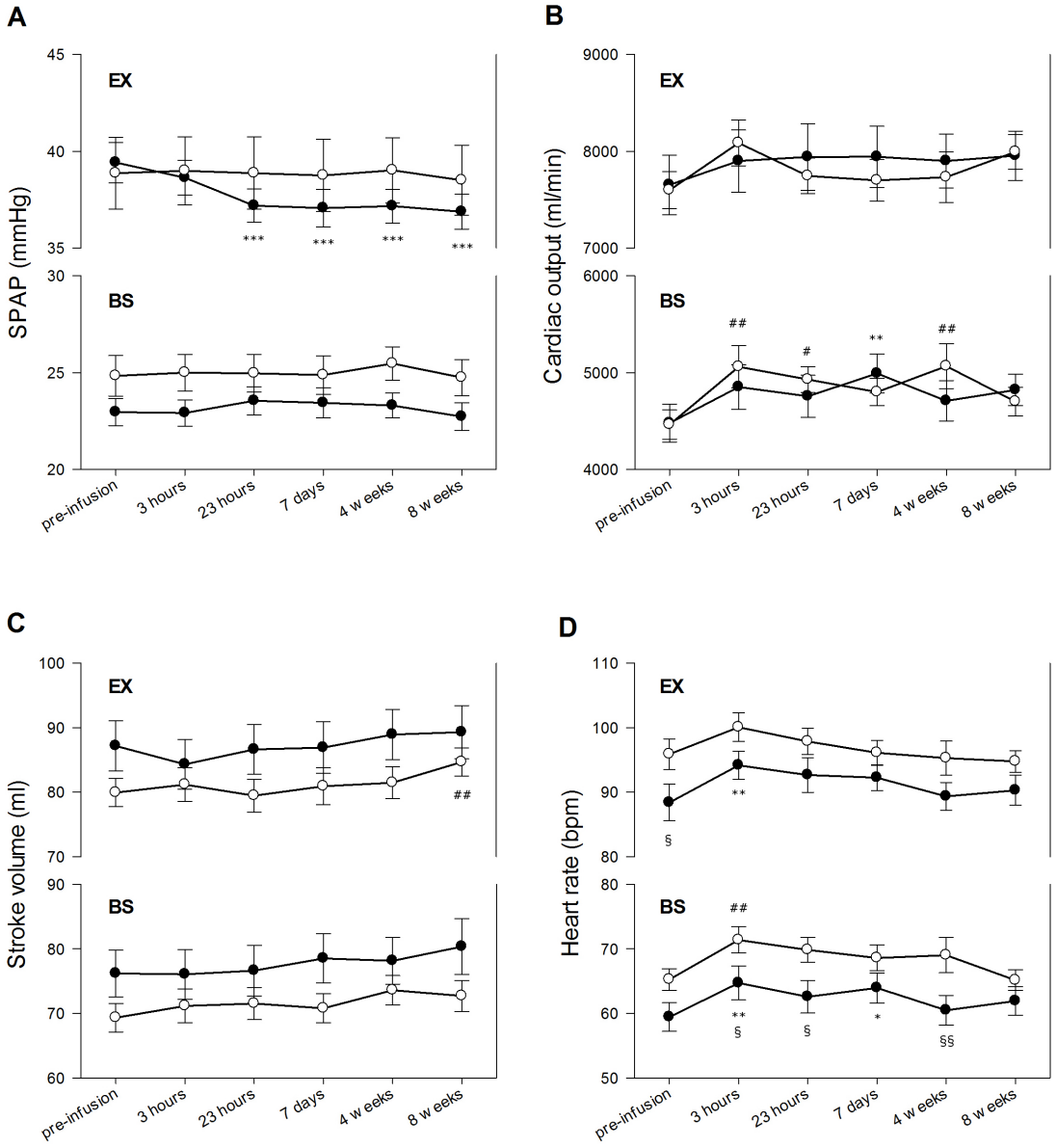

Figure. 6.3 **Measurement of echocardiography during light exercise. A**. Systolic pulmonary arterial pressure (SPAP); **B**. Cardiac output; **C**. Stroke volume; **D**. Heart rate. *Filled circles* represent responses in the iron group. *Empty circles* represent responses in the placebo group. Exercise (EX) and baseline (BS) values are shown in upper panels and lower panels respectively. Values are given as the mean ± SEM.

* Different from the pre-infusion in the iron group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Different from the pre-infusion in the placebo group; # $p < 0.05$, ## $p < 0.01$

§ Different between groups; § $p < 0.05$, §§ $p < 0.01$

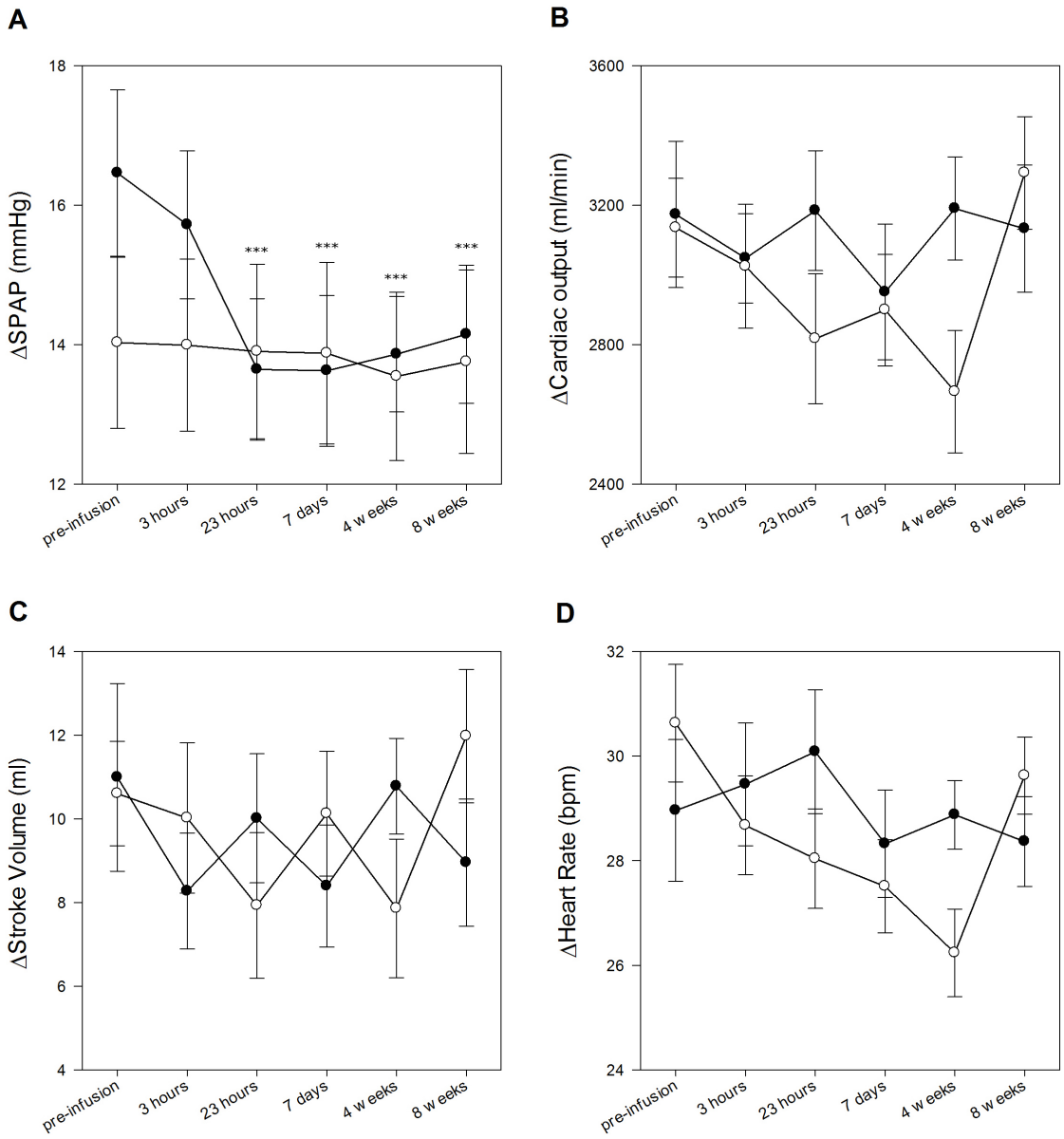

Figure. 6.4 **Difference between baseline and exercise values by echocardiography during light bicycle exercise.** **A**. Systolic pulmonary arterial pressure (SPAP); **B**. Cardiac output; **C**. Stroke volume; **D**. Heart rate. *Filled circles* represent responses in the iron group. *Empty circles* represent responses in the placebo group. Values are given as the mean ± SEM.
* Different from the pre-infusion in the iron group; *** $p < 0.001$

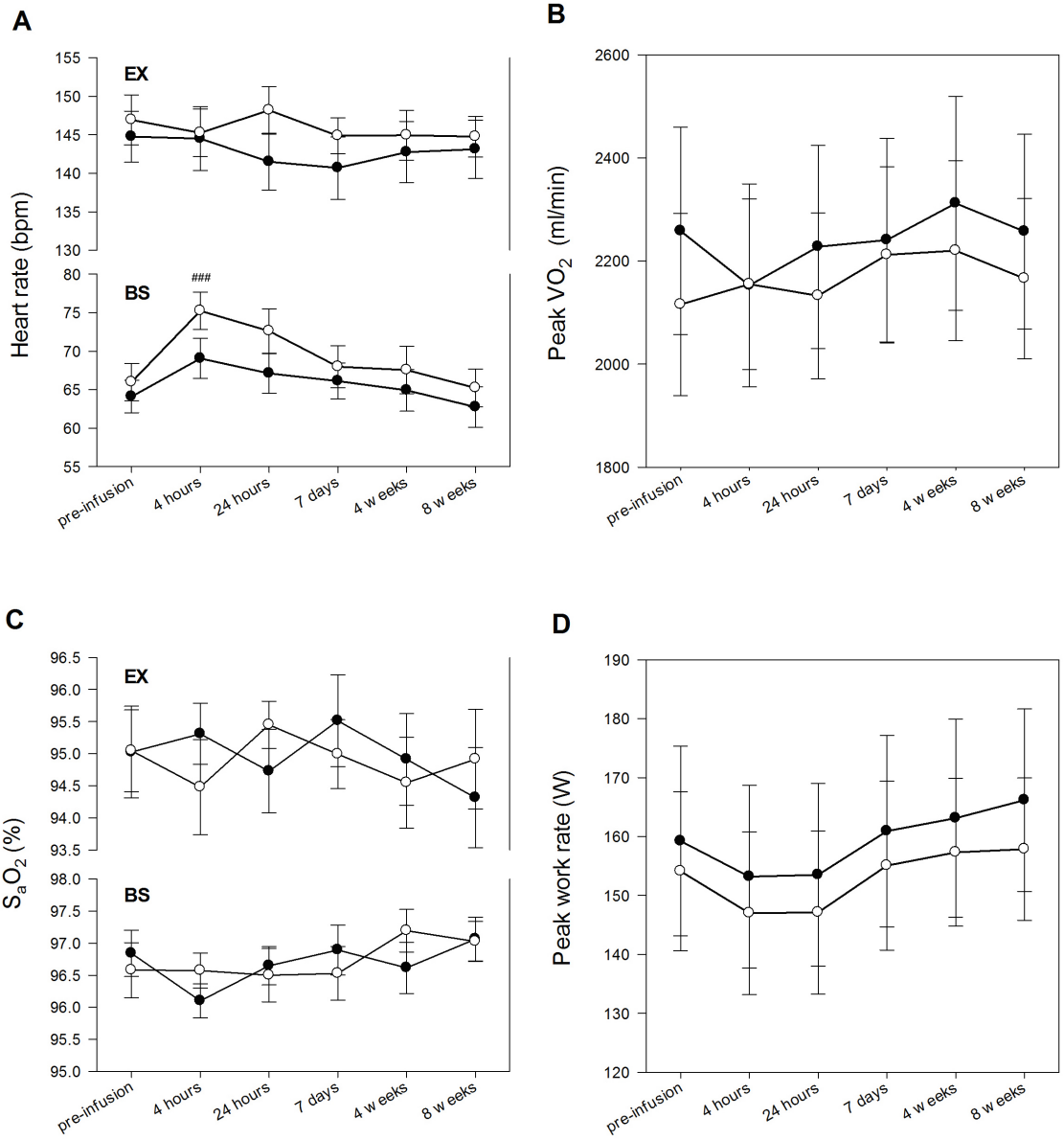

Figure. 6.5 **Responses to heavy execise.** **A**. Heart rate; **B**. $\dot{V}_{O_2Peak}$ consumption; **C**. Oxygen-haemoglobin saturation ($S_{a}O_2$); **D**. Peak work rate. *Filled circles* represent responses in the iron group. *Empty circles* represent responses in the placebo group. Exercise (EX) and baseline (BS) values are shown in upper panels and lower panels respectively. Values are given as the mean ± SEM.

[#] Different from the pre-infusion in the placebo group; [###] $p < 0.001$

6.4 Discussion

In this study, we observed that the increase in SPAP during exercise was coupled with age. After the iron infusion, SPAP during light exercise decreased between 23 hours and 8 weeks without significant changes in the resting SPAP. Despite the significant decrease in SPAP in the iron group during light exercise, these changes were not associated with any change in cardiac output or stroke volume in the study. Furthermore, the observed physical performance in the iron group, $\dot{V}_{O_2Peak}$ and peak work rate, did not change significantly during the 8 weeks following the infusion.

Several studies have noticed an exaggerated increase in SPAP with exercise with ageing (Mahjoub et al., 2009; Naeije and Chesler, 2012; Reeves et al., 2005). In this study, data from the pre-infusion analysis showed that ageing was associated with the increase in the magnitude of SPAP during light exercise. This is further confirmed by a comparison between this study and a previous study. Herigstad and colleagues used the same light exercise protocol in younger participants [mean age = 26.8 ± 6.9 (SD) years] and observed a rise in SPAP during light exercise by ∼7 mmHg (Herigstad and Robbins, 2009) while we observed the rise of ∼16 mmHg in our participants. The significant increase in exercising SPAP by age is roughly doubled, which is consistent with the results reported by Kovacs and colleagues (Kovacs et al., 2009). These results suggest that age indeed alters the responses of the pulmonary circulation to exercise.

It is unclear how ageing causes the increase in SPAP at exercise. One hypothesis suggests that arterial stiffening in the pulmonary circulation may be the main cause (Reeves et al., 2005). This hypothesis is supported by findings in a large longitudinal study (Lam et al., 2009). Lam *et al*. examined 2042 participants and found that

resting SPAP was correlated to age and arterial stiffening. The present study showed that resting SPAP did not show a strong correlation to age, which may result from a small number of participants. If resting heart rate and physical fitness are negatively associated with arterial stiffness, as suggested by Quan *et al*. (Quan et al., 2014), the observed strong negative correlation between resting SPAP and physical fitness indicators, such as resting heart rate and cardiac output during light exercise, can be an alternative evidence showing the correlation between resting SPAP and arterial stiffness. However, these will require further studies to confirm the relationships.

Our results suggest that the HIF signalling pathway may be involved in the increased SPAP during exercise with age since the increased SPAP was attenuated by iron loading. Although a limitation of this study is the lack of direct measurement of the HIF signaling pathway in the pulmonary circulation, the inhibition of erythropoietin (a downstream effector of HIF-2) was observed in this study after iron infusion. In addition, recent studies have shown that supplementary iron can restore or activate the HIF degradation (Jaakkola et al., 2001; Knowles et al., 2003; Nandal et al., 2011) and blunt hypoxia-induced pulmonary vasoconstriction (Smith et al., 2008a, 2009). These support that the decreased SPAP might result from the suppression of the HIF signalling pathway by iron infusion. Nevertheless, it is unclear why the effect of iron infusion seemed to last longer in the pulmonary circulation than in EPO production. It is possible that HIF function or iron transportation mechanism are variable between cell types (Stroka et al., 2001; Talks et al., 2000). However, this requires a further study to clarify.

Interestingly, even though a decreased SPAP by iron infusion was observed dur-

ing light exercise, subsequent changes in stroke volume and cardiac output were not seen. In addition, exercise performance, measured by $\dot{V}_{O_2Peak}$ and peak work rate, were identical for both groups over 8 weeks. Our results were not consistent with the study by Ghofrani *et al*., who sucessfully manipulated exercise capacity by reducing SPAP (Ghofrani et al., 2004). Ghofrani and colleagues conducted a cross-over, randomised and placebo controlled study examining the effects of sildenafil on PAP and exercise capacity, measured by work rate, at high altitude (5,245m) and during simulated altitude (10% of oxygen). During the simulated condition, a significant decrease in SPAP during exercise by 7.8 mmHg (median difference) was observed. This was associated with an increase of 1.3 l/min in the maximum cardiac output and 34.8 W in the maximum work rate in the exercise test. Compared with our results, a smaller decrease in exercising SPAP ($\sim$2 mmHg) was seen in the iron group. This small decrease might not big enough to change cardiac output and exercise capacity. Furthermore, individual variations and measurement variations from different visits/daily activities may have reduced the statistical power.

Although conventionally exercise capacity is tested by an incremental exercise test, this approach is somewhat problematic. The test itself is not determined only by anaerobic capacity but also by aerobic capacity. As lowering SPAP may improve aerobic capacity via improving cardiac output but not anaerobic capacity, such as strength of muscle, it would not be surprising to see unchanged exercise capacity in our participants. This would be overcome by examining exercise capacity via different ways, including time-trial and time-to-exhaustion exercise tests. On the other hand, exercise is a complex physiological event, which not only involves the cardiovascular system

but also the muscular, integumentary and nervous systems. As the impact of iron infusion on other systems was not examined, it is hard to rule out these other factors affecting the exercise performance, such as oxygen uptake efficiency in muscles. It would have been interesting to include other procedures, such as muscle biopsy, to examine other possible factors affecting the exercise capacity.

In conclusion, we have demonstrated that the increase in SPAP during light exercise can be attenuated by intravenous iron in healthy humans aged from 50 to 80 years. The effects of iron on SPAP can be observed from 23 hours to 8 weeks after the infusion and may involve the HIF signalling system. However, this decreased SPAP during exercise brought about by iron infusion did not induce significant changes in stroke volume, cardiac output during light exercise, and exercise capacity during heavy exercise in these healthy aged individuals.

Chapter 7

Cardiopulmonary responses to acute hypoxia in humans before and after 8-hour hyperoxia

7.1 Introduction

The mechanisms underlying HPV are controversial. One model proposes that ROS produced by mitochondria during hypoxia are central to this phenomenon (Waypa et al., 2001). During hypoxia, electron flow on the mitochondrial ETC is obstructed due to lack of oxygen as an electron acceptor. This results in an excessive flow of electrons in proportion to available oxygen and generation of ROS, such as hydrogen peroxide, oxygen ions and peroxides (Waypa et al., 2001). By using fluorescent dyes, increased levels of ROS have been directly detected during hypoxia in PASMC (Killilea et al., 2000; Liu et al., 2003). Further evidence comes from the use of inhibitors that

act on the ETC, such as rotenone and myxothiazol, mimicking the effects of hypoxia (Waypa et al., 2002). Hydrogen peroxide evokes intracellular signalling cascades that lead to sarcoplasmic reticulum calcium release (Lin et al., 2007) and rho-associated protein kinase activation (Knock et al., 2009). Taken together, these lines of evidence suggest that ROS act as second messengers to mediate HPV.

However, hypoxia is not the only way to generate ROS. It is established that hyperoxia leads to oxygen toxicity by the production of ROS *in vitro* and *in vivo* (Gore et al., 2010). For example, hydrogen peroxide release was found to be directly correlated with the oxygen concentration in porcine lung mitochondria (Turrens et al., 1982). Rat lung slices exposed to 85% oxygen for seven days had an increase in oxygen radicals from tissue and mitochondria (Freeman and Crapo, 1981). Similarly, antioxidant enzyme expression was also found to increase when rats were exposed to high levels of oxygen (Ho et al., 1996). Nevertheless, whether ROS produced by hyperoxia have the same effects as, or additive effects to, those produced by hypoxia is yet to be elucidated. As a high concentration of oxygen is commonly used in emergency conditions, for example in home oxygen treatment and cabin pressurisation systems, it is important to assess whether excessive oxygen produces ROS and causes unwanted physiological responses.

The aim of this study was to investigate the effects of hyperoxia on HPV at a level known to generate ROS. Specifically, this study was designed to test the following hypotheses: 1) 8-hour sustained hyperoxia would alter pulmonary vascular tone in healthy humans during the period of exposure; 2) 8 hour of sustained hyperoxia would augment HPV in healthy humans in response to an acute hypoxic challenge.

7.2 Methods

7.2.1 Participants

Eleven healthy volunteers (1 female, 10 males) were recruited to this study. Their average age was 22.5 years (SD = 3.4) with a range of 18 to 27 years. Average height and weight were 176.9 cm (SD = 5.4) and 67.9 kg (SD = 11.0). None had a history of respiratory, cardiac or other diseases. All volunteers visited the laboratory in advance of participation to confirm detectability of tricuspid regurgitation and discuss the experiment requirements and details. On each experimental day, participants were requested to refrain from alcohol and drinks containing caffeine. The female participant underwent in the experiment within the first 14 days of her menstrual cycle. Experiment information sheets were given in advance and consent was obtained from each volunteer before participation. This study was approved by The East Central London Research Ethics Committee 1 (Reference: 10/H0721/21)

7.2.2 Protocol

Figure 7.1 shows a schematic time course of the protocol. Each participant underwent two different 8-hour exposures in a customized chamber: 1) isocapnic hyperoxia, with $P_{ET}O_2$ held at 450 mmHg and $P_{ET}CO_2$ held at each volunteer's pre-exposure value; and 2) control, in which the volunteer's $P_{ET}O_2$ was held at 100 mmHg and $P_{ET}CO_2$ was held at volunteer's pre-exposure value. The details of gas control in the chamber are given in Chapter 2. The sequence of exposures was random to each volunteer and concealed from the volunteer. At least three days separated each individual's two exposures to

minimise any potential interactions.

The effects of sustained exposure were examined by measurements during the exposure and by acute hypoxic challenges before and after the exposure. Within 20 minutes of the start and end of each 8-hour exposure, the volunteer experienced an acute hypoxic challenge with three gas controlling steps: 1) isocapnic euoxia, with $P_{ET}O_2$ held at 100 mmHg and $P_{ET}CO_2$ held at the volunteer's pre-exposure value for 10 minutes; 2) isocapnic hypoxia, with $P_{ET}O_2$ held at 50 mmHg and $P_{ET}CO_2$ held at the volunteer's pre-exposure value for 10 minutes; 3) isocapnic euoxia, $P_{ET}O_2$ and $P_{ET}CO_2$ values were held as step 1 for 5 minutes. The details of gas control in the acute hypoxic exposure can be found in Chapter 2.

Throughout the acute hypoxic exposures each volunteer remained in the left lateral recumbent position and breathed through a mouthpiece with nose occluded to facilitate gas control and echocardiographic measurements.

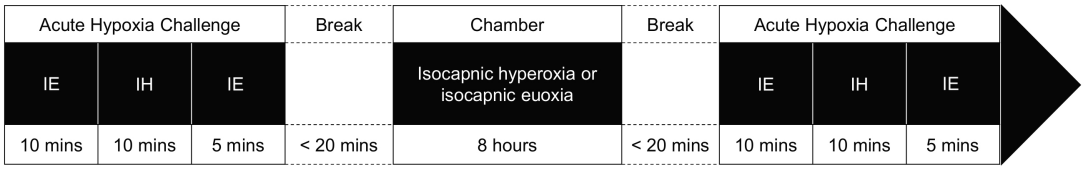

Figure. 7.1 **Schematic diagram of protocol.** IE, isocapnic euoxia; IH, isocapnic hypoxia.

7.2.3 Experimental techniques

SPAP, stroke volume and cardiac output were measured during the 8 hours of exposures, and acute hypoxic challenges, by echocardiography (Vivid i with 3S-RS transducer, GE Healthcare; Vivid q with M4S-RS transducer, GE Healthcare). These measurements were obtained in the chamber, and during the acute hypoxic challenges, at

hourly and minute intervals respectively. The measurements obtained at time 0 were called 'pre-exposure values'. In both chamber exposures and acute hypoxic challenges, S_{aO_2} and heart rate were measured by the same apparatus. S_{aO_2} was measured using a pulse oximeter (Ohmeda Biox 3740 Pulse Oximeter, BOC Healthcare) and a finger probe (OXY-F4-H Finger Sensor, GE Healthcare). Heart rate was measured through three-lead electrocardiogram (Micromon 7142 B ECG, Kontron Medical). Ventilation during the acute hypoxic challenges was measured by a turbine device (Ventilation Measurement Module, SensorMedics) and calculated by customized software, *BreathM*. All equipment was calibrated before each experiment.

7.2.4 Statistical Analyses

One-way ANOVA was used to examine the difference in studied variables between protocols (hyperoxia and control protocols) during 8-hour exposures. The same analytical method was applied to studied variables during acute hypoxic challenges before and after the exposures. The Bonferroni procedure was used in the multiple comparisons (post hoc analysis) with studied variables during acute hypoxic challenges before and after the exposures (hyperoxia and control protocols). The statistical significance level was set at 0.05. Values are mean ± SEM if not otherwise stated. The analysis was performed in IBM SPSS software package version 20.

7.3 Results

7.3.1 Participants

All of the 11 volunteers completed the study, and none reported significant discomfort from the chamber or acute hypoxic exposures.

7.3.2 Changes occurring during the 8-hour exposures

Gas control during the chamber is shown in Figure 7.2. In the hyperoxic protocol, volunteers' $P_{ET}O_2$ was increased to above 420 mmHg after 1.5 hours in the chamber. $P_{ET}O_2$ did not reach to the target level (450 mmHg) because of technical limitations. In the control protocol, volunteers' $P_{ET}O_2$ was maintained stably at 100 mmHg during 8 hours in the chamber. In both protocols, $P_{ET}CO_2$ of each volunteer remained constant during 8 hours, even though there was a slight fluctuation in the hyperoxic visit.

Cardiopulmonary responses to sustained hyperoxia are plotted in Figure 7.3. There was a significant difference between hyperoxic exposure and control in the S_{aO_2} (Hyperoxia: 98.1 ± 0.4%; Control: 96.8 ± 0.3%, p = 0.016) (Figure 7.3 E). However, no significant differences were found in SPAP, cardiac output, stroke volume or heart rate between two protocols (Figure 7.3 A to D).

7.3.3 Changes occurring during the acute hypoxic challenges before and after 8-hour exposure to hyperoxia

Gas control during acute hypoxic challenges prior to and post chamber exposures is shown in Figure 7.4. $P_{ET}O_2$ and $P_{ET}CO_2$ remained stable during acute hypoxic chal-

lenges.

Cardiopulmonary responses during acute hypoxic challenges prior to and post chamber exposures are shown in Figure 7.5. The SPAP during acute hypoxic challenges before and after 8-hour exposures appeared to be similar in both exposures (Figure 7.5 A). No differences were found between the hyperoxia and control protocols in cardiac output, stroke volume or heart rate (Figure 7.5 B to D).

After the 8-hour hyperoxic exposure, $S_{a}O_{2}$ during isocapnic euoxia was higher than before the exposure (Hyperoxia prior: 97.9 ± 0.3%; Hyperoxia post: 98.9 ± 0.3%, p = 0.045) (Figure 7.5 E). No significant difference was found in the control protocol. Although ventilation after 8 hours of hyperoxia exposures were higher than ventilation before the exposure, the difference was not statistically significant (Figure 7.5 F).

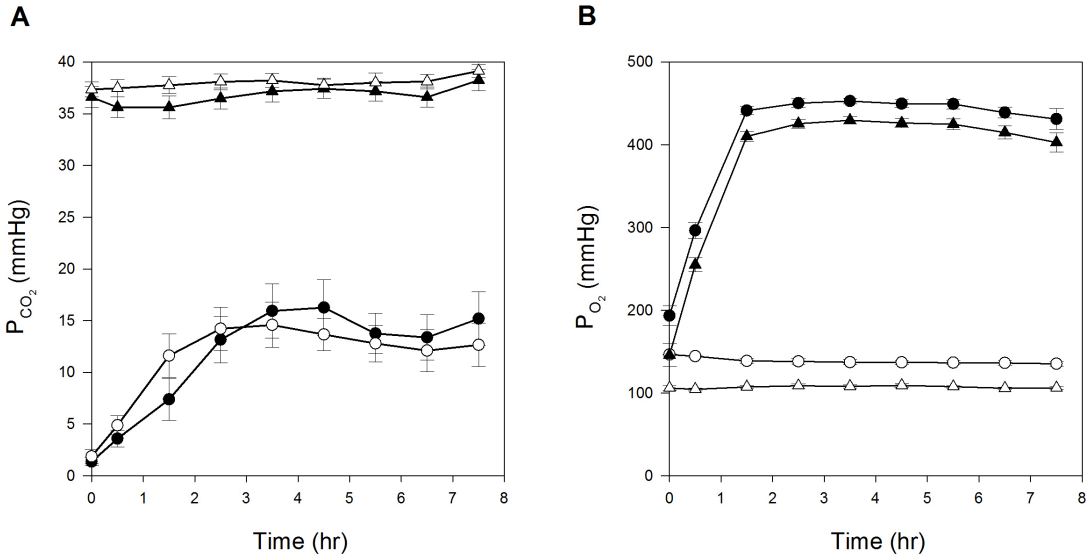

Figure. 7.2 **Control of end-tidal and inspired gases in chamber.** **A**. Gas control of P_{ICO_2} (*circles*) and P_{ETCO_2} (*triangles*); **B**. Control of P_{IO_2} (*circles*) and P_{ETO_2} (*triangles*). Data from hyperoxia and control protocols are coloured in black and white respectively. Values are means ± SEM.

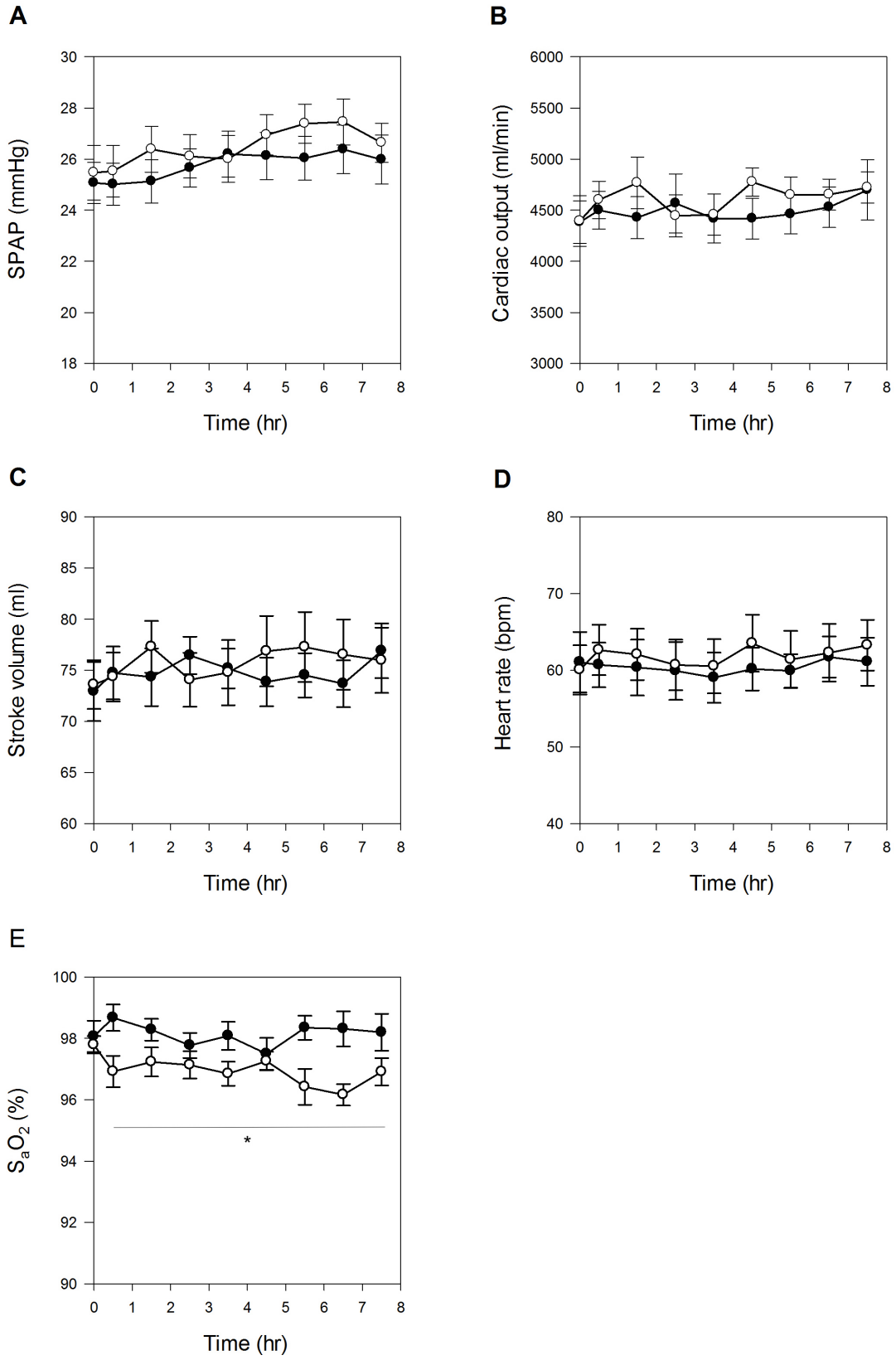

Figure. 7.3 **Cardiopulmonary responses during chamber exposures. A**. Systolic pulmonary artery pressure (SPAP); **B**. Cardiac output; **C**. Stroke volume; **D**. Heart rate; **E**. Oxygen saturation ($S_{a_{O_2}}$) were measured pre-exposure (0-hour) and at hourly intervals in hyperoxic (*filled circles*) and control (*empty circles*) exposures. Values are means ± SEM.
* $p < 0.05$

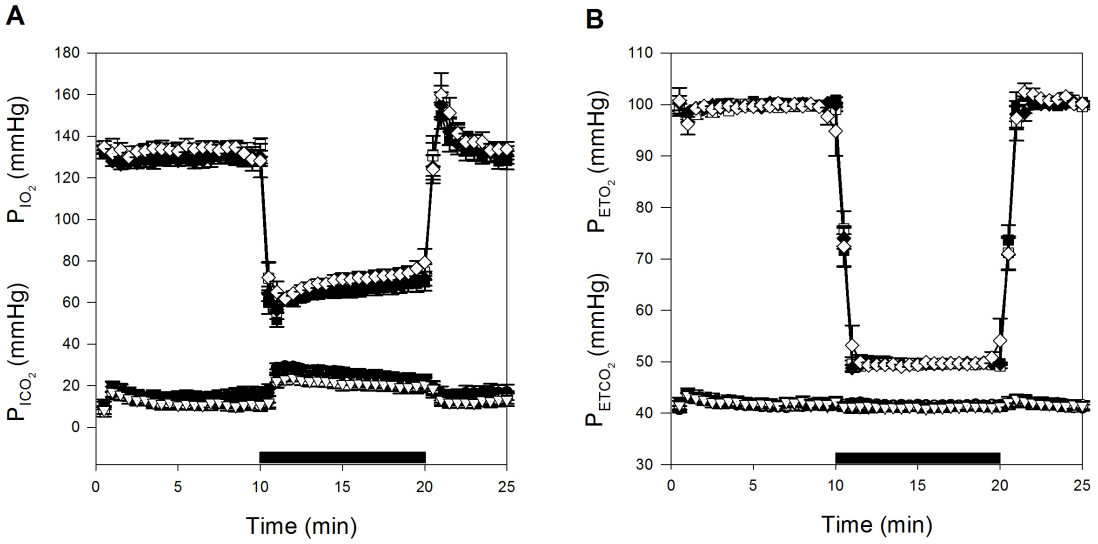

Figure. 7.4 **Control of end-tidal and inspired gases during acute hypoxic exposures before and after chamber. A**. Control of P_{ICO_2} (Prior: *circle*; Post: *triangle*) and P_{IO_2} (Prior: *square*; Post: *diamond*); **B**. Control of P_{ETCO_2} (Prior: *circle*; Post: *triangle*) and P_{ETO_2} (Prior: *square*; Post: *diamond*) were averaged at 30-second intervals during acute hypoxic exposures before and after the chamber exposure. Data from hyperoxia and control protocols are shown as *filled* and *empty* symbols respectively. The black bar shows the period of isocapnic hypoxia. Values are means ± SEM.

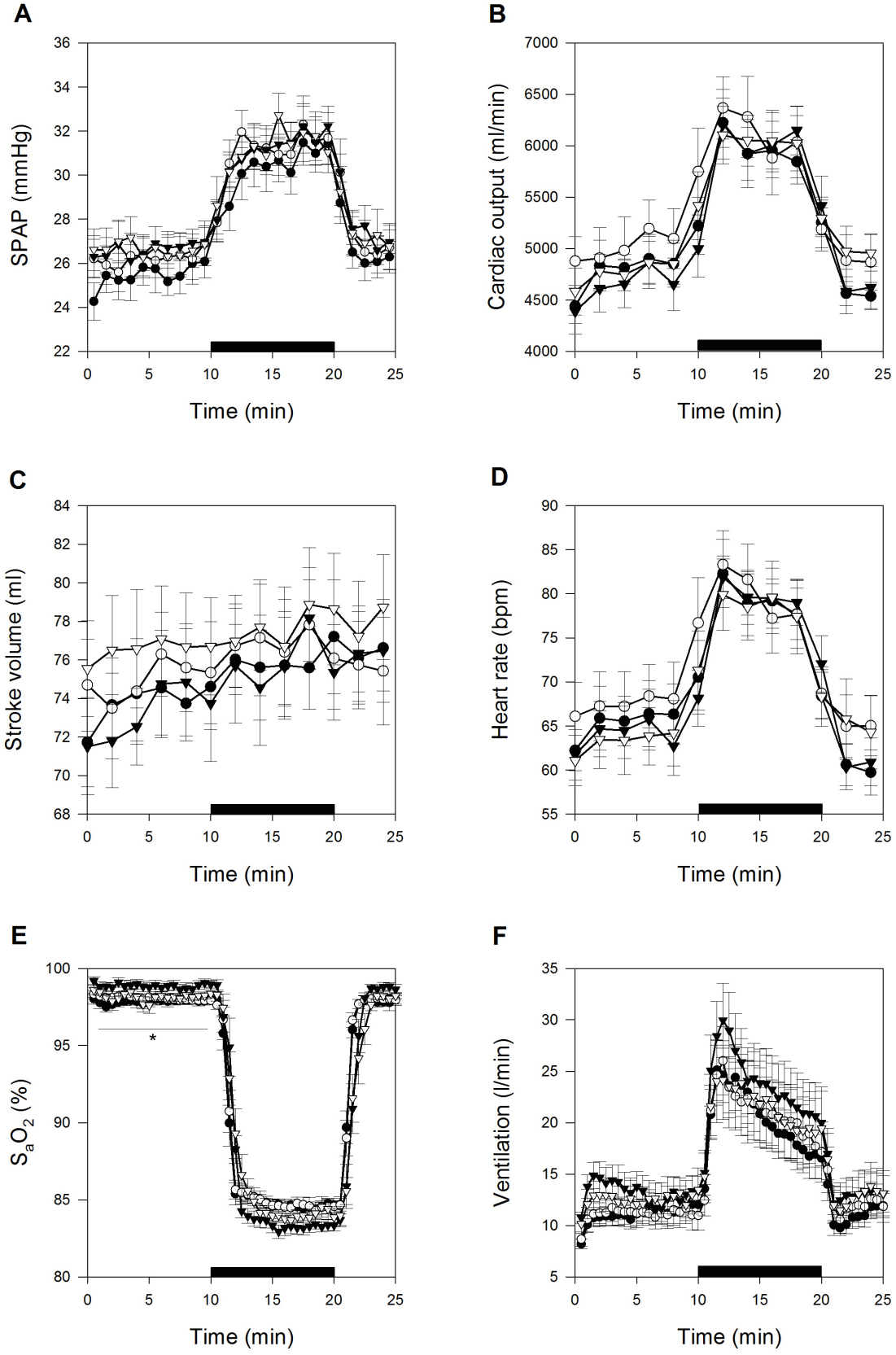

Figure. 7.5 **Changes in cardiopulmonary responses during acute hypoxic exposure before and after the chamber exposure.** **A**. Systolic pulmonary artery pressure (SPAP); **B**. Cardiac output; **C**. Stroke volume; **D**. Heart rate; **E**. Oxygen saturation ($S_{a}O_{2}$). Data from hyperoxia (*filled*) and control (*empty*) protocols are labelled by symbol filling; data from prior to (*circles*) and post (*triangles*) 8-hour exposures are labelled by symbol shape. The black bar shows the period of isocapnic hypoxia. Values are means ± SEM.

* $p < 0.05$ when compared between prior to and post hyperoxia exposures.

7.4 Discussion

In this study, we have shown that $S_{a}O_{2}$ was higher during an 8-hour isocapnic hyperoxia exposure than in an 8-hour euoxic exposure. However, no significant changes in SPAP, cardiac output, heart rate or stroke volume were detected during the exposure. Based on the mitochondrial ROS hypothesis, ROS are the second messengers mediating hypoxia signalling and hypoxic responses in the pulmonary circulation (Waypa and Schumacker, 2004). According to this scheme, if ROS were to increase, an increase in PAP might have been observed during the 8-hour hyperoxic exposure or a change seen in the acute hypoxic challenge after incubation with hyperoxia.

Previous studies have shown that inhaling oxygen has minimal effects on PAP in animals (Euler and Liljestrand, 1946), healthy humans (Marshall et al., 1961) and patients with ventricular septal defect (Marshall et al., 1961) and pulmonary vascular obstructive disease (Krongrad et al., 1973). Furthermore, patients with diseases that have been commonly thought to be mediated by ROS, such as chronic obstructive pulmonary disease (COPD) (Fletcher and Levin, 1984) and pulmonary hypertension (Shepherd et al., 1957), were found to have a slight decrease or no change in PAP while breathing oxygen. Similarly, no change in cardiopulmonary responses attributable to the hyperoxic exposure was detected in this study, despite changes in $S_{a}O_{2}$. In addition, the hyperoxic preconditioning did not change the responses of cardiopulmonary responses during the acute hypoxia challenges either. Together these question whether the excessive oxygen in the exposures has been transformed into ROS or ROS was eliminated by other pathways. A limitation of this study was the lack of direct measurement of ROS in the body. Moreover, ROS have a short half-life (Forkink et al.,

2010). A short break (less than 20 minutes) between the 8-hour exposure and the acute hypoxic challenges might have reduced the levels of ROS present in the body, which were not able to affect physiological responses to hypoxia. Nevertheless, a further study is required to clarify ROS metabolism and relevant pathways in humans.

Ventilation after the 8-hour hyperoxic exposure increased marginally during the isocapnic euoxia and hypoxia of acute hypoxia challenges, but this was not statistically significant. Several studies have noticed that isocapnic hyperoxia induces hyperventilation (Dean et al., 2004) and alters hypoxic ventilatory sensitivity (Ren et al., 2000a). The underlying mechanism was suggested by Becker and colleagues as the Haldane effect (Becker et al., 1996). The Haldane effect describes that nonoxygenated haemoglobin has a higher CO_2 transport capacity than oxygenated haemoglobin (Lumb and Nunn, 2010). As hyperoxia induces a degree of hypercapnia (Dean et al., 2004), isocapnic hyperoxic exposure thus induces a hyperventilatory response via distrupted CO_2 transport from excessively oxygenated haemoglobin. This seems to be consistent with our finding that a higher $S_{a}O_2$ and greater ventilation occur during isocapnic euoxia during the acute hypoxia challenges after the 8-hour hyperoxic exposure. However, the insignificant results can be caused by a small number of participants and short breaks between the 8-hour exposures and the acute hypoxia challenges. In addition, it would have been more clear if ventilatory responses were measured during the 8-hour exposures.

In conclusion, despite an isocapnic hyperoxic exposure leading to the increased $S_{a}O_2$, it did not have effects on SPAP, cardiac output, heart rate or stroke volume during the 8-hour exposure, or alter the responses of these variables during acute hypoxic

challenges. These results do not support a role for hyperoxia-induced ROS in HPV in humans. Future work will need to apply other methods or protocols to examine any role of ROS in the human pulmonary circulation.

Chapter 8

Conclusions and future work

8.1 Introduction

The main objective of this thesis has been to investigate the involvement of the HIF pathway in the increased RV afterload during exercise in healthy people aged between 50 and 80 years by considering & altering their iron status. This has been accomplished in a number of ways. Firstly, some of the basal relationships that may affect the HIF pathway and exercise capacity during ageing, i.e. erythropoiesis and iron homeostasis, were investigated in Chapter 3 and 4. Secondly, the inhibition of the HIF pathway by a single intravenous iron infusion (ferric carboxymaltose) in healthy people aged between 50 and 80 years was examined in Chapter 5. In Chapter 6, the effects of inhibition of the HIF pathway on the RV afterload during light exercise and exercise capacity during heavy exercise in these healthy people were then explored. A final minor objective of this thesis has been to examine the involvement of ROS in HPV in humans with hyperoxia exposure.

8.2 Ageing, iron homeostasis, erythropoiesis and exercise capacity

The results in Chapter 3 demonstrated that ageing did not significantly affect the concentrations of haematological variables or iron status indicators. These suggest that iron homeostasis and erythropoiesis are not altered by ageing in healthy participants or can be compensated for by other pathways. Although it may be the result of a selected population, this suggests that modern humans with sufficient nutrition and medical care can maintain a normal range of haematological variables and iron status indicators up to 80 years old.

Chapter 4 revealed that the impaired exercise capacity was possibly associated with age and the inflammatory indicators, CRP (both male and female participants) and ferritin (female participants only). In addition, the iron status indicator levels and haemoglobin concentration were not correlated with exercise capacity in our participants, which minimised the potential bias and hidden assumptions for the later studies shown in Chapter 5 and 6. Although there could be other reasons for the decline in exercise capacity with age, a future direction to take would be investigating the existence of a link between exercise capacity decline and inflammatory response.

8.3 HPV during exercise in older people

The results from Chapter 6 demonstrated that older people presented a significant increase in SPAP during exercise and that the increase was coupled with age. One of the possible causes for this is hypothesised as being the low oxygen tension in the mixed-

venous blood, suggesting that the underlying mechanism is similar to HPV. Marshall *et al*. have demonstrated that HPV does not only result from hypoxic gas exposure, but also low oxygenation of mixed-venous blood in anaesthetised dogs (Marshall et al., 1994). Furthermore, the observation of lower oxygenation of blood during exercise in older people (Prefaut et al., 1994; Proctor et al., 1998) supports the possibility of this hypothesis. A critique for this hypothesis is that a dose-response relationship between alveolar hypoxia and mixed venous hypoxaemia has not been established in humans. So the magnitude of HPV caused by the oxygen tension in the mixed-venous blood has not yet been confirmed. However, it is ethically difficult to manipulate mixed venous oxygenation in living humans and study the relationship. If a way can be found to do this, the results would be of great interest.

The investigation in this thesis applied a novel method, iron infusion, to examine whether the increase in SPAP during exercise in older people is HPV-related. If the underlying mechanism is similar to HPV, antagonists to the key underlying mechanism, such as the HIF pathway, for HPV should cause certain effects. In this study, iron infusion (ferric carboxymaltose) was chosen to antagonise the HIF pathway through acting on PHD or FIH, which has been demonstrated to blunt hypoxic pulmonary vascular responses by Smith *et al*. (Smith et al., 2008a). Indeed, the results from Chapter 6 demonstrated that a single intravenous dose of iron (ferric carboxymaltose) suppressed the increase in SPAP during light exercise in our participants, and that this effect lasted from 23 hours to 8 weeks. In addition, data from Chapter 5 showed that the production of EPO (HIF-2 downstream effector) was suppressed from 23 hours to 7 days. These findings support the idea that the increase in SPAP during exercise in

older people is at least partially modulated by the HIF pathway. A limitation of these studies is lack of direct measurement in the expression of the HIF and its regulators in the pulmonary circulation. Therefore, it is yet to be confirmed whether iron infusion acts on the proposed mechanism or other pathways.

However, there are a few possible directions that can be taken in the future. First to consider is the modulation of carbon dioxide and pH in pulmonary vascular tone during exercise. With increase in exercise intensity, hypocapnia and metabolic acidosis occur (MitchelI et al., 1958). Balanos *et al*. has demonstrated that hypocapnia causes a decrease in pulmonary vascular resistance in humans in a 4-hour gas-control chamber with Doppler echocardiographic measurement (Balanos et al., 2003). In addition, Loeppky *et al*. showed that metabolic acidosis enhanced the pulmonary vascular response to hypoxia (Loeppky et al., 1992). Furthermore, hyperventilation and metabolic acidosis during exercise is more severe in older people, compared with younger people (Prioux et al., 2000). The way that hypocapnia and metabolic acidosis interact with each other and thus modulate pulmonary vascular response during exercise in older people should also be explored.

A second factor to investigate in the future is nitric oxide involvement during exercise. Nitric oxide is a vasodilator, and inhaling nitric oxide reversed hypoxic pulmonary vasoconstriction in humans and animals (Frostell et al., 1993). Furthermore, systemic nitric oxide synthesis increases during exercise in humans (Bode-Boger et al., 1994). Whether the increase in nitric oxide synthesis during exercise affects pulmonary vasoconstriction in older people remains to be elucidated. Furthermore, ageing has been associated with marked impairment of NO generation by increas-

ing plasma concentrations of the NO synthase inhibitor, asymmetric dimethylarginine (ADMA) (Sverdlov et al., 2014). Whether increased SPAP during exercise in older people is related to defected nitric oxide synthesis should be clarified in the future.

8.4 Left atrial pressure during exercise in older people

Another main hypothesis in this thesis is that the significantly high levels of SPAP during exercise in older people limits their ability to exercise by reducing cardiac output. Although increase in SPAP during exercise in older people was attenuated by iron infusion as seen in Chapter 6, no relevant changes were seen in terms of stroke volume, cardiac output and exercise capacity. The conclusion for this cause is that the attenuation of SPAP by iron infusion was too small to cause an effect on cardiac output and exercise capacity. However, another cause for this could be the result of the venous side of pulmonary circulation (left heart) since we only observed and manipulated the arterial side of pulmonary circulation (right heart). In other words, left atrial pressure could remain high during exercise in older people, even though we managed to reduce the SPAP during exercise. Hence, although the SPAP was reduced, resistance across pulmonary circulation still stayed the same and cardiac output remained unchanged because left atrial pressure was high. It would have been clearer if this study could include left atrial pressure measurement. However, it would require invasive procedures, such as cardiac catheterisation, since there is no reliable non-invasive technique available at the moment. Future work should focus on the development of non-invasive techniques for left atrial pressure measurement.

8.5 Hyperoxia, ROS and HPV in humans

The results in Chapter 7 did not support the mitochondrial ROS hypothesis in the human model, which describes how ROS mediates HPV. Although participants were exposed to a high concentration of oxygen for a long period of time, a criticism of this study is the lack of direct measurement of ROS in the pulmonary circulation or blood. Therefore, it is hard to be convinced that the treatment of such hyperoxia causes the production of ROS in humans. Furthermore, it is yet to be confirmed whether ROS is metabolised by other pathways if hyperoxia induces ROS production in humans. A large amount of literature demonstrating the ROS hypothesis tends to related to *in vitro* work and this hypothesis has barely been addressed *in vivo* and in humans. Future work should concentrate on the investigation of detailed ROS metabolisms to clear the controversy over the relationship between cellular mechanisms and whole animal or human responses.

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Appendix A

Participant information sheet 1

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Participant Information Sheet and Letter of Invitation

Research study: Effects of iron on exercise capacity in men and women above 50. (Study A)

We would like to invite you to take part in our research study. Before you decide, we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. We'd suggest this should take about 15 minutes. Talk to others about the study if you wish. **Part 1** tells you the purpose of this study and what will happen to you if you take part. **Part 2** gives you more detailed information about the conduct of the study. This study is to detect whether there is a relationship between iron level in the blood and exercise capacity. Ask us if there is anything is not clear.

Part 1

1. What is the purpose of the study?

We would like to understand more about the relationship between the level of iron in the blood and exercise capacity in older people. Specifically, we are interested in whether iron levels in the body have an association with maximum work rate and maximum oxygen consumption during exercise in volunteers aged between 50 and 80. This study is also being undertaken as part of a PhD project.

2. Why have I been invited?

We are looking for 110 healthy male and female volunteers aged between 50 and 80 to take part in this study. The study requires volunteers who are not smokers, overweight, epileptic, sufferers of severe cardiac or respiratory disease, taking any medicines that may influence exercise ability, or having iron therapy recently.

3. Do I have to take part?

It is up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason.

4. What will happen to me if I take part?

We will discuss the information sheet with you, explain the experimental procedure to you and show you the equipment. To ensure that you are suitable for the experiment, the researcher in charge will ask some simple questions and then ask you to sign a consent form. Female participants up to age 55 will receive hCG exam before experiment to make sure whether you are pregnant. After that, we will take you through the following procedures:

Echocardiographic assessment

This will involve the use of an ultrasound machine to capture images of your heart. It is a non-invasive procedure, but will involve the use of some gel on your skin to help the conduction of the sound waves from the probe. This assessment is to tell us whether that we can obtain the measurements that form a later study B, which we expect to find in 70% of volunteers. You can decide not to do it without any reason.

Blood sampling

We will take a small blood sample (~20 ml = 4-6 teaspoonfuls) from a vein for blood analysis.

Exercise Capacity Examination

We will place three electrodes on your skin which we will record an electrocardiogram (ECG) to monitor your heart activity. Then we will ask you to sit on a cycle ergometer (a cycle machine used in the lab) and breathe normal air through a mouth piece while we take the ECG reading. During the experiment, we will ask you to maintain 40-60 revolutions per minute (RPM) on the ergometer, while the resistance is increased over time, until you are no longer able to maintain the RPM.

The study flow chart can be referred to the appendix on the last page.

The above procedures will take about one to two hours, depending on the duration of exercise. There is only one visit for this study.

5. Expenses and payments

We will pay you modestly £10 per hour for the time you have given up, and will reimburse any necessary travelling expenses.

6. What will I have to do?

You should discuss with one of our team whether you are eligible to take part in this research if you are taking part in other drug studies, or therapy, or have been in the recent past (less than three months) These drug studies and therapy may influence your iron level in the body and exercise capacity. In addition, female participants should inform us whether you are pregnant because pregnancy also influences your iron level and exercise performance. If you decide to take part, you will be asked to bring suitable clothes to exercise in and not to undergo any strenuous exertion, or consume any caffeine or alcohol on the day of the experiment. In addition, you should keep the same lifestyle and not attend any heavy exercise training on the day before the day of the experiment.

7. What are the possible disadvantages and risks of taking part?

The experiments should involve no major discomfort. You may feel uncomfortable during the blood sampling. However, if you begin to feel uncomfortable and at all unwell, you can tell us and we will stop the experiment.

8. What are the possible benefits of taking part?

There is no intended clinical benefit to participants in this research. However, the information we get from this study will improve our understanding of exercise physiology in people aged 50-80 years.

9. What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

10. Will my taking part in this study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

This completes Part 1.

Part 2

11. What will happen if I don't want to carry on with the study?

If you withdraw from the study, we will need to use your identifiable samples and the data collected up to your withdrawal.

12. What if there is a problem?

The University has arrangements in place to provide for harm arising from participation in the study for which the University is the Research Sponsor.

Complains statement:

If you wish to complain about any aspect of the way in which you have been approached or treated during the course of this study, you should contact Mr. Hung-Yuan Cheng (01865 272 487; hung-yuan.cheng@dpag.ox.ac.uk) or you may contact the University of Oxford Clinical Trials and Research Governance (CTRG) office on 01865 857939 or the head of CTRG, email: heather.house@admin.ox.ac.uk.

13. Will my taking part in this study be kept confidential?

Yes. All information which is collected about you during the course of the research will be kept strictly confidential. Your results will be coded with a participant number and no personal information will be attached to the data. This anonymisation will occur at the point of data collection. Your personal details will be stored in the laboratory on paper for contact. Only our team members, sponsors and regulatory authorities can access to this information. The overall results of the study may be published in scientific journals. However, all personal data will remain confidential, and no data relating to individual participants will be published. All information will be removed in 12 months after the study has ended.

14. Will my General Practitioner/Family doctor (GP) be informed that I am participating in the study?

We would only be in contact with your GP during the study if we had serious concerns about the state of your physical health or any adverse events occurring during the visit.

15. What will happen to any samples I give?

Part of samples you give will be sent to John Radcliffe Hospital, Oxford, for blood analyses and another part will be stored temporarily in the Department of Physiology, Anatomy and Genetics, Sherrington Building, University of Oxford for later analyses. These analyses will give us the information of red blood cell number, iron concentration, and other iron-related protein concentration in your blood samples. These samples will be disposed of or destroyed after the end of the study and will not be used for genetic research.

16. What will happen to the results of the research study?

We expect the results of this research to be published in an international scientific journal. You will not be identified in any report or publication. We could provide you with a copy of any such publication or a lay summary of the research results.

17. Who is organising the research and funding the study?

The study is being organised by researchers in the Department of Physiology, Anatomy & Genetics, which is part of the University of Oxford. The funding of this study is provided by Dunhill Medical Trust.

18. Who has reviewed the study?

All medical research in the UK is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by NRES Committee South Central-Oxford B (11/SC/0221).

Contacts for Further Information

If you would like any further information on this study, or if you have any concerns, please contact:

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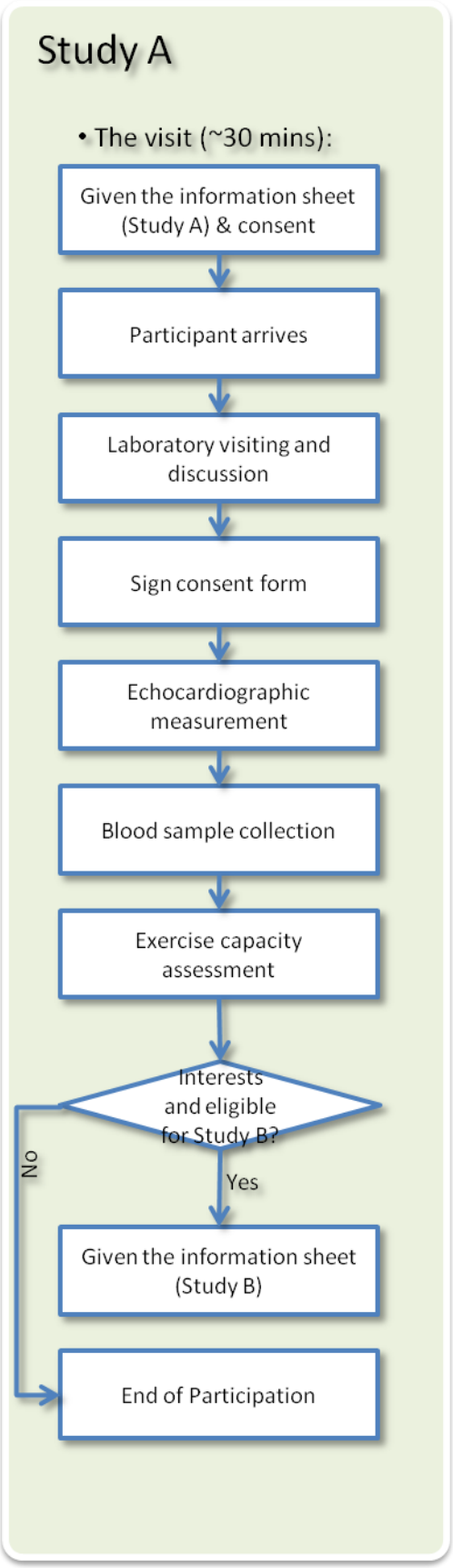
Thank you for your consideration.

Appendix
Study Flow Chart



Appendix B

Participant information sheet 2

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Participant Information Sheet and Letter of Invitation

Research study: Effects of iron on exercise capacity in men and women above 50. (Study B)

We would like to invite you to take part in our research study. Before you decide, we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. We'd suggest this should take about 15 minutes. Talk to others about the study if you wish. **Part 1** tells you the purpose of this study and what will happen to you if you take part. **Part 2** gives you more detailed information about the conduct of the study. This study is to detect whether increasing iron levels in the body is able to improve exercise capacity. Ask us if there is anything is not clear.

Part 1

1. **What is the purpose of the study?**
   We would like to understand more about the relationship between the level of iron in the blood and exercise capacity in older people. Specifically, we are interested in whether increasing iron levels in the body could alter maximum work rate and maximum oxygen consumption during the exercise in the group of human volunteers aged between 50 and 80. This study is also being undertaken as part of a PhD project.

2. **Why have I been invited?**
   We are looking for 60 healthy male and female volunteers aged between 50 and 80 to take part in this study. The study is looking for volunteers who attended Study A and whose echocardiographic assessment results and blood results meet the requirements of the experiment. The exclusion criteria are listed below.

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Exclusion Criteria

- The participant is a smoker.
- The participant is not able to perform normal cycling exercise.
- The participant has severe cardiovascular or pulmonary disease.
- The participant has recently lived at high altitude.
- The participant is taking iron supplements or drugs that might markedly influence exercise capacity and the pulmonary circulation, such as calcium channel blockers, nitrates, beta-blockers etc.
- The participant has iron repletion or iron overload (ferritin >800 µg/l, or 500-800 µg/l with transferrin saturation >50%).

3. Do I have to take part?

It is up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason.

4. What will happen to me if I take part?

You will be asked to visit the laboratory on five occasions, on the first day and on four other days at 23 hours, 1 week, 4 weeks and 8 weeks after an intravenous infusion on the first day. The study flow chart can be referred to on the last page.

First day at the laboratory

On the first day, we will ask you to allow us to measure heart rate, blood pressure, body temperature and take a small blood sample (~20 ml = 4-6 teaspoonfuls) from a vein. After that, we will take you through the following procedures:

Steady cycle exercise with echocardiographic measurement
You will be asked to exercise on a cycle ergometer (an exercise bike that is able to measure work output) with a work rate corresponding to an increase in heart rate of ~30 beats per minute above its resting value for 20 minutes. During the exercise, you will be asked to breathe normal air through a mouth piece with an ECG reading being made and an echocardiographic measurement being taken.

Exercise capacity examination with cardiac output measurement
One hour after undertaking the steady cycle exercise, you will be asked to do an exercise capacity examination on the cycle ergometer with cardiac output and breathe from a rubber bag filled with 20% of nitrous oxide in oxygen repeatedly for less than 30 seconds. This examination in total takes about 10-15minutes.

Iron or saline infusion
After the steady cycle exercise and exercise capacity examination, you will receive an infusion (a slow injection) which will last for about 30 minutes. It could contain iron (Ferric Carboxymaltose; Ferinject®) or be a simple salt solution (saline).

Steady cycle exercise with echocardiographic measurement & exercise capacity examination with cardiac output measurement
At 3 hours after the infusion, you will be asked to perform the steady cycle exercise again. At 4 hours after the infusion, you will be asked to do exercise capacity examination again.

At the end of the experiment, we will take another small blood sample (~20 ml = 4-6 teaspoonfuls) again. In all, the first visit may take around 8 hours. We shall provide you with light refreshments during your visit.

Second and subsequent visits at the laboratory
You will be asked to visit the laboratory on four more days, specifically 23 hours, 1 week, 4 weeks and 8 weeks after the iron infusion was given. Each of these visits will consist of taking of a blood sample (~20 ml = 4-6 teaspoonfuls), a period of steady cycle exercise with echocardiographic

measurement, followed by an exercise capacity examination with cardiac output measurement. Each visit may take around 3 hours.

5. Expenses and payments

We will pay you modestly £10 per hour for the time you have given up, and will reimburse any necessary travelling expenses.

6. What will I have to do?

You should discuss with one of our team whether you are eligible to take part in this research if you are taking part in other drug studies, therapy or have been in the recent past (less than three months) because these drug studies and therapy may influence your iron level in the body and exercise capacity. If you decide to take part, you must be able to come into the laboratory five times over an 8 week period for a number of hours each time, specifically 8 hours on the first day and then around 3 hours on four other days, which will be 23 hours, 1 week, 4 weeks and 8 weeks after the first day. In addition, you will be asked to bring suitable clothes to exercise in and not to undergo any strenuous exertion, or consume any caffeine or alcohol on the day of the experiment. You should keep the same lifestyle and not attend any heavy exercise training on the day before the day of the experiment.

Pregnancy (For women only)

Iron (Ferric Carboxymaltose; Ferinject®) crosses the placenta in animal studies and may influence skeletal development in the foetus. Therefore, you must not take part in this study if you are pregnant or you suspect that you might be pregnant, or you are trying to become pregnant. If you join the study and are aged 50-55 years, you will be asked to do a pregnancy test before the study begins.

7. What are the possible disadvantages and risks of taking part?

The experiments should involve no major discomfort. However, it is possible that cycle exercise could give you fatigue, and make you feel a bit sick. If you begin to feel unwell you should tell us and we can stop the experiment. In addition, taking part in this study is time consuming. It involves five visits to the Department of Physiology in Oxford.

8. What are the side effects of any treatment received when taking part?

It is unlikely that the intravenous iron infusions (Ferric Carboxymaltose; Ferinject®) would cause significant discomfort, although it has possible side-effects including gastro-intestinal disturbances, headache, dizziness, rash, injection-site reactions. Less commonly, hypotension (low blood pressure), flushing, chest pain, peripheral oedema (swelling), hypersensitivity reactions (including anaphylaxis), fatigue, paraesthesia (tingling sensations), malaise, pyrexia (fever), rigors, myalgia (muscle pain), arthralgia (join pain), back pain, pruritus (itching), and urticaria (skin swelling) have been reported, and dyspnoea (breathlessness) is happened rarely.

The re-breath of nitrous oxide in this study should not cause significant discomfort because the expose time is very short (less than 30 seconds with a few breathes). In addition, the toxicity of nitrous oxide to normal healthy humans is minimal unless there are chronic repeated exposes. However, this procedure may cause dizziness, headache, tachycardia (high heart rate), tachypnoea (fast breathing), nausea and vomiting (rarely).

9. What are the possible benefits of taking part?

There is no intended clinical benefit to participants in this research. However, the information we get from this study will improve our understanding of exercise physiology in people aged 50-80 years.

10. What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

11. Will my taking part in this study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

This completes Part 1.

Part 2

12. What if relevant new information becomes available?

Sometimes during the course of the study, new information becomes available on the drugs/condition being studied. If this happens, we will tell

you about it and discuss with you whether you want to or should continue in the study. If you decide to continue in the study, you will be asked to sign an updated consent form. On receiving new information, we might consider it to be in your best interests withdraw you from the study. If so, we will explain the reasons and arrange for your care to continue. If the study is stopped for any other reason, you will be told why.

13. What will happen if I don't want to carry on with the study?

If you withdraw from the study, we will destroy all your identifiable samples, but we will need to use the data collected up to your withdrawal.

14. What if there is a problem?

The University has arrangements in place to provide for harm arising from participation in the study for which the University is the Research Sponsor.

Complains statement:
If you wish to complain about any aspect of the way in which you have been approached or treated during the course of this study, you should contact Mr. Hung-Yuan Cheng (01865 272 487; hung-yuan.cheng@dpag.ox.ac.uk) or you may contact the University of Oxford Clinical Trials and Research Governance (CTRG) office on 01865 857939 or the head of CTRG, email: heather.house@admin.ox.ac.uk.

15. Will my General Practitioner/Family doctor (GP) be informed that I am participating in the study?

We will inform your GP of your participation before the experiment starts. In addition, we will be in contact with your GP during the study if we have serious concerns about the state of your physical health or any adverse events occurring during the visits.

16. Will my taking part in this study be kept confidential?

Yes. All information which is collected about you during the course of the research will be kept strictly confidential. Your results will be coded with a participant number and no personal information will be attached to the data. This anonymisation will occur at the point of data collection. Your personal details will be stored in the laboratory on paper for contact. Only our team members, sponsors and regulatory authorities can access to this information. The overall results of the study may be published in scientific journals. However, all personal data will remain confidential, and no data

relating to individual participants will be published. All information will be removed in 12 months after the study has ended.

17. What will happen to any samples I give?

Part of samples you give will be sent to John Radcliffe Hospital, Oxford, for blood analyses and another part will be stored temporarily in the Department of Physiology, Anatomy and Genetics, Sherrington Building, University of Oxford for later analyses. These analyses will give us the information of red blood cell number, iron concentration, and other iron-related protein concentration in your blood samples. These samples will be disposed or destroyed after the end of the study and will not be used for genetic research.

18. Will any genetic tests be done?

No.

19. What will happen to the results of the research study?

We expect the results of this research to be published in an international scientific journal. You will not be identified in any report or publication. We could provide you with a copy of any such publication or a lay summary of the research results.

20. Who is organising and funding the study?

The study is being organised by researchers in the Department of Physiology, Anatomy & Genetics, which is part of the University of Oxford. The funding of this study is provided by Dunhill Medical Trust.

21. Who has reviewed the study?

All medical research in the UK is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by NRES Committee South Central-Oxford B (11/SC/0221).

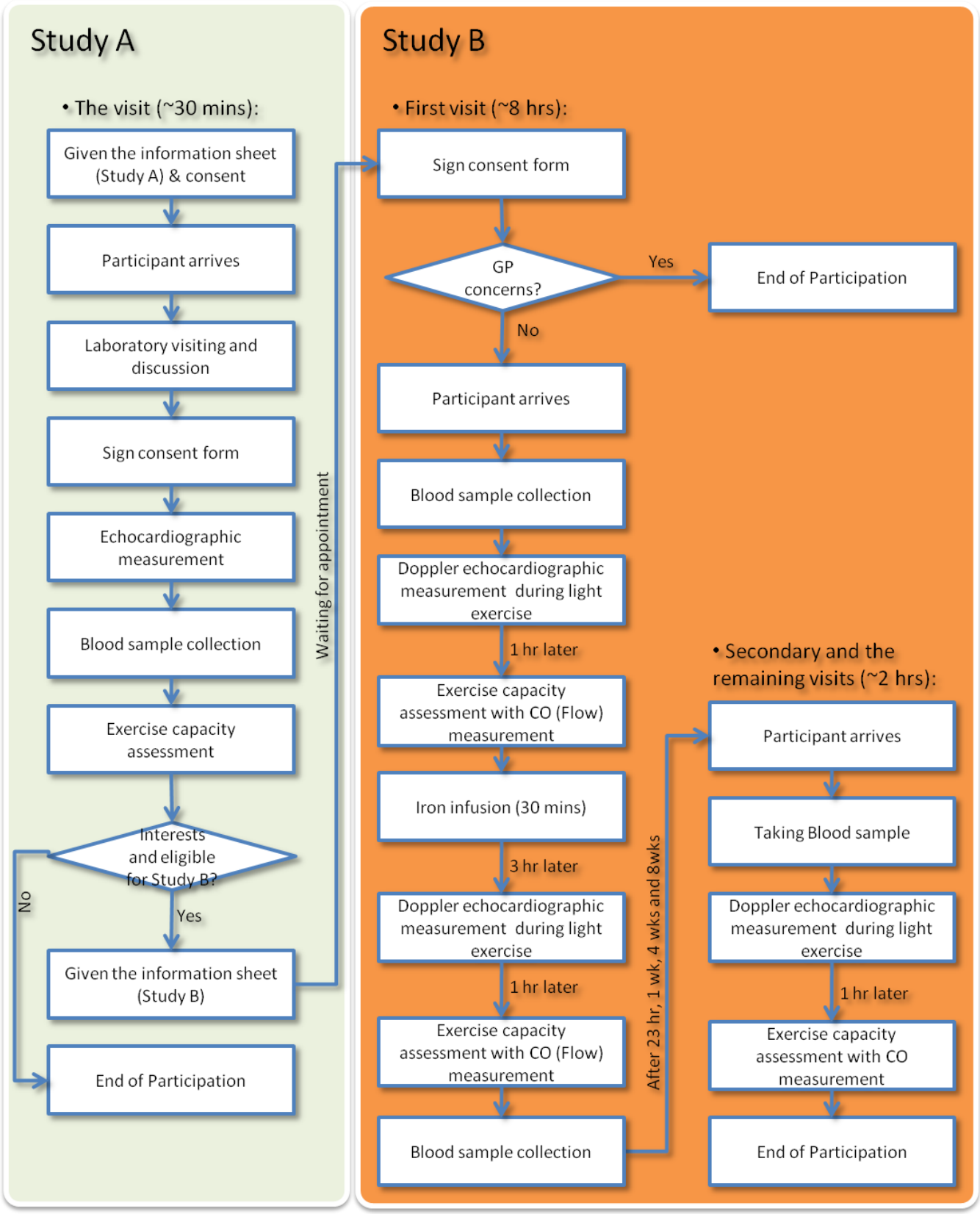
Contacts for Further Information

If you would like any further information on this study, or if you have any concerns, please contact:

Mr Hung-Yuan (Vincent) Cheng
Tel: 01865 272487
Email: hung-yuan.cheng@dpag.ox.ac.uk
Address: Department of Physiology, Anatomy and Genetics, Sherrington Building, Parks Road, Oxford OX1 3PT, UK

Thank you for your consideration.

Appendix
Study Flow Chart



Appendix C

Participant information sheet 3

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Quentin Croft
Web site: http://www.physiol.ox.ac.uk/~par/
E-mail: Quentin.croft@hotmail.com
01865 272487

Dear

A study of the pulmonary arterial response to hyperoxia

(A study of a lung response to high oxygen levels)

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. ***One of our team will go through the information sheet with you and answer any questions you have.*** *Talk to others about the study if you wish.*
If you agree to take part in the study you will be invited to come into the lab. three times: once for a preliminary assessment and on two full days for the main experiments.

What is the purpose of the study?
The purpose of the study is to measure the response of the blood vessels in your lungs to high oxygen levels.

Why have I been chosen?
You have volunteered for this experiment. The study requires 10 healthy volunteers who are not smokers, overweight, pregnant, epileptic, sufferers of sever cardiac or respiratory disease or taking alpha- or beta-blockers. Volunteers must be able to spare two days of their time for experiments.

What would happen on my first visit to the Laboratory?
We will discuss the experiment with you, including this information letter, show you the equipment and conduct an echocardiographic assessment of your heart. The ultrasound machine bounces sound waves off your heart that are detected and used to create an image of your heart as it beats. It is a non-invasive procedure, but will involve the use of some gel on your skin to help conduct the sound waves from the probe. This preliminary check is to ensure that we can obtain the measurements that we require; these are possible in roughly 50% of volunteers.

What would happen during the experiments?
You will be asked not to undergo any strenuous exertion, or consume any caffeine or alcohol, on the day you come into the laboratory. In addition, you will be asked to sit quietly for a few minutes before experiments start.

We will place three electrodes on your skin from which we will record an electrocardiogram (ECG: a recording of heart activity). We will then ask you to breathe low-oxygen gas through a mouthpiece for 30 minutes, during which we will take echocardiographic readings of your heart.

You will then be asked to sit in a sealed chamber for eight hours. The oxygen levels in your blood will be monitored using a clip on your finger called a pulse oximeter; this shines a light through your finger to measure changes in blood colour. You will have a small tube (a nasal catheter) attached under your nose to measure the gases you breathe out. On one day for all eight hours the gas in the chamber will have high oxygen levels; on the other day you will breathe normal air. We will also take ECG and echocardiographic recordings after the first half hour and at hourly intervals; you will wear the ECG electrodes throughout the day. Apart from these measurements you will be free to read, watch television

or otherwise relax. You may leave the chamber to go to the toilet. After the eight hours in the chamber we will repeat the 30-minute test on the mouthpiece.
The experiments should be done in the first half of all females' menstrual cycles, because the hormone changes can affect breathing patterns.

General Questions:

Will the experiments be uncomfortable?
The gas mixtures should not cause you any problems. However, if you begin to feel at all unwell you can tell us and we will stop the experiment. The experiment is neither harmful nor painful.

What happens if something goes wrong?
An experienced medical doctor supervises the experiments and works with the other researchers to conduct them. The laboratory has emergency equipment and medication for emergency use. The University of Oxford has appropriate insurance- related arrangements in place in respect of the University's role as Research Sponsor of this study. This insurance compensates a volunteer for any harm that comes to them from the experiment, without there being any need for negligence to be proved in a court of law.

How long will the experiments take?
The first experimental day should take half an hour of your time. The main experimental days should each take about ten hours of your time.

Will I be free to stop at any time?
Yes. You should understand that you are under no obligation to participate in this study, and also that you are free to withdraw at any time.

What are the risks and benefits of taking part?
There should be no long-term medical consequences of participating in this research. We will pay you £10 per hour for the time that you will have given up, and, within reason, reimburse any travelling expenses you may have incurred.

What will happen to my personal details?
Your personal details will be stored in the laboratory on paper so that we can contact you. All data collected from the experiments will be stored in an anonymised fashion. All information will be kept strictly confidential. Your GP will be informed of your involvement in this study.

What will happen to the results of the study?
The results of the study will be published in an international scientific journal. We can provide you with a copy of any such publication.

Who has reviewed the study?
The East Central London Research Ethics Committee 1.

Who is funding the study?
The study will be funded from a grant provided by the Dunhill Medical Trust.

Whom can I contact about the study?
We suggest that you keep this letter and show it to anyone concerned with your medical care as appropriate. If you have any questions or problems, please contact me. My telephone number is 01865 272487.

Yours sincerely,

Quentin Croft,
Department of Physiology, Anatomy and Genetics