

Iron metabolism and biomarkers in idiopathic pulmonary arterial hypertension

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By

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I declare that this thesis was conducted and written by myself and the work included within is my own unless otherwise stated.

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Abstract

Idiopathic pulmonary arterial hypertension (IPAH) is a progressive disease characterised by increased vascular resistance and remodelling of the pulmonary vasculature. This causes strain on the right ventricle, leading eventually to failure and death. Iron status is thought to influence pulmonary vascular tone, particularly in hypoxia, and may be important in IPAH.

Proteomic studies of lung tissues from IPAH patients versus control lobectomy samples revealed downregulated levels of the haemoglobin- and haem-scavenging proteins haptoglobin (Hp) and haemopexin. Plasma levels of Hp were also reduced in IPAH and related to the Hp genotype. Consistently low Hp levels were associated with dysregulated iron homeostasis in IPAH patients.

Iron deficiency, as defined by raised plasma soluble transferrin receptor (sTfR) levels, was prevalent in IPAH patients and related to increased levels of the master iron regulator hepcidin. Iron deficiency was also associated with poor exercise capacity, disease progression and mortality.

Circulating sTfR levels were compared against other iron-related and established prognostic biomarkers in IPAH, including N-terminal brain natriuretic peptide (NT-proBNP), red cell distribution width (RDW), growth differentiation factor-15 and interleukin-6. All predicted survival and related to disease severity in IPAH, but RDW and NT-proBNP provided the most information when clinical and haemodynamic indices were considered.

Dysfunctional bone morphogenetic protein (BMP) receptor type II (BMPRII) signalling contributes to idiopathic as well as heritable PAH. Downregulation of BMPRII, but not the other type II BMP receptors ActRIIA and ActRIIB, led to increased hepcidin expression and secretion in response to BMP-6 stimulation in hepatocellular carcinoma HepG2 cells. Hepcidin expression was also enhanced in rat lung tissue following 1 or 2 weeks exposure to hypoxia.

In conclusion, dysregulation of iron homeostasis is common in IPAH and appears to be important clinically. Increased hepcidin levels may contribute to this phenomenon and reflect the dysfunctional BMPRII signalling associated with the disease.

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List of abbreviations

5-HT	Serotonin	EPO	Erythropoietin
6MWD	Six minute walk distance	ET-1	Endothelin-1
ActRIIA/B	Activin type 2 receptors A and B	ET-A/B	Endothelin receptors types A and B
ALK1	Activin receptor-like kinase 1	FAIR-HF	Ferric carboxymaltose in heart failure and iron deficiency trial
ANOVA	Analysis of variance	GADD34	Growth arrest and DNA damage inducible-34
APAH	PAH associated with other conditions	GATA4	GATA-binding factor-4
BMP	Bone morphogenetic protein	GDF-15	Growth and differentiation factor-15
BMPR2	Bone morphogenetic protein receptor type 2	Hb	Haemoglobin
BNP-32	Brain natriuretic peptide-32	HIF	Hypoxia-inducible factor
BSA	Bovine serum albumin	HIV	Human immunodeficiency virus
CD163	Macrophage scavenger receptor for Hp	HO-1	Haem oxygenase-1
CHF	Chronic heart failure	Hp	Haptoglobin
CHOP	C/EBP homologous protein	HPAH	Heritable PAH
CO	Cardiac output	HRP	Horseradish peroxidase
CPET	Cardiopulmonary exercise testing	IL	Interleukin
CTD	Connective tissue disease	INR	International normalised ratio
CTEPH	Chronic thromboembolic PH	IPAH	Idiopathic pulmonary arterial hypertension
DMT-1	Divalent metal transporter-1	IQR	Interquartile range
DNA	Deoxyribonucleic acid	IRE	Iron response element
DPX	Dipthalate and xylene mount	IRE1	Inositol-requiring protein-1
EDTA	Ethylene-diamine-tetraacetic acid	IRP	IRE-binding protein
eIF	Eukaryotic initiation factor	LC	Liquid chromatography
ELISA	Enzyme-linked immunosorbent assay	LDH	Lactate dehydrogenase

LDS	Lithium dodecyl sulphate	PVOD	Pulmonary veno-occlusive disease
ln	Natural logarithm	PVR	Pulmonary vascular resistance
m/z	Mass/charge ratio	RDW	Red cell distribution width
MCT	Monocrotaline	RNA	Ribonucleic acid
mPAP	Mean pulmonary artery pressure	ROC	Receiver operating characteristic
mRAP	Mean right atrial pressure	RT-PCR	Reverse transcription-PCR
mRNA	Messenger RNA	RV	Right ventricle
MS	Mass spectrometry	RV/LV+S	Right ventricular over left ventricle plus septum
mTOR	Mammalian target of rapamycin	SD	Standard deviation
NO	Nitric oxide	SDS	Sodium dodecyl sulphate
NT-proBNP	N-terminal proBNP	SELDI-TOF	Surface-enhanced laser desorption ionisation-time of flight
NYHA	New York Health Association	Smad	Mothers against decapentaplegic homologue
PAGE	Polyacrylamide gel electrophoresis	sTfR	Soluble transferrin receptor
PAH	Pulmonary arterial hypertension	SU5416	Sugen, VEGF inhibitor
PBS	Phosphate buffered saline	TGF- β	Tumour growth factor beta
PBS-T	PBS with Tween-20	TNF- α	Tumour necrosis factor alpha
PCH	Pulmonary capillary haemangiomatosis	TWSG1	Twisted gastrulation 1
PCR	Polymerase chain reaction	UPR	Unfolded protein response
PDE	Phosphodiesterase	VEGF	Vascular endothelial growth factor
PERK	Protein kinase R-like endoplasmic reticulum kinase	WHO	World Health Organisation
PH	Pulmonary hypertension	WT	Wild type

Summary of thesis structure

Chapter 1 contains an overall introduction to the thesis, which concludes with the **hypothesis and objectives**.

Chapter 2 contains detailed **methodologies** with material sources covering the studies described in Chapters 3-6.

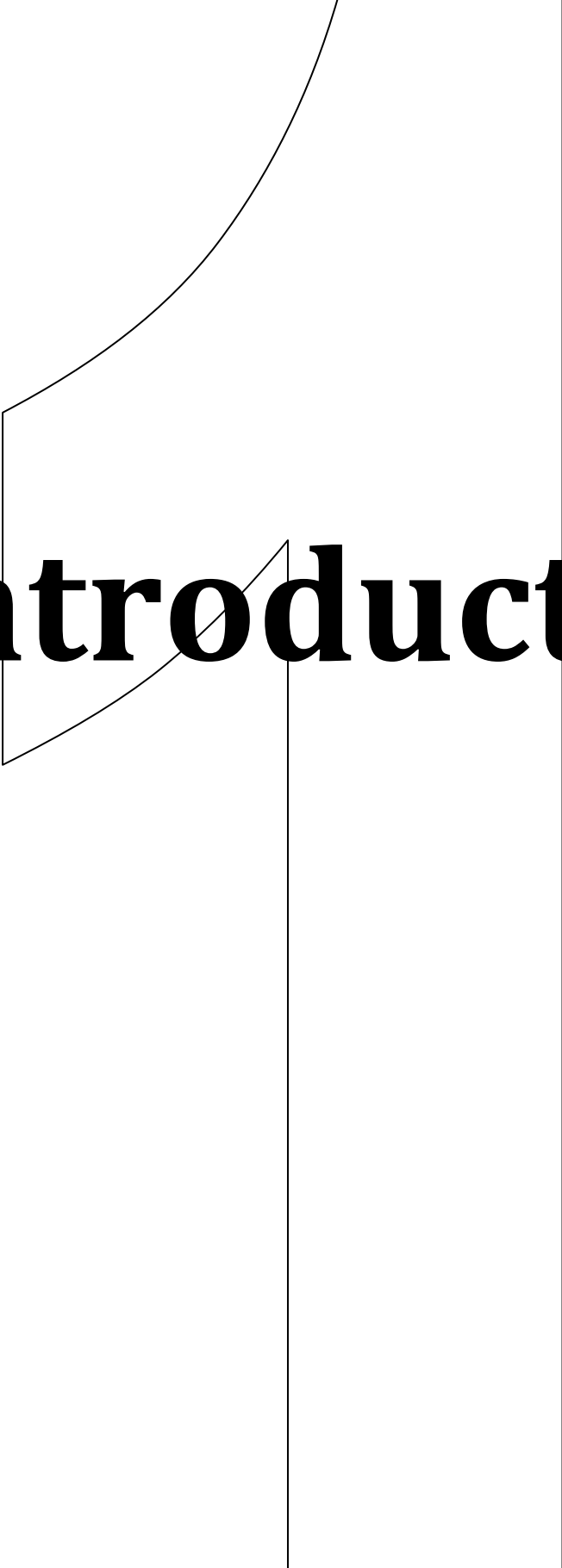
Chapter 3 focuses on the discovery of downregulated haptoglobin and haemopexin proteins in the lung tissue of IPAH patients compared to controls. Analysis of haptoglobin expression and genotypes in animal model and plasma samples are used to further investigate this finding. The conclusion that **iron homeostasis is dysfunctional in patients with idiopathic pulmonary arterial hypertension (IPAH)** leads onto the studies described in Chapter 4.

Chapter 4 describes the analysis of the iron status of patients with IPAH, with evidence to indicate that **iron deficiency is prevalent and related to functional capacity and survival in these patients**. Dysregulation of the “master iron regulator” hepcidin is identified and these data lead to the studies described in chapters 5-7.

Chapter 5 builds on the prognostic value of iron deficiency in IPAH identified in Chapter 4 to analyse the **prognostic capabilities of other markers related to iron status** in comparison with other proposed biomarkers in IPAH.

Chapter 6 investigates further the **mechanism behind the dysregulation of hepcidin** identified in Chapter 4, primarily the possible relationship with dysfunctional BMPR2 signalling that is characteristic of IPAH, but also possible local dysregulation.

Chapter 7 outlines a proposed **clinical study of intravenous iron therapy**, following the demonstration that iron deficiency is prevalent and clinically important and that raised hepcidin levels which may inhibit dietary iron uptake are present in IPAH in Chapter 4.



Introduction

Chapter 1 – Introduction

1.1 The Pulmonary Vasculature

Blood vessels are composed of three layers – the intima, the media and the adventitia. The intima comprises endothelial cells, which in monolayer form the basic structure of the vessel. Smooth muscle cells form a media of varying depth dependent on the pressures that are to be withstood from blood flow. The adventitia comprises primarily fibroblasts and connective tissue, which hold the vessel in position. These layers are separated by the internal and external elastic laminae, which are formed from elastin, and again vary in thickness with vessel size and function.

The airways of the lung and the pulmonary vasculature develop in synchrony *in utero* (Hislop 2002). The airways branch to form bronchi, bronchioli, terminal bronchioli and respiratory bronchioli leading to the alveolar ducts which incorporate the majority of the huge surface area of the lung. The pulmonary vessels also branch to form smaller, less muscularised arteries leading to the capillary bed, where the primary function of the lung - gas exchange - can occur. Pulmonary vessels are formed through a combination of angiogenesis and vasculogenesis (Hislop 2002). Growth factors such as the fibroblast growth factors, platelet-derived endothelial cell growth factor and vascular endothelial growth factor (VEGF) stimulate the growth of the new endothelium in a bed of extracellular matrix (Stenmark & Mecham 1997). Larger vessels are supported by the development of a smooth muscle layer whereas more distal vessels remain completely non-muscularised (Hislop & Pierce 2000). Layers of elastin provide vessels with distensibility and collagen provides rigidity. The healthy adult pulmonary circulation is a high-flow low-pressure system with relatively thin-walled blood vessels in comparison to the systemic circulation (Naeije & Rodehorst 2004).

1.2 The Pathology of Pulmonary Arterial Hypertension

Pulmonary hypertension (PH) is defined as an elevation of the resting mean pulmonary artery pressure (PAP) above 25mmHg. Increased pulmonary vascular resistance (PVR) that requires the right ventricle to raise PAP to maintain cardiac output causes the failure of the afterload-intolerant right ventricle, ultimately leading to death. PH is divided into several subgroups (Table 1.1) (Simonneau et al. 2009). PH in the presence of a normal pulmonary capillary wedge pressure (<15 mmHg) is termed pulmonary arterial hypertension (PAH). PAH is a multifactorial, progressive disease, with substantial mortality and morbidity. It is characterised by increased pulmonary

vasoconstriction and structural remodelling of the pulmonary vasculature, which vary in degree of importance in individual patients. Thrombotic lesions are frequently found in pulmonary arteries in PAH. Inflammation is also a component of the pathology of PAH (Tuder & Voelkel 1998) and raised levels of inflammatory cytokines are present and relate to survival (Humbert et al. 1995; Soon et al. 2010).

PAH with unknown cause is termed idiopathic PAH (IPAH, (Galie & Rubin 2004)), and PAH with an inherited genetic cause is termed familial or heritable PAH (HPAH). Median survival time for untreated IPAH in a historical series of PAH was 2.8 years (D'Alonzo et al. 1991). PAH associated with various other diseases including human immunodeficiency virus (HIV) infection, connective tissue diseases, portal hypertension, schistosomiasis, chronic haemolytic anaemia and congenital heart disease is termed APAH, and the prognosis varies with the underlying association (McLaughlin et al. 2004). According to the updated World Health Organisation (WHO) Dana Point classification of pulmonary hypertension, the other classes of PH are: PH associated with pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis, PH associated with left heart disease, PH associated with lung disease and/or hypoxemia (including chronic obstructive pulmonary disease and chronic exposure to high altitude), pulmonary hypertension due to chronic thrombotic and/or embolic disease (CTEPH) and miscellaneous causes (e.g. sarcoidosis – see Table 1.1) (Simonneau et al. 2009).

The increased vasoconstriction of the pulmonary arteries in PAH has been attributed to reduced expression/activity of voltage-gated potassium channels and dysfunction of the endothelium and platelets, leading to imbalances in the production and release of both vasodilators such as nitric oxide (NO) and prostacyclin and vasoconstrictors such as endothelin-1 (ET-1) and serotonin (5-HT) (Humbert et al. 2004). Many of these regulators of vascular tone also have roles in the control of cell proliferation and apoptosis and can contribute to the characteristic remodelling of the pulmonary vasculature. Tissue hypoxia can also contribute via inhibition of smooth muscle cell voltage-gated potassium channels, leading to depolarisation of cell membranes, increased calcium influx through voltage-gated channels, and hence further vasoconstriction (Morrell et al. 2009).

The structural changes seen in the pulmonary vessels in PAH include medial thickening due to smooth muscle cell hyperplasia and hypertrophy (Hislop & Reid 1976), formation of a neointima composed of smooth muscle cells or myofibroblasts embedded in a mucopolysaccharide matrix and the occurrence of plexiform and other obstructive lesions due to endothelial and smooth muscle cell proliferation (Yi et al. 2000). This vascular remodelling leads to occlusion of pulmonary arteries, increasing PVR and PAP. Muscularisation of distal arteries, normally non-muscular, also occurs due

to recruitment, proliferation and differentiation of intermediate cells, pericytes and fibroblasts (Chan et al. 1987). This remodelling has become a key target for therapy. A treatment protocol that can halt or reverse this process as well as addressing the imbalance of vascular tone chronically is the goal of many experimental therapeutic approaches.

1 Pulmonary arterial hypertension (PAH)

- 1.1 Idiopathic PAH
- 1.2 Heritable
 - 1.2.1 BMPR2
 - 1.2.2 ALK1, endoglin (with or without hereditary hemorrhagic telangiectasia)
 - 1.2.3 Unknown
- 1.3 Drug- and toxin-induced
- 1.4 Associated with
 - 1.4.1 Connective tissue diseases
 - 1.4.2 Human immunodeficiency virus (HIV) infection
 - 1.4.3 Portal hypertension
 - 1.4.4 Congenital heart diseases
 - 1.4.5 Schistosomiasis
 - 1.4.6 Chronic haemolytic anaemia
- 1.5 Persistent pulmonary hypertension of the newborn

1' Pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary hemangiomatosis (PCH)

2 Pulmonary hypertension owing to left heart disease

- 2.1 Systolic dysfunction
- 2.2 Diastolic dysfunction
- 2.3 Valvular disease

3 Pulmonary hypertension owing to lung diseases and/or hypoxia

- 3.1 Chronic obstructive pulmonary disease
- 3.2 Interstitial lung disease
- 3.3 Other pulmonary diseases with mixed restrictive and obstructive pattern
- 3.4 Sleep-disordered breathing
- 3.5 Alveolar hypoventilation disorders
- 3.6 Chronic exposure to high altitude
- 3.7 Developmental abnormalities

4 Chronic thromboembolic pulmonary hypertension (CTEPH)

5 Pulmonary hypertension with unclear multifactorial mechanisms

- 5.1 Hematologic disorders: myeloproliferative disorders, splenectomy
- 5.2 Systemic disorders: sarcoidosis, pulmonary Langerhans cell histiocytosis: lymphangioleiomyomatosis, neurofibromatosis, vasculitis
- 5.3 Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
- 5.4 Others: tumoural obstruction, fibrosing mediastinitis, chronic renal failure on dialysis

Table 1.1 - Updated Clinical Classification of Pulmonary Hypertension (Dana Point, 2008). Adapted from Simmonneau *et al.* 2009. The WHO diagnostic classes are bolded. BMPR2, bone morphogenetic protein receptor type 2; ALK1, activin receptor-like kinase 1.

The familial segregation of PAH points to genetic susceptibility to the disease. The most common genetic mutation leading to PAH affects the gene for type 2 bone morphogenetic protein receptor –

BMPR2 (Newman et al. 2008). *BMPR2* mutations are found in around 60% (50-80%) of HPAH (Cogan et al. 2006; Deng et al. 2000; Lane et al. 2000; Machado et al. 2006; Morisaki et al. 2004), 10-20% of IPAH patients (Newman et al. 2008), and rarely in secondary PAH associated with anorexic drugs or congenital heart disease (Humbert et al. 2002; Roberts et al. 2004). The mutations vary but all predict a reduction in *BMPR2* signalling (Atkinson et al. 2002). Patients with *BMPR2* mutations present with PAH approximately 10 years earlier than PAH patients without a defined *BMPR2* genomic perturbation, and have more severe haemodynamic compromise leading to younger age at death (Sztrymf et al. 2008) and are also less likely to respond to vasodilator therapy (Rosenzweig et al. 2008). Penetrance of PAH in *BMPR2* mutation carriers is generally around 20% but can vary greatly (from 33% to 80%) depending on single nucleotide polymorphisms of tumour growth factor (TGF)- β which alter gene expression (Phillips, III et al. 2008). The current thesis is that *BMPR2* mutations predispose to PAH, but there is a requirement for a “second hit” to express the disease. Recent studies suggest that *BMPR2* mutations result in failure of the protein to exit the endoplasmic reticulum following synthesis and studies with chemical chaperones have suggested that this phenotype can be reversed, suggesting a novel strategy for treating patients with heritable PAH (Sobolewski et al. 2008). Two other members of the TGF- β receptor superfamily, activin receptor-like kinase 1 (Alk1) and endoglin have also been causally linked to the development of PAH in hereditary haemorrhagic telangiectasia (Harrison et al. 2003).

1.3 Current Treatments for PAH

Many patients with PAH receive warfarin, diuretics, digoxin and oxygen. These treatments are often referred to as background or conventional therapy. Diuretics and digoxin provide symptomatic relief but are not thought to affect the course of the disease. Warfarin might provide a survival advantage but its contribution is difficult to estimate. Its use is based on retrospective analysis (Fuster et al. 1984; Johnson et al. 2006). Calcium channel blockers, such as nifedipine, offer considerable benefit to the small number of patients that respond to them, although these account for only around 6% of PAH patients (Rubin 1985).

The last 25 years has seen the introduction of treatments targeted more directly at the pathology of PAH. The three main categories of approved PAH therapies (Table 1.2), prostanoid analogues, endothelin receptor antagonists and phosphodiesterase-5 inhibitors, primarily act through inducing pulmonary vasodilatation, although they may also have other effects. Epoprostenol (Flolan®) was the first of these to be studied, and in addition to symptomatic improvement, it is the only

treatment that has been shown to offer a survival advantage (Badesch et al. 2000; Barst et al. 1996; McLaughlin et al. 1998; McLaughlin et al. 2002; Rubin et al. 1990; Sitbon et al. 2002). However, its administration (intravenous, via an indwelling catheter) is complex and it causes adverse effects such as headache, nausea and diarrhoea. Other prostanoid analogues have since been studied, such as iloprost (i.v., oral, Ilomedine®; or inhalation, Ventaris®), treprostinil (i.v. or subcutaneous, Remodulin®; or inhalation, Tryvasco®) and beraprost (oral), with data from clinical trials supporting short-term benefits (Barst et al. 2003; Galie et al. 2002; Galie et al. 2004; McLaughlin et al. 2006; McLaughlin et al. 2010; Olschewski et al. 2002; Simonneau et al. 2002; Tapson et al. 2006) but their cost-effectiveness has been challenged in the past by the National Institute for Health and Clinical Excellence (N.I.C.E. 2008). Since then, the appraisal has been stopped with the following statement “A significant number of commentators were of the view that [the National Institute for Health and Clinical Excellence] could not add value by continuing its appraisal. This was because [National Health Service] specialised commissioners have now put in place agreed arrangements for the supply of targeted therapies for all patients affected at the most severe stages of the condition” (N.I.C.E. 2009).

Endothelin-1 is a vasoconstrictor which acts via two receptors, ET_A and ET_B, in the pulmonary vasculature to both regulate vascular tone and cell proliferation. Both receptor subtypes are found on vascular smooth muscle cells and mediate vasoconstriction, while the ET_B receptor on endothelial cells mediates nitric oxide and prostacyclin release. Lung and circulating levels are increased in PAH patients (Giaid et al. 1993). Endothelin receptors antagonists, such as bosentan (Tracleer®), ambrisentan (Letairis®, Volibris®) and sitaxsentan (Thelin®) have been shown to improve pulmonary haemodynamics, exercise capacity (6-minute walk distance) and symptoms (Barst et al. 2004; Barst et al. 2006; Channick et al. 2001; Galie et al. 2005a; Galie et al. 2008a; Galie et al. 2008b; Rubin et al. 2002). In the absence of head-to-head comparisons, it is difficult to judge whether selective ET_A antagonism, which spares the endothelial ET_B receptor, offers advantages in efficacy over combined ET_A and ET_B antagonism but current opinion is that their effects are similar in magnitude. Suspected differences between the 3 endothelin antagonists in terms of liver toxicity have been noted (Raja & Dreyfus 2008) and indeed Thelin® was withdrawn in December 2010 due to hepatotoxicity. While comparison with historical control data suggests that bosentan monotherapy increases survival (McLaughlin et al. 2005), there are no robust data from appropriately designed clinical trials.

Phosphodiesterase (PDE) inhibitors increase cyclic mononucleotides (cyclic adenosine monophosphate and cyclic guanosine monophosphate) by inhibiting their degradation (Corbin & Francis 1999). This leads to vasorelaxation and inhibition of pulmonary arterial smooth muscle cell

proliferation (Wharton et al. 2005). PDE5 is abundant in the pulmonary vasculature (Hanson et al. 1998) and levels are raised in the lungs and right ventricles of PAH patients (Corbin et al. 2005; Murray et al. 2002; Wharton et al. 2005). A large (222 patients) study of the PDE-5 inhibitor sildenafil (Revatio®) in PAH (Galie et al. 2005b) demonstrated a significant increase in 6 minute walking distance (6MWD), improvement in WHO functional class and decrease in mean PAP (mPAP) after 12 weeks. Sildenafil also reduces right ventricle hypertrophy (Wilkins et al. 2005) and is associated with increases in circulating endothelial progenitor cells (Diller et al. 2008). Two further PDE5 inhibitors, tadalafil (Adcirca®) and vardenafil (Levitra®) have also shown positive results in clinical trials (Aizawa et al. 2006; Galie et al. 2009a; Jing et al. 2009) and tadalafil was approved for the treatment of PAH in 2009.

Therapies	Examples	Notes
Background therapies	Anti-coagulation – warfarin Diuretics Digoxin Oxygen	INR 1.5-2.5. Survival benefit (Fuster et al. 1984; Johnson et al. 2006) Reduce RV volume overload Modest CO improvements (Rich et al. 1998) Maintain PaS _{O2} > 90%
Calcium channel blockers	Nifedipine	Only benefit ~6% of PAH patients (Rubin 1985)
Prostanoid analogues	Epoprostenol (Flolan®) Iloprost (Ilomedine®, Ventaris®) Treprostinil (Remodulin®, Tryvasco®) Beraprost	Improves symptoms and survival (Badesch et al. 2000; Barst et al. 1996; Barst et al. 2003; Galie et al. 2002; Galie et al. 2004; McLaughlin et al. 1998; McLaughlin et al. 2002; McLaughlin et al. 2006; McLaughlin et al. 2010; Olschewski et al. 2002; Rubin et al. 1990; Simonneau et al. 2002; Sitbon et al. 2002; Tapson et al. 2006)
Endothelin receptor antagonists	Bosentan (Tracleer®) Ambrisentan (Letairis®, Volibris®) Sitaxsentan (Thelin®)	Improves symptoms (Barst et al. 2004; Barst et al. 2006; Channick et al. 2001; Galie et al. 2005a; Galie et al. 2008a; Galie et al. 2008b; Rubin et al. 2002) Benefits of receptor-specific inhibition unclear Sitaxsentan withdrawn in December 2010 due to hepatotoxicity
Phosphodiesterase-5 inhibitors	Sildenafil (Revatio®) Tadalafil (Adcirca®) Vardenafil (Levitra®)	Improves symptoms, reduces right ventricular hypertrophy to a similar extent as bosentan (Aizawa et al. 2006; Galie et al. 2005b; Galie et al. 2009a; Jing et al. 2009; Wilkins et al. 2005)

Table 1.2 – Current therapeutic options for the treatment of PAH. INR, international normalised ratio

A meta-analysis (Macchia et al. 2007) has been critical of the short-term nature of the studies that have investigated the efficacy of pharmacological treatments for PAH, calling into question the true benefit of the current treatments. In response to this, a more recent meta-analysis incorporating several additional studies found more positive outcomes, suggesting improvements in survival were achieved with targeted therapies (Galie et al. 2009c). Although they offer some hope for patients, they also confer various adverse effects, but more importantly, they do not offer a cure. While the time to clinical worsening of symptoms is increased, the progression of the disease is inevitable and improved treatments must be developed.

As discussed above, new therapeutic strategies are required to target the pulmonary vascular remodelling that occurs in PAH. Several novel therapeutic targets have been proposed and are under active investigation, including soluble guanylyl cyclase, phosphodiesterases (other than PDE5), tetrahydrobiopterin, 5-HT_{2B} receptors, vasoactive intestinal peptide, receptor tyrosine kinases, adrenomedullin, Rho kinase, elastases, endogenous steroids, bone morphogenetic protein and its receptors, potassium channels, metabolic pathways, and nuclear factor of activated T cells. Tyrosine kinase inhibitors, statins, 5-HT_{2B} receptor antagonists and soluble guanylyl cyclase activators are among the most advanced, having produced encouraging results in animal models, and human trials are underway. Endothelial progenitor cells and immune cells also represent a possible target in PAH (Rhodes et al. 2009). Simvastatin therapy produced a transient reduction in right ventricular hypertrophy after 6 months which was not maintained at 12 months, suggesting statin therapy would not add significant clinical benefit to current guidelines (Wilkins et al. 2010).

1.4 Blood Biomarkers in PAH

Identification of PAH usually follows an abnormal result from an echocardiogram performed due to suspicion of PH based on patient history, physical examination, chest X-ray and electrocardiogram. Confirmation of the diagnosis of PAH by right heart catheterisation is required (McLaughlin et al. 2009a). This is an invasive and expensive procedure, which is not suitable for regular follow-up monitoring of disease progression. Circulating biomarkers may represent a potential solution to this issue.

A 'biomarker' can be defined as follows:-

"Any characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (The Biomarker Definitions Working Group 2001).

Any signal that informs about the state of health of a biological system may hold some value as a biomarker and this includes biochemical and physical measurements (Ventetuolo et al. 2008), as well as more qualitative measurements such as questionnaires (Chen et al. 2008). Soluble (or biochemical) biomarkers have a number of advantages. Among these are that they use samples, blood and urine, that can be obtained relatively easily, and are therefore easy to deploy at the bedside and can be repeated serially at follow-up. They can also be quantified objectively and should be relatively low cost.

For any biomarker to be useful, however, it has to be qualified as fit for purpose. The evidence needed to qualify a biomarker depends upon the nature of the decisions that will be made based on the information it provides. Clearly the supporting evidence will be less where the biomarker is reporting on a proposed mechanism of drug effect (for example, to aid an in-house decision on whether to proceed with the development of that drug), than where it is used as a surrogate endpoint in a clinical trial and used as a basis on which to licence a drug for a clinical indication. In the latter case, the biomarker needs approval from the regulatory authorities. The key steps in this pathway are the collection of pre-clinical data to identify a novel exploratory biomarker, demonstration of the clinical relevance of an exploratory biomarker, characterisation of the reproducibility of a biomarker's clinical utility with adequate sensitivity and specificity, and validation that a biomarker can act as a surrogate for an established clinical endpoint (Wagner et al. 2007; Wang 2011).

The general conditions that need to be satisfied when considering the utility of a biomarker include:

Biological plausibility: There should be a reasonable scientific argument that links the biomarker with the disease process.

Strength of association: There should be a strong statistical correlation between changes in the biomarker and the event it is reporting.

Consistency: The association should hold irrespective of the intervention.

Specificity: The biomarker should be associated with the disease of interest and not confounded by co-existing disease or other factors. This is measured practically as the percentage of individuals without the corresponding disease state correctly identified.

Sensitivity: The biomarker should be able to clearly distinguish between those with or without the disease of interest. This is measured practically as the percentage of individuals with the corresponding disease state correctly identified.

Temporality: Changes in the biomarker should reflect changes in the disease in a suitable timeframe.

Biological gradient: The biomarker should have a reasonable dynamic range that enables grading of the disease or its response to interventions.

Experimental evidence: The clinical utility of a biomarker needs to be supported by data from clinical scenarios. This is most robust when gathered in the context of a clinical trial and is an iterative process, refined by the findings from the latest study.

Robustness of the assay: The biomarker assay should be accurate and reliable and reproducible in other laboratories.

There are a variety of potential uses of biomarkers for example screening, diagnosis, disease grading, determining prognosis and in guiding treatment decisions, in all cases these remain unmet clinical needs in PH. Our current understanding of the natural history of IPAH suggests individual variation in susceptibility to the condition, with a genetic basis in some cases, and a sub-clinical phase before presentation. There are therefore opportunities for screening for the disease and for its detection at an early stage. The diagnosis of PH is often delayed as other diagnoses are considered, and it is dependent upon an invasive procedure, cardiac catheterisation. While at present the latter remains essential for a definitive diagnosis, it has been replaced in some centres in the regular follow up of patients by skilled imaging techniques. This is an important development but cannot match the potential of a suitable blood or urine test for ease of deployment and cost. The clinical classification of PH into subtypes assists with treatment choice but there is considerable room for refinement through a better understanding of the disease processes at the molecular level (Archer et al. 2010). The translation of that molecular understanding into biomarkers could provide a classification or algorithm that enables more personalised prescribing. Studies to evaluate the efficacy of new treatments for PH are highly dependent upon surrogate clinical endpoints to filter out ineffective and unsafe interventions and enable resource to be given to more promising approaches. Soluble biomarkers have great appeal in this regard and once accepted, can be incorporated into goal-oriented treatment programmes for PH patients based upon repetitive monitoring during follow up (Hoeper et al. 2005).

Arguably, at the present time, *BMPR2* genotype is the only biomarker of PAH universally recognised (Aldred et al. 2006; Cogan et al. 2006; Humbert et al. 2002; Lane et al. 2000; Machado et al. 2006; Morisaki et al. 2004; Phillips, III et al. 2008; Roberts et al. 2004; Rosenzweig et al. 2008; Sztrymf et al. 2008), although brain natriuretic peptide-32 (BNP-32) and N-terminal (NT) pro-BNP have been studied extensively and have utility in clinical trials, if not day-to-day patient management (Blyth et

al. 2007; Daniels & Maisel 2007; Fijalkowska et al. 2006; Leuchte et al. 2004a; Leuchte et al. 2004b; Leuchte et al. 2005; Leuchte et al. 2006; Leuchte et al. 2007; Nagaya et al. 1998; Nagaya et al. 2000; Souza et al. 2005; Souza et al. 2007; Wilkins et al. 2005; Yamaguchi et al. 2004). Other potential biomarkers which have been and are actively under investigation include troponin, growth and differentiation factor (GDF)-15, uric acid, endothelin, creatinine, interleukin (IL)-6, red cell distribution width (RDW), angiopoietins and apelin (Bendayan et al. 2003; Fijalkowska et al. 2006; Goetze et al. 2006; Hampole et al. 2009; Kumpers et al. 2010; Nagaya et al. 1999; Nagaya et al. 2000; Nickel et al. 2008; Park et al. 2004; Rubens et al. 2001; Shah et al. 2008; Soon et al. 2010; Torbicki et al. 2003).

1.5 Biomarker discovery and qualification

The availability of new high-throughput technologies in genomics, proteomics, transcriptomics and metabolomics has opened up approaches for novel biomarker discovery. Screening blood samples, circulating cells and tissue from well-phenotyped patient groups is an active area of research in a number of centres (Abdul-Salam et al. 2010; Bull et al. 2004; Bull et al. 2007; Kwapiszewska et al. 2008; Moreno-Vinasco et al. 2008; Terrier et al. 2008; Yu et al. 2007). In principle, the pulmonary vascular bed should be a rich source of new biomarkers. The techniques for identifying markers that discriminate between disease and health are well established and the prospects for innovation exciting, but the effort required to qualify a biomarker for a particular purpose should not be underestimated. By way of example, BNP-32 has been proposed as a biomarker of value in the management of heart disease almost since its isolation in 1989 but its acceptance by regulatory authorities for specific indications is very recent.

A simple, cheap and non-invasive blood biomarker could be very useful in a number of clinical scenarios in PH, including identifying susceptible individuals, screening, diagnosis, risk stratification and therapeutic monitoring. Despite the clear value of easily-measurable biomarkers, only two have entered clinical practice at present and both have limited utility. *BMPR2* and *ALK1* gene profiling is valuable in identifying at risk relatives of patients with established mutations. BNP-32/NT-proBNP measurements are markers of right heart dysfunction in PH and have value in assessing group responses to interventions in clinical trials. BNP-32/NT-proBNP measurements carry information on prognosis and can be useful in individual patient management when integrated into the complete package of clinical information gathered about that patient. The possibility that BNP measurements might be more useful in patient management in combination with other blood biomarkers, for

example, GDF-15, merits prospective study. New high-throughput technologies and the vast surface area of the pulmonary vasculature offer the potential for new discoveries and a biomarker that reflects vascular damage rather than cardiac stress. The qualification of new biomarkers in PH is no small feat and requires access to large well-phenotyped patient populations, with serial sampling. Clinical studies to confirm that biomarkers improve outcomes in a randomised controlled setting are necessary before biomarkers can be adopted as true surrogates of established clinical measures (Wang 2011).

1.6 Liquid chromatography and mass spectrometry

Greater understanding of the pathophysiology of PAH could help in the development of new treatments or biomarkers with diagnostic or prognostic value. Proteomic approaches to studying diseases whereby combining gel electrophoresis (1D or 2D) and liquid chromatography (LC - 1D or 2D) with mass spectrometry (MS) can lead to the identification and even quantification of hundreds or thousands of proteins in any one sample, have become popular in the hunt for disease biomarkers (Bons et al. 2007; Cutillas & Vanhaesebroeck 2007; Kolker et al. 2006; Li et al. 2007; Mayr et al. 2006; Terrier et al. 2008; Yu et al. 2007; Zhu et al. 2008a; Zhu et al. 2008b). The Wilkins laboratory recently utilised an LC-MS/MS-based method to compare the proteomes of lung tissue samples from IPAH patients with those from lobectomy patients (Abdul-Salam et al. 2010), with the hope of identifying and quantifying proteins which could either become useful biomarkers or help elucidate a disease-altered signalling pathway which could be modified in a novel treatment rationale. One preliminary result to come from these proteomic studies (see Chapter 3) was the downregulated expression of the iron regulatory proteins haptoglobin and haemopexin in the lung tissue of patients with idiopathic pulmonary arterial hypertension in comparison with normal lung tissue taken from lobectomy samples. The largest reduction observed with the highest confidence was in haptoglobin.

1.7 Haptoglobin

The main function of haptoglobin (Hp) is the sequestration of cell-free haemoglobin (Hb), which is released from erythrocytes during haemolysis. Once the Hp-Hb complex is formed, it exposes a neo-epitope, which massively increases the affinity of Hb for the macrophage CD163 scavenger receptor (Kristiansen et al. 2001; Schaer et al. 2006b). Upon binding this receptor, the whole complex is internalised and degraded and the iron released from haem – see Figure 1.1. Cell-free haemoglobin

has been shown to be a scavenger of nitric oxide (Azarov et al. 2008; Wang et al. 2004) and is believed to contribute to the NO-resistant state seen in the vasculature of sickle cell anaemia patients (Wood et al. 2008). Haptoglobin has been identified as an acute phase protein (Theilgaard-Monch et al. 2006) and one function of its upregulation is in preventing the scavenging of iron by infecting bacteria (Francis, Jr. et al. 1985; Zakaria-Meehan et al. 1988). Haptoglobin has also been identified as an angiogenic factor in sera from patients with systemic vasculitis (Cid et al. 1993) and may have a role in vascular remodelling, dose dependently stimulating endothelial cells to form tubes and vascular smooth muscle cells to migrate (Lohr et al. 2005).

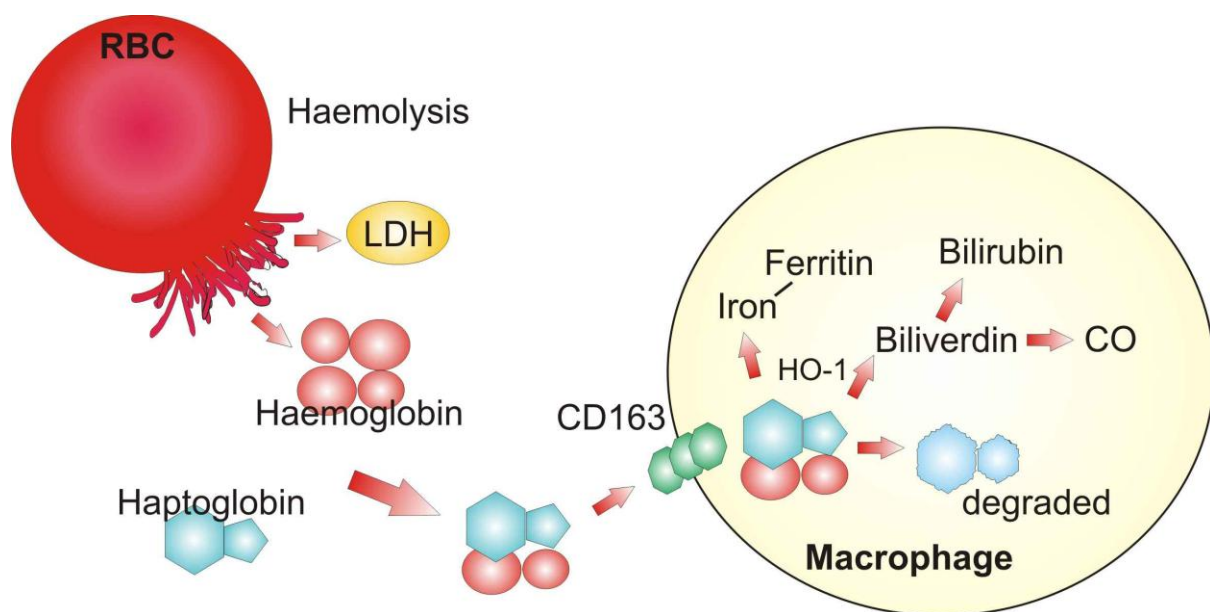


Figure 1.1 – The function of haptoglobin. The primary role of haptoglobin in iron regulation is to bind cell-free haemoglobin, which is released from red blood cells during haemolysis. Haptoglobin can then return haemoglobin to cell stores for example in macrophages, where it can be broken down and the iron stored, bound to ferritin. Lactate dehydrogenase (LDH) is also released when red blood cells lyse. Haem oxygenase-1 (HO-1) breaks down intracellular haem to biliverdin, which is broken down further to bilirubin and carbon monoxide.

Haptoglobin is synthesised as a 45 kDa peptide and cleaved into alpha (9-18 kDa) and beta (38 kDa) chains, which form a tetrameric $\alpha_2\beta_2$ protein. In humans there exist two alleles of haptoglobin, which encode either 18 kDa or 9 kDa alpha chains, Hp1 and Hp2, respectively (Figure 1.2). This is probably due to a gene duplication of exons 3 and 4, and the beta chains encoded are the same in

both alleles. Two allelic variants of *Hp1* (*Hp1^s* and *Hp1^f*) have also been identified that differ by 4 amino acids, thereby altering their electrophoretic mobility (Brune et al. 1984; Maeda et al. 1984). These alleles produce 3 major haptoglobin phenotypes in humans; homozygous *Hp1-1* or *Hp2-2*, and heterozygous *Hp2-1*. The distribution of haptoglobin genotypes in north-western European populations are approximately 16% *Hp1-1*, 48% *Hp2-1* and 36% *Hp2-2*, but particular polymorphisms have been linked with a variety of inflammatory, autoimmune and cardiovascular diseases (Carter & Worwood 2007; Langlois & Delanghe 1996). These include diseases associated with PAH, such as HIV infection, sarcoidosis and systemic lupus erythematosus, where the *Hp2-2* genotype may be more frequent and carry a worse prognosis (Bons et al. 2007; Delanghe et al. 1998; Pavon et al. 2006). Haptoglobin phenotypes have also been found to be differentially expressed in patients with toxic oil syndrome, which is associated with the development of PAH (Quero et al. 2004; Rodriguez et al. 2006).

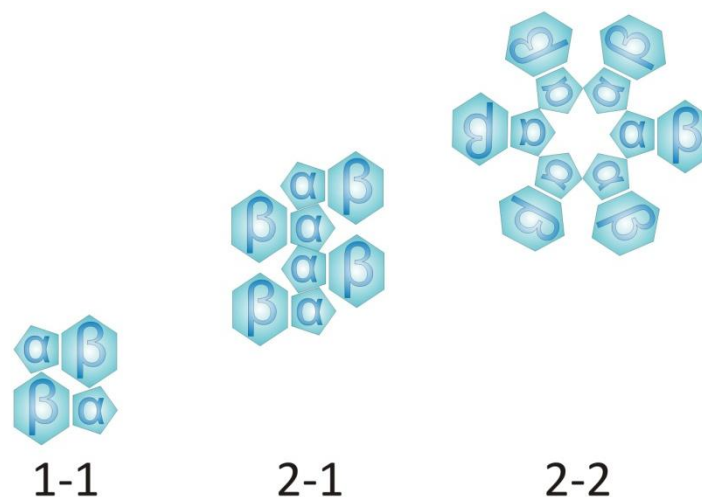


Figure 1.2 – Haptoglobin phenotypes. Various structures of haptoglobin are possible dependent on the phenotype, due to an extra cysteine residue in the alpha-2 chain producing an extra disulphide bridge in the quaternary structure.

The different haptoglobin alleles are functionally as well as structurally distinct. An extra cysteine residue in the α_2 chain causes the formation of long polypeptide chains in *Hp2-1* individuals and large cyclical polymers in *Hp2-2* individuals – see Figure 1.2. These larger polypeptides have lower affinities for Hb and the complexes formed are internalised less efficiently by the CD163 receptor. In addition to this, individuals with the *Hp2-2* phenotype have the lowest circulating levels of haptoglobin, with *Hp2-1* intermediate and *Hp1-1* levels the highest (Langlois & Delanghe 1996). The *Hp2-2* phenotype has been identified as a cardiac risk factor in patients with diabetes mellitus (Levy et al. 2002) and haptoglobin phenotypes predict 30-day mortality and heart failure in patients with

diabetes post-myocardial infarction (Suleiman et al. 2005). The Hp2-2 genotype has recently been shown to be associated with increased iron deposition in atherosclerotic carotid plaques (Lioupis et al. 2011).

1.8 Haptoglobin and iron regulatory proteins in pulmonary hypertension

Haptoglobin gene expression is not confined to the liver. Significant expression also occurs in the lung, where haptoglobin mRNA has been localised to airway epithelial cells and alveolar macrophages in diseased or inflamed lung (Abdullah et al. 2009) and found to reduce lung injury associated with exposure to blood (Yang et al. 2000; Yang et al. 2003). Haptoglobin is also reported to be expressed in the arterial wall, where it has been implicated in vascular remodelling, adventitial cell migration and angiogenesis (Cid et al. 1993; De Kleijn et al. 2002; Smeets et al. 2003), to be induced by inflammatory mediators (D'Armiento et al. 1997) and modulated by factors such as hypoxia. Polycythemia and haemoglobinemia accompany hypoxia-induced PH and depletion of plasma haptoglobin has been described in hypoxic rats (Bonkovsky et al. 1986), where changes in circulating haptoglobin levels may occur despite the apparent normal survival of erythrocytes (Ou & Smith 1978). Two other studies have examined the effect of hypoxia on haptoglobin expression in isolated cells, describing reduced expression in human adipocytes (Wang et al. 2007) and increased expression in human hepatocellular carcinoma HepG2 cells (Wenger et al. 1995). Thus, it is possible that the hypoxia-sensitive regulation of haptoglobin may vary in different tissues. Hypoxia and inflammatory mediators have also been found to modulate other iron regulatory proteins, including the peptide hormone hepcidin that acts to limit the mobilisation of iron into plasma from intracellular stores (Merle et al. 2007).

The CD163-mediated endocytosis of Hb is linked to the induction of haem-oxygenase-1 (HO-1), which protects against Hb-induced oxidative damage. HO-1 is a rate-limiting enzyme in haem catabolism and the products of haem degradation (iron bound to ferritin, carbon monoxide, biliverdin, bilirubin) have anti-oxidative, anti-apoptotic, and anti-inflammatory activities (Fredenburgh et al. 2007; Idriss et al. 2008; Wagener et al. 2003). In the lung, microvascular endothelial HO-1 and ferritin production is upregulated by methaemoglobin (Balla et al. 1995). Additional stimuli of HO-1 synthesis include CD163-mediated Hb-Hp uptake, IL-10 secretion and corticosteroids (Philippidis et al. 2004; Schaer et al. 2006a; Yamazaki et al. 2007). In fact, induction of this cytoprotective enzyme occurs in several settings and protects against the development of PH and cardiovascular remodelling in animals exposed to chronic hypoxia or treated with monocrotaline

(Carraway et al. 2000; Christou et al. 2000; Goto et al. 2002; Minamino et al. 2001; Zhou et al. 2006). Thus, induction of HO-1 has been proposed as new therapeutic strategy in cardiovascular disease (Ito et al. 2007; Stocker & Perrella 2006) and the products of its enzymatic activity as potential therapies for pulmonary vascular disease (Ryter et al. 2007), emphasising the potential importance of iron homeostatic pathways in PAH. One immunohistochemical study has indicated that both endothelial HO-1 and caveolin expression may be depleted in patients with severe PH (Achcar et al. 2006), but the co-distribution of haptoglobin and associated iron regulatory proteins is unknown.

1.9 Iron homeostasis

The control of circulating and intracellular iron concentrations is important to both enable effective erythropoiesis and the activity of iron-dependent biochemical reactions, including many links of the respiratory chain, and to prevent the toxic effects of excessive iron, including production of reactive oxygen species via the Fenton reaction (Peyssonnaud et al. 2007; Peyssonnaud et al. 2008). Around two-thirds of the body's 3-4g of iron is found in haemoglobin in erythrocytes, and under normal iron-replete conditions, the majority of the rest is stored intracellularly, bound to ferritin or hemosiderin molecules (Finch et al. 1970). Erythrocyte iron is effectively recycled by macrophages of the reticuloendothelial system and haptoglobin and haemopexin molecules mop up the cell-free haemoglobin and haem, respectively. Retention of iron in the macrophages is tightly regulated in response to stimuli such as infection or erythropoiesis. Small daily losses of 1-3mg iron, primarily through gastrointestinal bleeding, are balanced by dietary iron absorption. Excessive bleeding has not been noted in IPAH patients (Ruiter et al. 2010). Transport of ferrous iron from enterocytes or iron-storing cells into the plasma is dependent on the sole iron exporter ferroportin. Iron released into plasma is rapidly bound to transferrin molecules, which can bind to cell surface transferrin receptors and be internalised in cells where iron is required.

Iron and oxygen homeostasis are interconnected with erythropoiesis, acting via hypoxia inducible factors (HIFs) to modulate iron regulatory proteins that mediator cellular responses to anaemia and hypoxia (Figure 1.3). This is based upon the requirement of the HIF-degrading enzymes (HIF hydroxylases) for iron and oxygen as co-factors (Ivan et al. 2001; Jaakkola et al. 2001). Circulating erythrocytes have a mean lifespan of 120 days. These cells are normally removed extravascularly, by macrophages in the reticuloendothelial system, but can also be broken down in the circulation (intravascular haemolysis). If haptoglobin is depleted (e.g. during haemolytic anaemia) Hb uptake can also occur via a low affinity haptoglobin-independent CD163 receptor mechanism (Schaer et al.

2006b), but oxidative damage may interfere with Hb clearance and thereby contribute to Hb-mediated toxicity in diseases such as PAH that are linked to oxidative stress (Vallelian et al. 2008).

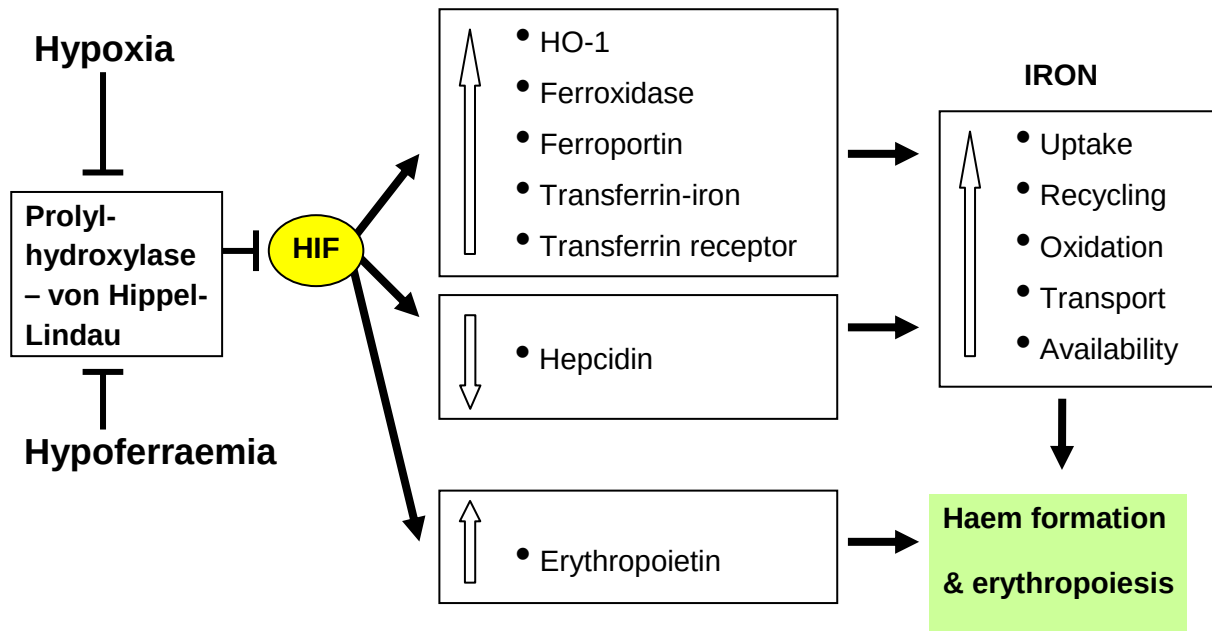


Figure 1.3 - Oxygen and iron homeostasis. Hypoxia inducible factors (HIFs) have a key role in pulmonary hypertension and pulmonary vascular remodelling and regulate target genes involved in iron metabolism. Iron and oxygen are both implicated in the post-translational modification of HIF. Low iron and hypoxia reduce prolyl-hydroxylase activity and von Hippel-Lindau-mediated degradation of HIF. Stabilization of HIF promotes selective changes in the levels of iron regulatory proteins and erythropoietin, thereby coordinating iron availability and erythropoiesis activation in order to normalise iron homeostasis and tissue oxygenation. These changes contrast with the acute phase response to inflammation, which involves upregulation of proteins that promote iron sequestration (hepcidin, haptoglobin, haemopexin) and suppression of proteins promoting iron accumulation (transferrin, ferroportin).

Large amounts of cell-free Hb are a feature of severe haemolytic diseases, such as sickle cell disease, and associated with marked depletion of circulating haptoglobin, haemopexin formation and methaemalbuminaemia. Indeed, haemolysis has also been implicated in the pathophysiology of sickle cell disease, where PH is a risk factor for early mortality and correlates with markers of haemolysis (Gladwin et al. 2004; Rother et al. 2005). Intravascular haemolysis has adverse effects on vascular homeostasis, with the release of Hb and arginase from erythrocytes resulting in scavenging of endothelium-derived NO and breakdown of its precursor arginine, impairing NO bioavailability. In

addition, the vascular endothelium is at risk of oxidative damage by exposure to free Hb, which reacts with NO to produce the oxidised (ferric) methaemoglobin. This in turn activates endothelial production of inflammatory chemokines and induces neutrophil recruitment (Balla et al. 2007).

The significance of haemolysis in relation to PAH is underlined by the observation that inherited haemoglobinopathies and acquired forms of haemolytic anaemia are associated with pulmonary hypertension, whereas anaemia occurring with chronic diseases or iron-deficiency is not (Barnett et al. 2008). Several murine models of haemolysis have also demonstrated that haemolytic anaemia is sufficient to produce global endothelial dysfunction and NO dysfunction (Frei et al. 2008; Hsu et al. 2007). Importantly, this was accompanied by spontaneous pulmonary hypertension and right ventricular hypertrophy, but not remodelling of the pulmonary vasculature. Earlier case reports have suggested that in some patients with IPAH or PAH associated with congenital heart disease, haemolysis occurs due to physical damage to erythrocytes in the diseased pulmonary microvascular bed (Jubelirer 1991; Pare et al. 1983; Stuard et al. 1972). There has been no systematic study of this phenomenon in PH and so the extent and clinical significance is at present unclear.

The acute phase response to inflammation and insult to tissues is also associated with the modulation of key regulators of iron homeostasis. This involves upregulation of proteins that promote iron sequestration (hepcidin, haptoglobin, haemopexin, lactoferrin and ferritin) and suppression of proteins that promote iron accumulation (transferrin and ferroportin). A number of cytokines (e.g. IL-6, tumour necrosis factor (TNF)- α , IL-10) stimulate these changes and the net effect is to induce hypoferremia, limiting the availability of iron essential for the growth of microorganisms and neoplastic cells and minimising its capacity to catalyse the production of reactive oxygen species (Lagan et al. 2008).

1.10 Iron and the pulmonary vasculature

Several human physiology studies conducted by Robbins and colleagues have demonstrated the importance of iron availability in the control of pulmonary artery pressure and pulmonary vascular responses to hypoxia. They propose that iron deficiency may mimic the effects of hypoxia through normoxic stabilisation of HIF. Infusion of the iron chelator desferrioxamine in healthy subjects over 8 h mimicked hypoxia by inducing increases in tricuspid valve maximum pressure gradient. Although the increases seen were about a quarter of the magnitude of those seen with hypoxia, the time course and individual variation in responses were similar, leading the authors to conclude that HIF

stabilisation may be at least in part responsible for the pulmonary vasoconstriction seen in response to hypoxia (Balanos et al. 2002).

Subsequently it was shown that the infusion of iron prevented the increased acute hypoxic pulmonary vasoconstrictive response normally induced by pre-exposure to hypoxia (~10% oxygen) for 8 hours, whereas infusion of desferrioxamine exacerbated hypoxic pulmonary vasoreactivity (Smith et al. 2008). Similarly, iron infusion partially reversed the pulmonary hypertensive response to hypoxia in healthy sea-level resident men at high altitude (altitude 4340m), where air pressure is reduced such that it is equivalent to breathing ~12% oxygen at sea level (Smith et al. 2009). In 11 high altitude residents with chronic mountain sickness, progressive iron deficiency induced by staged venesection of two litres of blood also increased pulmonary artery systolic pressure, although no acute effect of iron replacement was detected. Together, these data indicate that variation of iron availability in the normal physiological range can affect pulmonary vascular tone and hypoxic pulmonary hypertension and clinical iron deficiency could exacerbate pulmonary hypertensive disease (Joyner & Johnson 2008).

1.11 Iron in chronic heart failure

Anaemia is a powerful and independent prognostic indicator in chronic heart failure (CHF) (Al-Ahmad et al. 2001; Horwich et al. 2002; Mozaffarian et al. 2003) and five key studies have attempted to address the potential therapeutic value of intravenous iron replacement therapy in these patients (Anker et al. 2009; Bolger et al. 2006; Okonko et al. 2008; Toblli et al. 2007; Usmanov et al. 2008). These studies are summarised in Table 1.3 and have demonstrated that iron replacement therapy can improve haemoglobin levels, exercise capacity, functional class, quality of life and biochemical markers of myocardial stress and inflammation.

The culmination of these studies was the large multi-centre Ferric Carboxymaltose in Patients with Heart Failure and Iron Deficiency (FAIR-HF) study by Anker and colleagues (Anker et al. 2009). They recruited 459 patients with New York Heart Association (NYHA) class II or III CHF and iron deficiency (defined by ferritin < 100 or 100-299 µg/L if transferrin saturations were <20%) and a haemoglobin between 9.5 g/dL and 13.5 g/dL, and randomised them 2:1 to ferric carboxymaltose or placebo. Both primary endpoints of patient self-reported global assessment and NYHA class showed significant improvements at 24 weeks, and significant differences were observed in several primary and secondary endpoints (including quality of life questionnaires and exercise capacity) at all time points including 4 weeks and 12 weeks. Surprisingly, improvements were seen irrespective of the presence

of anaemia and no significant difference in haemoglobin levels at baseline or 24 weeks were observed between the treatment and placebo arms. This implicates iron deficiency itself as an important therapeutic target in cardiovascular diseases and emphasises the clinical relevance of the other roles of iron discussed earlier.

Study	Iron therapy	Patient group (n)	Placebo?	Duration	Primary effects	Secondary effects
Bolger <i>et al.</i> 2006 (Bolger <i>et al.</i> 2006)	Up to 1g iron sucrose	Anaemic heart failure (16)	No	Up to 3 months	Haemoglobin improvements	Improved symptoms and 6MWD (average change >40m) No adverse events
Toblli <i>et al.</i> 2007 (Toblli <i>et al.</i> 2007)	5 weekly 200 mg doses iron sucrose	Anaemic chronic heart failure (40)	1:1 randomisation	6 months	Reduced N-terminal pro-brain natriuretic peptide and C-reactive protein	Improved symptoms, LVEF, exercise capacity. All 5 hospitalisation in placebo.
FERRIC-HF (Okonko <i>et al.</i> 2008)	200 mg/wk until ferritin>500, then fortnightly (average total 1433+/-365 mg)	Chronic heart failure (35)	2:1 randomisation Iron:placebo	12 weeks	Peak O ₂ consumption only up in anaemic	Improved NYHA class. Adverse events similar.
Usmanov <i>et al.</i> 2008 (Usmanov <i>et al.</i> 2008)	100 mg three times a week for 3 weeks and then once weekly for 26 weeks (total dose 3200 mg)	CHF NYHA class III/IV and persistent anaemia (32)	No	26 weeks	Significant improvements in LV function and structure	Half of class III patients improved to class II. No improvement from class IV.
FAIR-HF (Anker <i>et al.</i> 2009)	Fortnightly 200 mg ferric carboxymaltose until Ganzoni iron deficit replenished	NYHA class II or III CHF with iron deficiency and Hb 95-135 g/l (459)	2:1 randomisation iron:placebo	24 weeks	Significant improvements in patient self-reported global assessment and NYHA class	6MWD up ~40m vs. placebo. Quality of life improved. Improvements irrespective of anaemia. Adverse events similar.

Table 1.3 – Intravenous iron replacement therapy studies in left heart failure patients. NYHA, New York Health Association.

1.12 Iron and prognostic biomarkers in pulmonary hypertension

Aside from its role in regulating pulmonary vascular tone and its efficacy in improving symptoms of heart failure, iron can be implicated in the pathology of pulmonary hypertension through studies of prognostic markers of this disease.

RDW is a measure of the variability of red blood cell size and is used clinically to distinguish iron deficiency anaemias. Its levels are predictive of cardiovascular mortality in otherwise healthy older adults (Patel et al. 2010) and its prognostic power in chronic heart failure has been linked to iron deficiency and ineffective erythropoiesis (Forhecz et al. 2009). RDW predicted mortality in 162 patients with pulmonary hypertension, outperforming the more established NT-proBNP and blood urea nitrogen measurements in multivariable models (Hampole et al. 2009). More recently, anaemia itself has been shown to be associated with higher mortality in 145 patients with PH of mixed aetiologies (Krasuski et al. 2010). 38 (26%) were found to be anaemic and this was associated with a significantly poorer survival. This was shown to be independent of age, WHO functional class, diagnosis, PVR and tricuspid regurgitation grade. The authors suggested reduced production of nitric oxide from nitrites by deoxygenated haemoglobin may affect pulmonary vascular haemodynamics.

Another prognostic marker in PH is GDF-15 (Nickel et al. 2008). GDF-15 levels are raised in patients with IPAH (Nickel et al. 2008) and can also be induced in several cell types by the iron chelating agent desferrioxamine. This effect was independent of iron regulatory proteins and HIF. Infusion of the desferrioxamine led to significant induction of GDF-15 expression in the plasma of 8 healthy volunteers 8-12 hours following infusions (Lakhal et al. 2009). The relationship of these markers, whose levels are modulated by tissue iron status, to prognosis in IPAH suggests iron deficiency may contribute to the prognostic information derived from these markers.

1.13 Iron deficiency in IPAH

Iron deficiency defined by reduced serum iron and transferrin saturations has been characterised in 30/70 patients with IPAH (Ruiter et al. 2010). Iron deficiency with or without anaemia was associated with reduced 6MWD. Interestingly, oral iron therapy was only effective at increasing ferritin levels in 8/18 patients treated. This suggested that dietary iron uptake may be dysfunctional in these patients. Conversely, studies in rats suggest that iron might have detrimental effects on the right ventricle (RV). Iron-catalysed carbonylation of annexin A1 leads to its degradation (Park et al. 2010). Degradation of annexin A1 prevents its repression of nuclear transcription factor Y, activation of which leads to increased expression of the *Gata4* gene (Mantovani 1999). Transgenic mice with cardiac-specific overexpression of GATA-binding factor-4 (GATA4) exhibit concentric hypertrophy of atria and well as both ventricles (Liang et al. 2001). Therefore iron might contribute to cardiac hypertrophy through GATA4. In support of this hypothesis, it was demonstrated that inhibition of hypoxic-PH mediated RV hypertrophy mediated by the iron chelator desferrioxamine was coincident

with the repression of *Gata4* mRNA induction (Park et al. 2010). GATA4 has also been implicated in the transcription of the S100 calcium-binding protein S100A4 (Lawrie et al. 2005), which is overexpressed in occlusive lesions of patients with pulmonary vascular disease and can drive the development of occlusive pulmonary vascular lesions in ~5% of transgenic animals (Greenway et al. 2004). These mechanisms implicate a possible negative role for excess free iron in PH, emphasising the importance of maintaining iron levels within the normal range.

The question of how to define iron deficiency is an important one. Traditional markers of iron status – serum iron, ferritin and transferrin saturation – do provide important information about the bioavailability, storage and handling of iron in the healthy subject, but when dealing with patients with chronic diseases or acute inflammatory episodes, they may be confounded. Iron is an important growth factor for many bacteria and part of the inflammatory response is to limit serum iron availability, therefore inhibiting bacterial growth. The master controller of this system is hepcidin (Nemeth & Ganz 2009). Hepcidin is a 25-amino acid peptide, which acts to internalise ferroportin (Hentze et al. 2010), leading to its degradation in lysosomes, and hence inhibition of dietary iron absorption and release of body iron stores (Figure 1.4). This leads to reduced serum iron concentrations and transferrin saturations, which reduce the availability of iron to parasites as part of the immune response.

Soluble transferrin receptor (sTfR) levels offer an alternative quantification of tissue iron availability that is unaffected by inflammation (Cook et al. 1990; Huebers et al. 1990) and therefore represent the best available marker of iron deficiency in diseases such as PAH with an inflammatory component. sTfR levels in the normal range reflect erythropoietic activity (i.e. the uptake of iron in the bone marrow by transferrin receptors), whereas in iron deficiency, sTfR levels are disproportionately raised (Huebers et al. 1990).

Chronically raised hepcidin levels underlie the anaemia of chronic disease (Weiss & Goodnough 2005). Although predominantly found in the liver hepcidin expression has been demonstrated elsewhere, including alveolar macrophages (Nguyen et al. 2006) and the heart, where, unlike the liver, it is upregulated by hypoxia (Merle et al. 2007). Interestingly, bone morphogenetic protein (BMP) and Smad (mothers against decapentaplegic homologue proteins) signalling are closely involved in regulating hepcidin expression (Babitt et al. 2006; Nemeth 2008; Yu et al. 2008) and another member of the TGF- β superfamily, GDF-15, represses hepcidin expression (Tanno et al. 2007). Other factors that may affect hepcidin expression in PAH include inflammatory cytokines, erythropoietic activity and sTfR (Hentze et al. 2010).

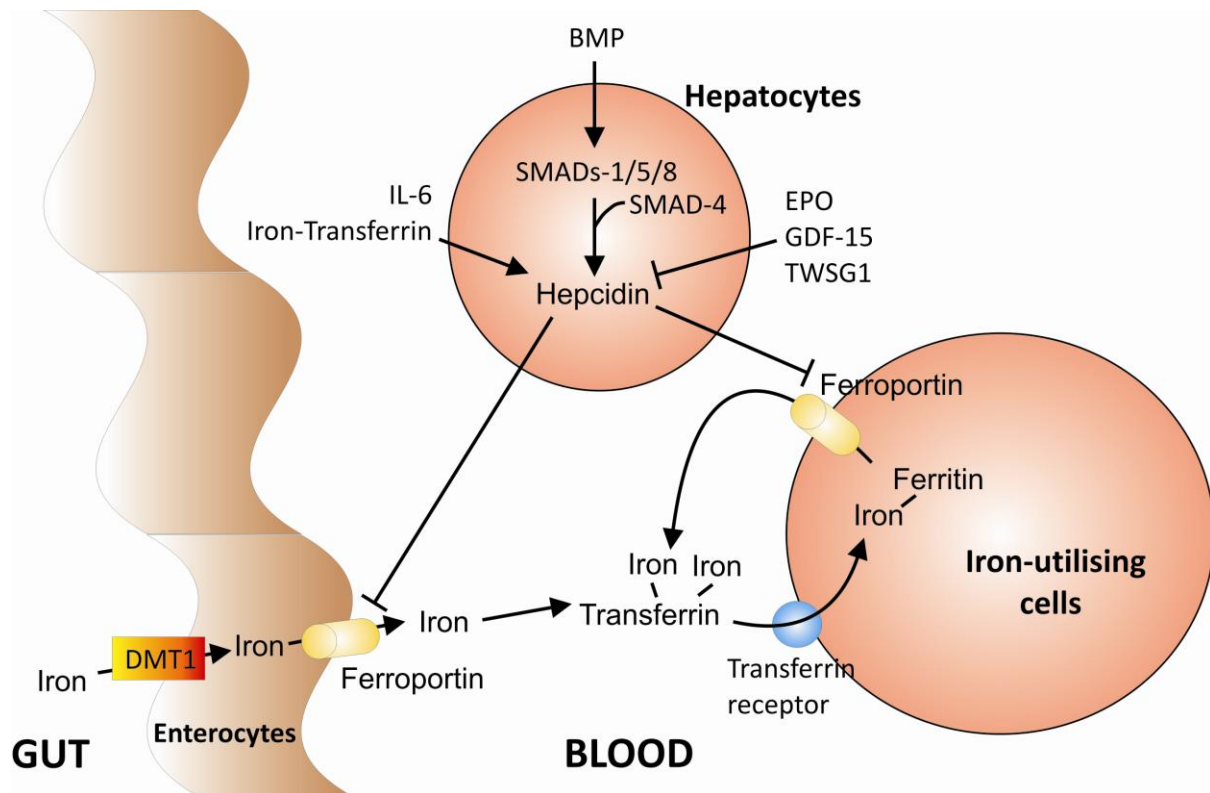


Figure 1.4 – Regulation of dietary iron uptake and release from iron-utilising cells by hepcidin.

Hepcidin acts to internalise ferroportin, leading to its degradation and inhibition of dietary iron uptake and release of iron from iron-utilising or –storing cells. Hepcidin is regulated by several factors, though bone morphogenetic protein (BMP) signalling through the Smad transcription factors, which can be modulated by hemojuvelin, appears to be of particular importance. Hepcidin expression can be stimulated by inflammation (through interleukin-6, IL-6) and circulating iron (Fe-transferrin). Erythropoiesis (and erythropoietin, EPO) indirectly inhibits hepcidin, and circulating erythroid factors such as growth differentiation factor-15 (GDF-15) and twisted gastrulation-1 (TWSG1) have been proposed to directly inhibit hepcidin. Divalent metal transporter-1 (DMT-1) is responsible for uptake of dietary iron into enterocytes.

1.14 Hypothesis

Dysfunctional iron homeostasis in patients with PAH could contribute to the vascular pathology, functional capacity and symptoms of this disease, and therefore represents a novel therapeutic target in PAH.

1.15 Objectives

1. To confirm the dysregulation of the iron regulatory protein haptoglobin in patients with IPAH and explore its regulation in animal models of PH
2. To investigate iron status and its clinical importance in patients with IPAH
3. To investigate the prognostic power of biomarkers related to iron regulation in comparison with other novel biomarkers in patients with IPAH
4. To investigate the regulation of important iron regulatory molecules such as hepcidin in IPAH



Materials & Methods

Chapter 2 – Materials and methods

2.1 Declaration of responsibilities

All live animal procedures were performed by Dr. Lan Zhao or Dr. Sven van Eijl.

Plasma samples were collected with the assistance of Dr. Luke Howard and Dr. Simon Gibbs and PH nurses in the National Pulmonary Hypertension service, and the staff of the Sir John McMichael Centre, both at Hammersmith Hospital, London, UK.

Proteomics experiments were performed in collaboration with Dr. Waheedah Abdul-Salam and Dr. Rob Edwards. Mr. Ka Hou Lao performed the final BMPR2 western blot used in figure 6.6.

Genomic DNA was obtained from Dr. Rajiv Machado at King's College London, UK.

All other data was collected, produced and analysed by myself.

2.2 Tissues and models available for the investigation of pulmonary hypertension

As pulmonary hypertension is a disease of the lung vasculature with secondary effects on the heart, investigations into its pathology can require samples of lung tissue from patients with PH. These become available when end-stage patients unresponsive to PAH medicinal therapies undergo lung transplantation. These procedures are relatively rare, with most centres in the UK only performing one operation per year in PAH patients. Therefore, tissue sample availability is very limited. Appropriate control tissues are also difficult to obtain, and the most commonly used samples are taken from apparently healthy sections of lung obtained from patients undergoing a lobectomy, usually with lung cancer. Tissues from unused healthy donors are also sparsely available and may not be handled in optimum conditions before samples can be collected and frozen for storage. Disease controls such as lung tissue from patients with chronic diseases such as cystic fibrosis might also serve as a useful control in these studies. A much more readily available source of patient samples is blood sampling. Measurements of circulating biochemical indices and studies involving isolated, cultured circulating cells (for example, endothelial progenitor cells (Diller et al. 2008)) can provide insights into the pathophysiology of PAH.

Animal models of PAH are available and different models may reflect better different aspects of the pathology. The monocrotaline (MCT)-injected and chronically-hypoxic rat models are the most

commonly used models of pulmonary hypertension (Campian et al. 2006; Naeije & Dewachter 2007). A single injection of 60 mg/kg MCT intraperitoneally or sub-cutaneously leads to the development of severe pulmonary hypertension (Lalich & Merkow 1961). MCT is converted to pyrrole-MCT, which is toxic to the pulmonary vascular endothelium (Mattocks 1968). This leads to imbalance in vasoactive factors such as endothelin-1 (Mathew et al. 1995) and nitric oxide (Mathew et al. 1997) mirroring the endothelial dysfunction seen in human PAH, and is associated with considerable inflammation in the first 2 weeks, which subsides by 4 weeks. Mean PAP increases consistently over the 4 weeks following MCT injection, and death occurs at 6-8 weeks.

Acute hypoxia leads to pulmonary vasoconstriction primarily due to increased levels of mitochondrial reactive oxygen species leading to release of ryanodine-sensitive calcium stores (Ward & McMurtry 2009). Chronic hypoxia (10% O₂) triggers muscularisation of previously non-muscularised distal branches of the pulmonary vasculature, further contributing to the development of pulmonary hypertension in this model (Zhao et al. 1996a; Zhao et al. 1996b; Zhao et al. 2001). In most rat strains, mPAP doubles to around 35 mmHg over the first 2 weeks then stabilises. Hypoxia is a less severe PAH model as animals generally survive and PAH can be reversible upon return of the animals to a normoxic atmosphere. This may take days to weeks, reflecting the contribution of vascular remodelling to increased mPAP.

While both these models do display some vascular remodelling, this is primarily vessel muscularisation. Key histopathological features of human PAH such as formation of plexiform lesions and neointimal lesions are not replicated in these models. More severe remodelling can be seen in other animal models of PH, including MCT combined with unilateral pneumonectomy (Okada et al. 1997) or MCT-injection in endothelin B receptor-deficient rats (Ivy et al. 2005). Combination of hypoxic exposure with the VEGF receptor antagonist SU5416 results in marked neointimal formation (Taraseviciene-Stewart et al. 2001), and vascular remodelling may be further augmented in rats lacking T-cells (Taraseviciene-Stewart et al. 2007). Another tool in the investigation of PAH pathology is genetic manipulation. For example, *BMPR2* haploinsufficient or dominant-negative mice develop mild PAH with little pulmonary vascular remodelling (Beppu et al. 2004; West et al. 2004), but nevertheless provide an important window for understanding the relevance of *BMPR2* dysfunction in different aspects of the pathology of PAH. While the majority of studies published thus far have focussed on chronic hypoxic and MCT models of PAH, emphasis on the importance of models that develop a more accurate representation of the human condition is growing.

2.3 Animals

Dark Agouti and Sprague-Dawley rats (Charles River, UK) were used for the hypoxia (10% oxygen for 2 days, 1 week or 2 weeks) studies. Sprague-Dawley rats (Charles River, UK) were used for MCT (3 weeks) studies. Wild type and BMPR2 heterozygous mice were used for preliminary studies of isolate primary hepatocytes and liver tissues. Animals were fed a regular diet (3.3% fat, 21.3% protein, and 58% carbohydrate) and maintained at a constant temperature (20°C to 22°C) with a 10-hour on/14-hour off light cycle. All procedures were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 (PP270/6593).

At each time point, animals were weighed and anaesthetised (Hypnorm 0.25 mL·kg⁻¹; midazolam 25 mg·kg⁻¹ *i.p.*). Pulmonary artery pressure was measured *via* a pre-curved catheter inserted through the right jugular vein, passed by the right ventricle and into the pulmonary artery. Following sacrifice hearts were harvested and ventricular chambers dissected carefully and weighed. RVH was assessed from the ratio of the right ventricle to the left ventricle, plus the septum mass (RV/LV+S). Left lung mass was also measured prior to freezing for storage.

BMPR2 mice were genotyped by PCR as seen in (Beppu et al. 2000), using 3 primers A: 5'-GCTAAAAGCCGCATGCTCCAGACTGCCTTG-3', B: 5'-TCACATCATFAACATFATFFAFCFF-3', C: 5'-AGGTTGGCCTGGAACCTGAGGAAATC-3' with 35 cycles at 60°C. The wildtype BMPR2 gene gives a 220 bp product, whereas the dysfunctional BMPR2 gene gives a 260 bp product (Figure 2.1).

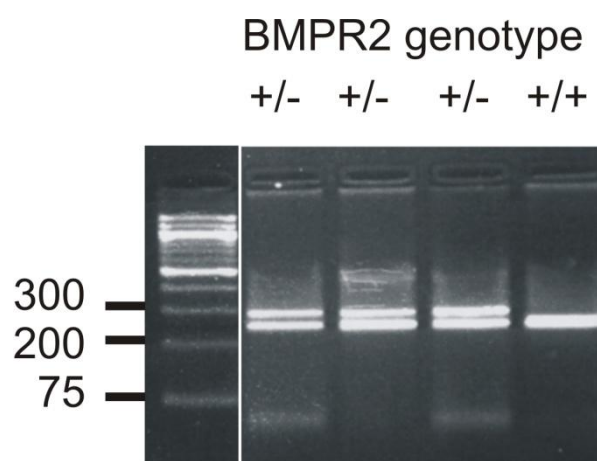


Figure 2.1 – Genotyping of BMPR2 in mice. Image courtesy of Dr. Lan Zhao.

2.4 Tissues, Serum and Plasma

Tissue samples suitable for protein extraction, proteome analysis and Western blotting were obtained with the informed consent of patients and the approval of the Brompton, Harefield & National Heart & Lung Institute and Hammersmith Hospitals Ethics Committees (Ethics ref: 01-210K/2001/6003).

Diagnosis	Sex	Age, y	Disease Duration, mo	mPAP, mm Hg	Vasodilator Therapy
PAH patients					
<i>Proteomic and Western blot analysis</i>					
IPAH	Female	41	18	61	Naïve
HPAH	Female	48	46	60	PGI2 tested
IPAH	Female	28	42	52	Naïve
IPAH	Male	28	14	50	Naïve
IPAH	Female	51	96	60	Naïve
IPAH	Male	48	NA	70	Naïve
IPAH	Female	10	NA	64	Naïve
IPAH	Male	42	40	69	Naïve
IPAH	Female	38	NA	NA	NA
IPAH	Male	3	6	NA	Naïve
IPAH	Female	41	NA	54	Naïve
IPAH	Female	41	60	58	PGI2 tested
IPAH	Female	25	NA	NA	NA
IPAH	Female	28	NA	NA	NA
<i>Immunohistochemical analysis</i>					
IPAH	Female	56	3	82	PGI2 tested
IPAH	Female	46	NA	65	Naïve
IPAH	Female	17	NA	73	Naïve
IPAH	Female	11	NA	NA	Naïve
IPAH	Female	33	NA	NA	Naïve
IPAH	Female	34	NA	NA	Naïve
Control subjects					
<i>Proteomic and Western blot analysis</i>					
Cancer	Female	50			
Cancer	Female	61			
Cancer	Male	63			
Cancer	Male	56			
Cancer	Female	63			
Cancer	Male	59			
Cancer	Male	52			
Cancer	Male	27			
Cancer	Male	67			
Cancer	Female	79			
Cancer	Male	68			
Cancer	Female	78			
Cancer	Male	55			
Cancer	Female	45			
<i>Immunohistochemical analysis</i>					
Donor	Male	33			
Donor	Male	57			

Table 2.1 – IPAH and lobectomy control subject details. Adapted from (Abdul-Salam et al. 2010)

Fresh surgical specimens of explanted lung were collected either at transplantation or at lobectomy/pneumonectomy for carcinoma of the bronchus. All tissues were transported chilled on ice, processed in a similar manner within 0.5-4 hours of surgery and stored under identical conditions. Adjacent tissue samples were either snap frozen, processed for cryostat sectioning or fixed in 10% formal-saline prior to embedding in wax. This provided comparable tissue samples from controls (lobectomy) and patients with IPAH. Subject characteristics are shown in Table 2.1.

Small pieces (~100-200mg) of snap-frozen peripheral lung tissue were used for proteomic and Western blot analysis. Tissues were homogenised in ice cold 100mM potassium phosphate buffer (1.5 ml per 100 mg tissue) containing 1mM ethylenediaminetetraacetic acid (EDTA) and 1mM dithiothreitol, aliquoted and frozen immediately after protein concentration was measured by the Bradford method (BioRad, Hemel Hempstead, Herts. UK).

Human liver tissue samples were used to demonstrate hepatic BMPR2 expression. Samples were obtained from unused transplant donor tissues, kept on ice and then stored frozen at -80°C until use.

Plasma samples with sufficient volume for the required analyses were obtained from the Imperial College Pulmonary Hypertension biorepository. All samples were obtained with the informed consent of patients and the approval of the Hammersmith Hospitals & Charing Cross Hospital Research Ethics Committees (Ethics ref 2001/6157 & 09/H0711/4). This repository comprises blood samples from consenting patients with IPAH attending Hammersmith Hospital, London, between 2002 and 2009. The diagnosis of IPAH was based on standard criteria with confirmation by right heart catheterization and exclusion of other forms of PH (Galie et al. 2009b). The inclusion of both incident and prevalent patients in survival modelling has its limitations; one of the main assumptions of Cox regression analysis is that the hazard ratio for each variable is constant over time; however the mortality rate in IPAH is not consistent over time. Over the first few months mortality rates are much higher and patients selected after this period may be considered “survivors” of this high risk time (McLaughlin & Suissa 2010). To attempt to take this into account treatment status and time since diagnosis were included as covariables in the modelling. The inclusion of both incident and prevalent cases is also consistent with two recently published registry studies analysing survival in IPAH populations in the US (Benza et al. 2010) and France (Humbert et al. 2010) though the complex bootstrapping techniques applied in the French study could not be reproduced in this smaller cohort study.

Haemodynamic data were acquired at catheterization. Patients with co-morbidities that might affect iron homeostasis including haemolytic anaemias, genetic disorders of haemoglobin, diabetes and systemic cardiovascular disease, were excluded. Survival status was censored on July 26, 2010. For survival analysis, all-cause mortality was defined as the primary endpoint, and no patient was lost to follow-up. Samples were also analysed from healthy volunteers from Imperial College London, UK. Patient blood samples were coincident with measurements of WHO functional class, six minute walk distance and oxygen saturation. Healthy controls were recruited from Imperial College London, UK. All plasma samples were collected into EDTA (ethylenediaminetetraacetic acid, as an anticoagulant) tubes (chilled on ice) and stored at -80°C.

2.5 Label-free LC-MS/MS proteomic profiling

1D Gel electrophoresis LC-MS/MS proteomics as an approach

One of the main advantages of the proteomics methodology we have employed (Figure 2.2) is the simultaneous detection, quantification and identification of peptides that is achieved. Other proteomics methodologies including 2D gel separation and surface-enhanced laser desorption/ionisation-time of flight (SELDI-TOF) mass spectrometry are also quantitative but do not simultaneously identify the protein from which detected peptides originated. In terms of furthering understanding of pathophysiology or identifying possible new biomarkers, the identification of the protein itself is vital in terms of continuing the investigation into the biology of any result. However it is also important to note that different proteomic methodologies have differing sensitivities and different techniques used to separate the proteins present in these samples will lead to different subsets of the whole proteome being measured. No one experiment will capture all the tens or hundreds of thousands of possible proteins and this is a limitation of any of these methods.

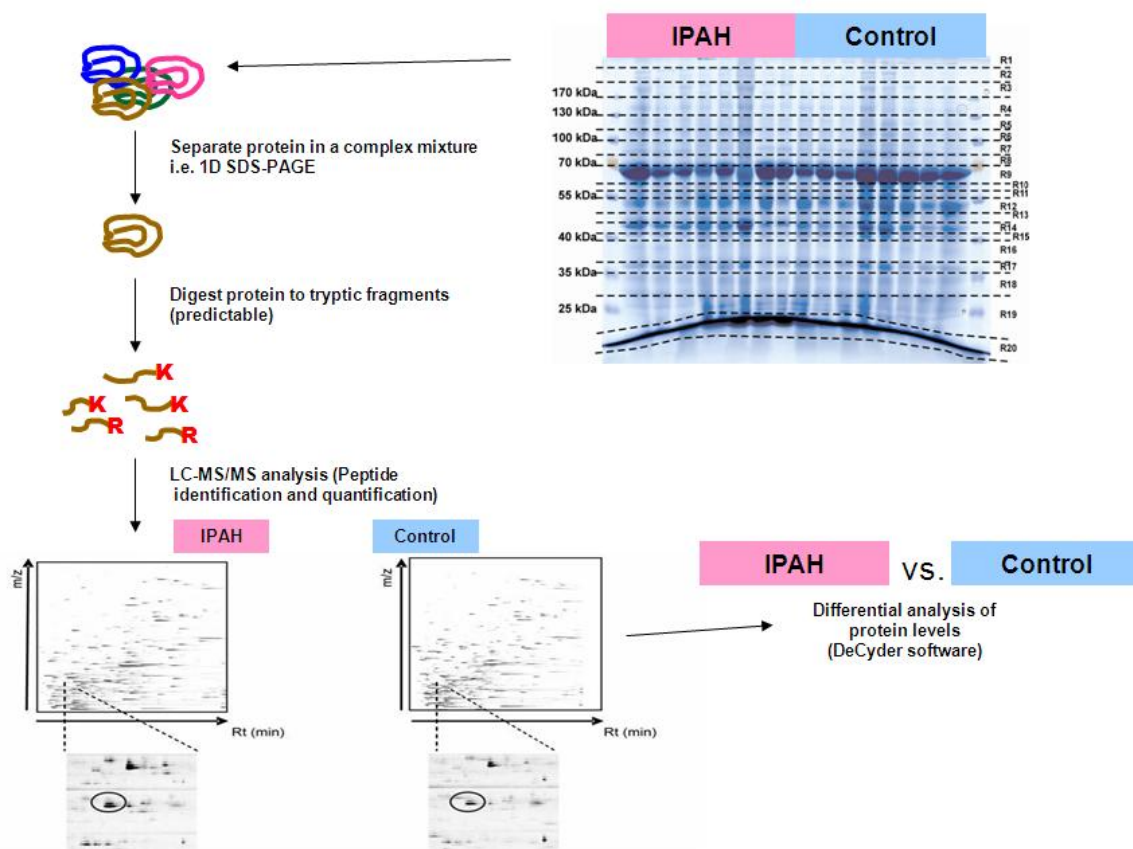


Figure 2.2 – Proteomic profiling workflow. Proteins are first separated by molecular weight on a 1D gel, and regions of the gel (e.g. 0-10 kDa, 11-25 kDa etc.) cut and separated. The individual gel pieces containing the proteins are then digested with trypsin to form small predictable peptides. These peptides are then separated further by liquid chromatography (LC) and mass spectrometry (MS) analysis, visualised on the DeCyder software. Tandem mass spectrometry data is collected for peaks to provide sequences through peptide fragmentation patterns to identify the proteins of origin. Peptide peaks can be quantified and thus proteins compared between groups.

Materials

A protein assay (bicinchoninic acid) kit, containing bovine serum albumin (BSA) standards, was purchased from Perbio Science (Northumberland, UK). Protein mixture digest, containing carboxymethylated, trypsin digested and lyophilised peptides derived from cytochrome c (bovine), lysozyme (chicken), alcohol dehydrogenase (yeast), serum albumin (bovine), apo transferrin (bovine) and beta-galactosidase (*Escherichia coli*) (100 pmole each), was obtained from LC Packings (Cumberly, UK). HPLC grade acetonitrile, formic acid and methanol were obtained from VWR International Ltd. (Lutterworth, UK) and trifluoro acetic acid was from Rathburn Chemicals Ltd.

(Walkersburn, UK). Precast Nupage® Novex 4-12% bis-tris gels, SeeBlue® pre-stained molecular markers, Novex 2-(N-morpholino)ethane sulphonic acid (MES) SDS running buffer, lithium dodecyl sulphate (LDS) sample buffer and Nupage® antioxidant were from Invitrogen Ltd. (Paisley, UK). InstantBlue® was obtained from Novexin Ltd (Cambridgeshire, UK) and sequencing grade modified trypsin was from Promega UK (Southampton, UK). All other reagents were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK).

SDS-polyacrylamide gel electrophoresis (PAGE) and extraction of digested peptides.

Lung tissues were denatured in LDS buffer and reduced with 5 mM dithiothreitol by heating at 100°C for 2 min. Once cooled, they were carboxyamidomethylated with 25 mM iodoacetamide in the dark for 30 min. Samples (20 µg protein) were loaded onto pre-casted Nupage® 10% gradient bis-tris sodium dodecyl sulphate (SDS) gels and proteins stained with InstantBlue®. The gel lanes for each control and IPA sample were divided into seven distinct molecular weight bands and destained with 100 mM ammonium bicarbonate in 50% acetonitrile. After a rinsing with water, the gel pieces were dehydrated with acetonitrile for 30 min. Acetonitrile was then removed and the gel pieces were allowed to air dry before being re-hydrated with 50 mM ammonium bicarbonate. Proteins present in the gel pieces were digested with trypsin (1.2 ng/µL) in 250 µL of 50 mM ammonium bicarbonate at 37°C overnight. Peptides were extracted in 250 µL of 0.1% formic acid in 2% acetonitrile solution for 30 min and a fixed volume was removed; freeze dried and stored at -20°C until further analysis.

LC-MS/MS analysis

The high performance LC system consisted of a dual solvent pump combined with an autosampler (Ettan MDLC; GE Healthcare UK Ltd, UK). Lyophilised samples were reconstituted in 30 µL of 0.1% TFA and 10 µL of the solution was injected onto a C18 trap-column (Zorbax; Packing: 5µm, Pore size: 300Å; Column dimensions: 0.3 x 5mm; Agilent, USA) followed by a high resolution reverse phase separation on a C18 column (PepMap; 3 µm, 100Å, 75 µm x 150 mm; Dionex/LC Packings, USA). The mobile phases were 0.1% formic acid in water (buffer A) and 84% acetonitrile, 0.1% formic acid in water (buffer B). A linear gradient of 0-54% buffer B was introduced at a flow rate of 250 nL/min for 48 min. The peptides eluting from the RP column were analysed on-line using Finnigan's LTQ linear ion trap MS equipped with a dynamic nano-spray interface (Thermo Electron Corporation, Waltham, USA). The MS method consisted of a cycle combining one full MS scan (m/z 400-1600)

with three data dependent MS/MS events (35% collision energy at each event) for 70 min. Dynamic activation time was set at 30 s with scans at every 2 μ s.

Protein identification and quantification

MS data was assessed on the basis of ion intensity of peptide ions with coincident LC retention time and mass/charge (m/z) values. These data were displayed as an intensity map by DeCyder MS (2.1) software (GE Healthcare UK Ltd, Buckinghamshire, UK). The software was used to measure the relative abundance (intensity) of peptides with the same retention time and m/z value by integrating the volume of each peak. The PepDetect module of DeCyder MS was used for peptide detection, charge-state assignment, de-convolution of retention time versus m/z values and quantification. A filter was applied to only include non-overlapping peptides with its charge assigned (up to +4) and a signal to noise ratio of above 2.

A highly reproducible chromatographic profile between runs allowed matching of peptides from intensity maps of the different samples (retention time tolerance = 0.8 min and m/z tolerance = 0.5 Da) using the PepMatch module. Manual insertion of peptide intensities based on PepDetect values were performed when PepMatch missed the cross detection of peptides in all samples. The identity of peptides was determined by exporting the MS/MS output files from the matched analysis into Turbo SEQUEST search engine (BioWorks Browser 3.2; Thermo Electron Corporation, Waltham, USA) and interrogating it against the NCBI human UNIPROT database. Modifications were set to allow for the detection of carboxyamidomethylated cysteine (+57 Da).

2.6 Western blotting

Lung tissue homogenates were denatured in LDS loading buffer and reducing agent (Invitrogen Paisley, UK) by heating at 70°C for 10 minutes and separated by SDS-PAGE on pre-cast 10% gels (Invitrogen) at 200V. Rat purified haptoglobin was bought from Lee Biosolutions Inc. (USA) and diluted 1:200 in phosphate buffer, 6.5 μ l/well was used. MagicMark XL (Invitrogen) was used to show migration of marker proteins. Proteins were transferred onto nitrocellulose membranes (GE Healthcare, Bucks, UK) and blocked for 1 hour in 5% non-fat milk in PBS-T (phosphate-buffered saline (PBS) with 0.1% Tween-20 (Sigma)). Human haptoglobin was detected by incubation with a polyclonal anti- haptoglobin antibody raised in mouse at 1:1000 for 2 hours (Sigma) followed by incubation with an anti-mouse IgG antibody (GE Healthcare) at 1:2500 dilution in 5% milk-PBS-T for 1

hour, washing 6 times for 5 minutes with PBS-T after each incubation period. Rat and mouse haptoglobin was detected using a chicken polyclonal anti- haptoglobin antibody at 1:2000 (Abcam) followed by anti-chicken IgY antibody at 1:30000 (Sigma). Anti-beta-actin antibody (Sigma) was used at 1:2000 for 1h followed by anti-mouse IgG secondary antibody (GE Healthcare) at 1:2500 for 1h. BMPR2 protein was detected using a mouse monoclonal antibody for human BMPR2 at 1:400 dilution overnight at 4°C (BD Biosciences, Oxford, UK) followed by anti-mouse IgG (GE Healthcare) at 1:2000 for 1 hour at room temperature. Visualisation was achieved with enhanced chemiluminescence reagents (GE Healthcare) and exposure to radiography film (Fisher). Semi-quantitative analysis of protein expression was performed using densitometry and proteins of interest were normalised to loading control proteins (e.g. actin). Oxyblot (Millipore, Billerica, MA, USA) was used to detect protein oxidation as per manufacturer's guidelines.

2.7 Polymerase chain reaction (PCR) and reverse transcription (RT)-PCR

RNA was extracted from tissue samples using the Trizol method (Invitrogen). 1ml of Trizol was used to extract RNA per 100 mg tissue and homogenised using either in-tube pestles (VWR, Leicestershire, UK) or ceramic beads. Ceramic beads were more efficient as a method of homogenisation, but RNA was quantified by measurement of absorption at 260 nm in a spectrophotometer to ensure 2 µg of RNA was used in each RT-PCR reaction. Concentration in µg/µl was calculated by $\text{Abs}_{260\text{nm}} \times \text{dilution factor} \times 40 / 1000$. 2 µg RNA in up to 10 µl RNase/DNase-free water (Invitrogen) were added to 4 µl 5X cDNA synthesis buffer, 1 µl 0.1M DTT, 1 µl RNaseOUT™, and 1 µl ThermoScript™ reverse transcriptase (Invitrogen) with 50 ng random hexamers, and 2 µl 10 mM dNTP mix. RT-PCR conditions were as follows: 25°C for 10 min, followed by 50 min at 50°C, terminated at 85°C for 5 min. 20 µl samples were diluted in RNase-free water to 100 µl and 2 µl of diluted product was added to 4 µl RNase-free water, 2 µl 10 mM of each primer and 10 µl PCR Master Mix (M750B, Promega). PCR conditions were as follows: 94°C for 2 min, 94°C for 30 s, annealing temp. for 1 min. 72°C for 1 min – underlined repeated for 28 (beta-actin), 30-34 (haptoglobin) cycles, 72°C for 10 min. 5 µl of PCR product were run on 1% agarose in Tris-acetate-EDTA gel in Tris-acetate-EDTA running buffer at 100V for 20-45 min. Gene ladder 1000bp Plus ladder (Fermentas, York, UK) was run alongside. Primer sets used for PCR reactions are shown in Table 2.2.

To ensure PCR reactions were not saturated or exhausted different cycle numbers were run, showing linear increases, and different levels of starting cDNA with the same cycles also gave linear

relationships. Control blanks were run with the RT step excluded, to ensure genomic DNA/contaminant DNA was not being replicated. Gene expression was normalised to actin.

Target gene	Species	Forward primer	Reverse primer	Product size, bp	Annealing temp. °C	Cycles	Source
Actin	Human	CATGCCATCCTGC GTCTGGA	CCGTGGCCATCTC TTGCTCG	172	60	27-29	(Si & Liu 2008)
Haptoglobin	Human	CTGTGCTGGCATG TCTAA	CAGCTATGGTCTT CTGAAC	178	50	32-34	(Xia et al. 2008a)
Hepcidin	Human	GGCTCTGTTTTCCC ACAACAG	TCCTTCGCCTCTG GAACATGG	102	60	33	(Zhang et al. 2009)
Ferroportin	Human	GGAGATCACAACC GCCAGA	AAGGAATTTTGCA GAGGTCAGGT	69	58	30	(Nahdi et al. 2010)
BMPR2	Human	CTGCGGCTGCTTC GCAGAA	TGGTGTGTGTCA GGAGGTGG	347	53	35	(Upton et al. 2009)
ActRIIA	Human	GCAAAATGAATAC GAAGTCTA	GCACCCTCTAATA CCTCTGGA	435	56.5	30	(Upton et al. 2009)
ActRIIB	Human	ACACGGGAGTGC ATCTACTAC	GGCAAATGAGTG AAGCGCTCG	274	56.5	34	(Upton et al. 2009)
BMP-6	Human	CAACAGAGTCGTA ATCGCTCTACC	TTAGTGGCATCCA CAAGCTCT	399	62	27	(Mi et al. 2010)
Haptoglobin	Rat	GAAAGGCGCTGT AAGTCCTG	GGACCCAGTCCTT CAGATCA	236	55	34	Designed on invitrogen.com
Hepcidin	Rat	TTCCCATATGCCT CTTCTG	GGCAGTGTGTTGA GAGGTCA	108	55	34	Designed on invitrogen.com
Ferroportin	Rat	CCGTGAACCTGAA TGTGAACAAG	CGGAAGGGTCTG CGATCT	60	58	30	(Jenkitkasemwong et al. 2010)
Hepcidin1	Mouse	CTGAGCAGCACCA CCTATCTC	TGGCTCTAGGCTA TGTTTTGC	205	59	33	(Krijt et al. 2010)
Hepcidin1	Mouse	TTCCAGTGTGGT ATCTGTTGC	GGTCAGGATGTG GCTCTAGGC	51	62	33	(Fatih et al. 2010)
Actin	Mouse	AGGCCAGAGCA AGAGAGG	TACATGGCTGGGG TGTTGAA	229	56	33	(Ramey et al. 2009)
S18 RP	Mouse	ACTTTTGGGCCT TCGTGTC	GCCCAGAGACTCA TTTCTTCTTG	96	59	35	(Truksa et al. 2006)
Hepcidin	Mouse	TTGCGATACCAAT GCAGAAAGA	GATGTGGCTCTAG GCTATGTT	124	59	33	(Truksa et al. 2006)

Table 2.2 – Primer sequences used for polymerase chain reactions.

2.8 Phenotyping and genotyping of haptoglobin in patient samples

Serum or plasma samples were phenotyped by immunoblotting as described above, with presence or absence of alpha-1 and alpha-2 chains of haptoglobin used to determine phenotype (Figure 2.3A). Genotyping was used for patients from whom DNA but not plasma or serum samples were available and achieved by the method of Koch *et al.* (Koch et al. 2002). This method amplifies genomic DNA within and containing the exons duplicated to produce the alpha-2 allele. This method established a 100% agreement between haptoglobin phenotype from serum and haptoglobin genotype from genomic DNA. Presence or absence of amplified sequences from the alpha-1 and alpha-2 chains of

haptoglobin was used to determine genotype (Figure 2.3B). Oligonucleotide primers A (5'-GAGGGGAGCTTGCTTTCCATTG-3') and B (5'-GAGATTTTGGAGCCCTGGCTGGT-3') were used for amplification of a 1757-bp Hp 1 allele-specific sequence and a 3481-bp Hp 2 allele-specific sequence. Primers C (5'-CCTGCCTCGTATTAAGTGCACCAT-3') and D (5'-CCGAGTGCTCCACATAGCCATGT-3') were used to amplify a 349-bp Hp 2 allele-specific sequence. Primers were synthesized by Invitrogen.

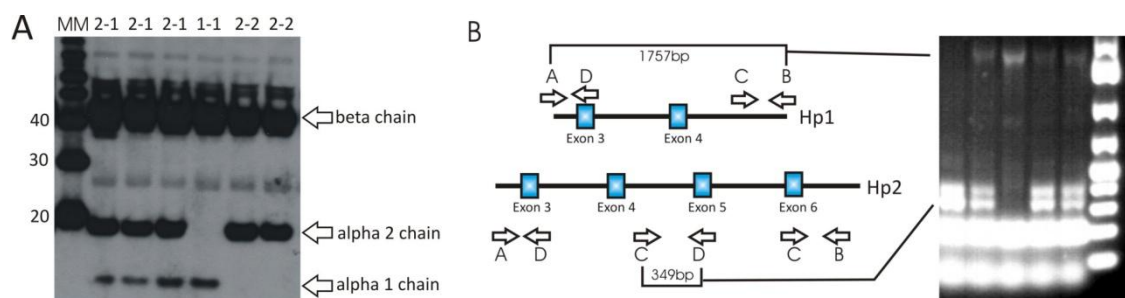


Figure 2.3 - Haptoglobin allele determination. A. Phenotyping of patient serum or plasma. B. Genotyping of patients by PCR using genomic DNA from whole blood samples. Adapted from Koch *et al.* 2002.

2.9 Circulating protein and biochemical measurements

Circulating haptoglobin levels were determined using a single-plex Haptoglobin Luminex assay (Millipore) and a Bio-Plex™ machine (Bio-Rad Laboratories, Inc.). This assay depends upon fluorescent microsphere beads, which are coated in antibodies raised against haptoglobin. Incubation of 25 µl of 1:40000 diluted plasma/serum with these beads is followed by incubation with detection antibody, which binds to haptoglobin bound to these beads. Further incubation with Streptavidin-Phycoerythrin completes the preparation of the samples. The beads used in these assays vary in the levels of two types of fluorescence that allows up to 100 different bead sets to be simultaneously used in one well. Two lasers in the Bio-Plex™ machine detect these fluorescence levels to identify the beads and the breakdown of the streptavidin by the detection antibody can be detected to assign each individual bead an activity reading, which should be relative to the antigen (haptoglobin in this case) concentration. In this assay, 50 beads per well were measured giving an excellent fit on the standard curve (Figure 2.4).

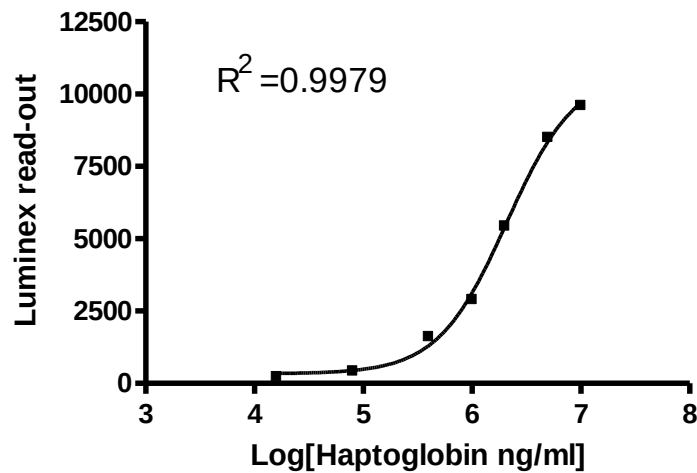


Figure 2.4 – Haptoglobin luminex assay normal curve. Representative standard curve for Luminex assay, based on one plate with duplicates.

Lactate dehydrogenase was measured by a standard clinical pathology-accredited hospital assay. The assay is a kinetic UV test for clinical chemistry analysers using an Olympus system reagent (Olympus Diagnostica GmbH, Lismeehan, Co. Clare, Ireland).

Circulating iron and biomarker measurements

sTfR, GDF-15 and IL-6 levels were determined by enzyme-linked immunosorbant assay (ELISA, R&D Systems Europe, Abingdon, Oxfordshire, UK). Hepcidin levels were determined by a competitive radioimmunoassay, as previously described (Busbridge et al. 2009). Erythropoietin (EPO) was measured using an Immulite-2000 analyser (Siemens, Frimley, Surrey, UK). All other measures were determined by standard clinical pathology accredited hospital assays. Iron deficiency was defined by high levels of sTfR (normal range 8.7-28.1 nmol/L). Serum iron (normal range: Male: 9-29; Female 7-27 µmol/L), transferrin saturation (<20% indicates iron deficiency), and ferritin (<30 µg/L indicates iron stores deficient) were also measured using standard clinical pathology-accredited hospital assays. The normal range of hepcidin was previously determined at 2-55 ng/mL and independently validated in a second group of healthy volunteers (Ashby et al. 2009). EPO had a normal range of 3.3-16.6 mIU/mL. Anaemia was assessed by haemoglobin measurements (lower limits: Females: 12 g/dl, males: 13 g/dl). RDW indices were measured using a Sysmex-XE-2100 auto-analyser (Sysmex, Wymbush, Milton Keynes, UK); a method based upon electrical impedance using hydrodynamic

focusing. Creatinine was measured by standard Clinical Pathology Accreditation-approved hospital assay. NT-proBNP levels were measured using a non-competitive assay as previously described (Omland et al. 2002).

2.10 Determination of circulating BMP-6 levels

BMP-6 levels were determined by ELISA (Insight Biotechnology, Wembley, UK) with a modification to the manufacturer's protocol incubating both samples and detection antibody overnight at 4°C. This assay is a sandwich ELISA, in which the wells of the plate are first coated with antigen-specific primary antibody, washed, blocked with BSA, washed, incubated with sample containing antigen, washed and secondary antigen-specific antibody added. The secondary antibody is biotinylated and is detected using streptavidin conjugated to horseradish peroxidase (HRP), and a substrate solution - a mixture of H₂O₂ and tetramethylbenzidine. The reaction is stopped with 2M sulphuric acid and absorbance of the yellow substrate product is then detected at 450 nm.

The manufacturers claim the minimum detectable dose of BMP-6 is typically less than 150 pg/ml and that the assay provides good measurements in plasma, serum and cell culture media with good linearity across a range of concentrations (from 200 – 10000 pg/ml). They claim good reproducibility with intra-assay: coefficient of variation (SD/mean) <10% and inter-assay coefficient of variation <12%. They claim the ELISA kit shows no cross-reactivity with at least 45 other cytokines tested.

Optimisation of sandwich ELISAs can involve alterations of any one or several of the protocol steps: increased concentration, volume or incubation time of capture antibody, sample, detection antibody or streptavidin-HRP can be attempted to maximise the signal produced. Following the first ELISA plate, only 1 of the 20 control samples had measurable levels, with the lowest detectable dose in the standard curve at 247 pg/ml, and estimated background of ~100 pg/ml (Figure 2.5A). I then increased the sample incubation time from 2.5 hours at room temperature to overnight at 4°C, and the detection antibody to 1.5 times the original concentration, incubated overnight at 4°C instead of 1 hour at room temperature. To minimise background signal I increased each washing step from 4 washes to 6-7 washes. These changes improved the assay sensitivity, with the lowest dose on the standard curve (40 pg/ml) providing a signal above background (Figure 2.5B). Despite this improvement, BMP-6 levels were still undetectable in the samples with undetectable levels in the first assay. Increasing the capture antibody concentration 5-fold, detection antibody 4-fold and streptavidin-HRP 7.5-fold produced no further improvements in the assay sensitivity (Figure 2.5C).

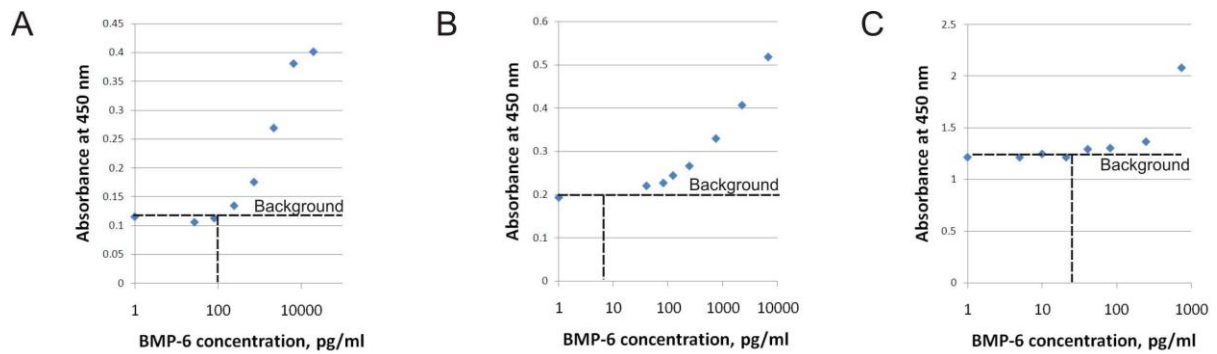


Figure 2.5 – Improving BMP-6 ELISA sensitivity. A. Baseline sensitivity of BMP-6 ELISA following manufacturer’s instructions. B. Modified protocol using higher detection antibody concentration and longer (overnight) incubation of samples and detection antibody. C. Second modified protocol using higher coating and detection antibody concentrations and higher streptavidin-HRP concentrations with overnight incubations as in B.

2.11 Immunohistochemistry

Immunostaining utilised a chicken antibody against human Hp (Abcam Ltd, Cambridge, UK). Formal-saline fixed, wax embedded sections of lung were subjected to antigen retrieval: 0.1% trypsin in 0.1% calcium chloride (pH 7.6) at 37°C for 8 min, and immunostained using a Vectastain™ Elite avidin biotin complex kit (Vector Laboratories, UK). Sections were blocked in 0.3% (vol/vol) H₂O₂ in PBS for 30 minutes at room temperature, rinsed 3x in PBS for 5 minutes, incubated in 3% (v/v in PBS, 0.1% wt/vol BSA and 0.01% wt/vol sodium azide) normal serum from secondary antibody species for 30 minutes. Primary antisera were incubated @4°C in a humid atmosphere for 16-24h in PBS w/0.1% (w/v) BSA, 0.01% sodium azide. Sections were rinsed then incubated with secondary biotinylated antisera (Vector Laboratories, UK) 1:100 in PBS containing 0.1% (wt/vol) BSA for 30 minutes at room temperature. Sections were rinsed, incubated in avidin biotin complex diluted 1:200 in PBS containing 0.1% BSA for 1h at room temperature and rinsed again. Sections were then immersed in 0.025% (w/v) 3,3' diaminobenzidine and 0.03% H₂O₂ in PBS for 2-3 minutes, rinsed briefly and counter-stained with Harris’ Haematoxylin diluted 1:4 in water for 5-10 s. Sections were rinsed in tap water and dehydrated in alcohol series, cleared in xylene and mounted in DPX (dibutyl phthalate and xylene). All materials unless otherwise stated were obtained from Sigma.

2.12 Cell culture

HepG2 (human hepatocellular carcinoma) cells (LGC Standards, Teddington, Middlesex, UK) were seeded at 5×10^5 cells per well in 6-well plates in minimum essential medium (MEM, Sigma-Aldrich) containing 10% foetal bovine serum (Invitrogen) and antibiotics/antimicrobials (Invitrogen). siRNA transfections were performed the following day in reduced serum medium (OPTI-MEM, Invitrogen), using Oligofectamine (Invitrogen) and siRNA duplexes that targeted a scrambled sequence or the type II BMP receptors BMPR2, ActRIIA and ActRIIB (Invitrogen). Three days post-transfection, cells were treated with vehicle or 10 ng/well recombinant human BMP-6 (R&D Systems). Protein was extracted in radio-immuno-precipitation assay buffer (Sigma-Aldrich, Gillingham, Kent, UK). Pulmonary arterial endothelial cells and smooth muscle cells were obtained from PromoCell (Heidelberg, Germany) and cultured in the recommended endothelial cell and smooth muscle cell growth media with supplement mixes (PromoCell). RNA was extracted using the Trizol method (Invitrogen), as described above.

2.13 Isolation of primary hepatocytes

The primary hepatocytes isolation protocol was developed from published reports (Berry & Friend 1969; Truksa et al. 2006). The hepatocytes were isolated by perfusion of the liver via the inferior vena cava with 0.05% collagenase (Sigma) in Hank's Buffered Saline solution (Sigma), single cells isolated with a 70- μ m nylon filter (VWR) and allowed to attach to collagen-coated ($5 \mu\text{g}/\text{cm}^2$, BD Biosciences) plates overnight. Cells were seeded at 6×10^5 to 6×10^6 per well in 6-well plates. Dulbecco's modified Eagle's medium (containing 10% FBS) was then refreshed twice at 24h and 48h. At 48h BMP-6 (0-100 ng/ml) or PBS was added and mRNA was extracted after 16 h incubation.

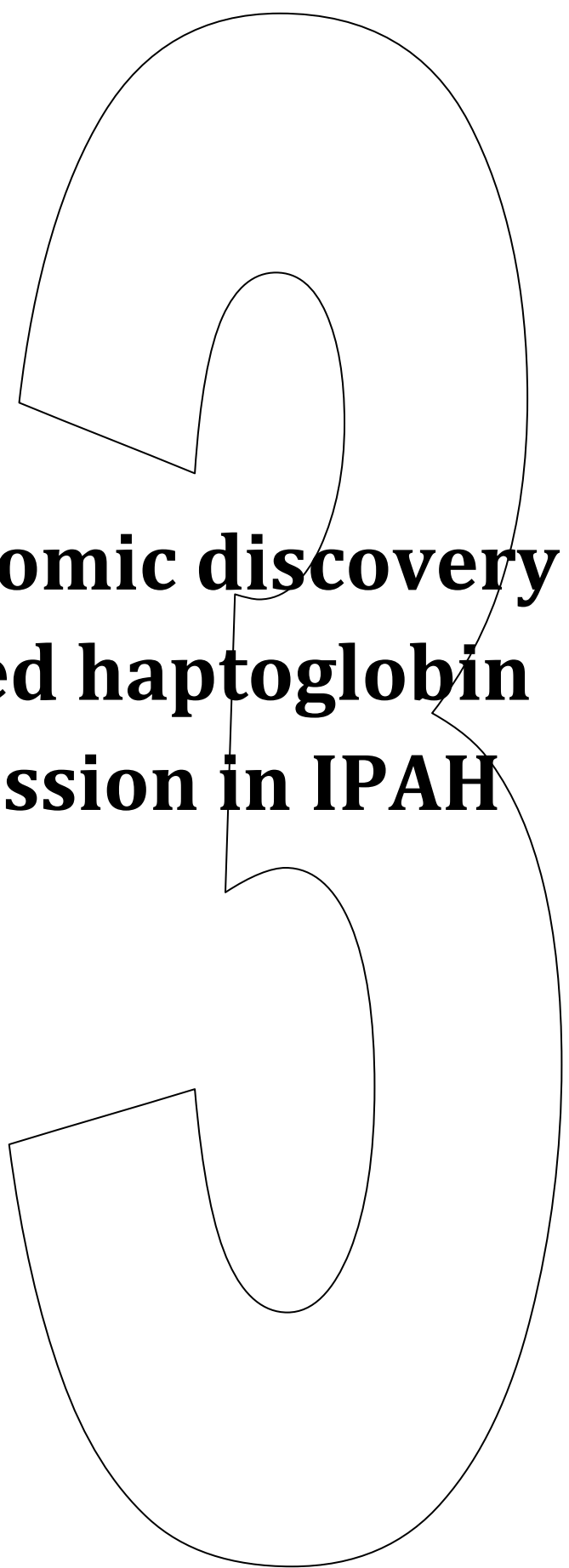
2.14 Data presentation and statistical analysis

Peptide data is presented as means of IPAH and lobectomy peaks. Proteomic and patient age data are presented as box and whisker plots, displaying means and quartiles. Haptoglobin measurements by patient group and haptoglobin phenotype are displayed as scatter-plots with individual measurements displayed. Bars indicate means. Clinical and haemodynamic data is presented as means of group, with standard deviation, unless they were not normally distributed, in which case they are presented as medians with interquartile ranges (IQR). Circulating protein and biochemical measurements are displayed as dot-plots, bars indicate means.

A quantitative comparison was made between the control and the IPAH groups based on MS signal intensities of individual LC-MS analyses or densitometry values from Western blotting and PCR using an unpaired student's t-test. Population distributions of haptoglobin phenotype were compared with a chi-squared test. The relationship between patient characteristics and biomarkers were assessed by Spearman's rank test or Mann-Whitney U and Kruskal-Wallis tests for categorical variables.

Receiver Operating Characteristic (ROC) curves assessing 2-year all-cause mortality and all possible biomarker cut-offs and their area-under-the-curve were used to compare prognostic values of biomarkers. 2-years was selected as the time point for ROC analysis as this provided a good event rate (29 deaths) without excluding too many patients (n=101). Highly sensitive (>80%) cut-offs were selected by the maximum combined sensitivity and specificity. Kaplan-Meier survival estimates from time of sampling were produced and compared using a log rank test. Several parameters were not normally distributed, as shown by the Kolmogorov-Smirnov test, and were therefore respectively transformed to their natural logarithm (ln) and square root, as appropriate to best normalize the data, before Cox analysis (Altman 1991). Univariate Cox regression survival analysis was used to analyse single parameters as predictors of survival. Multivariable Cox regression survival analysis by the backwards stepwise hazard ratios method was used to analyse multiple parameters with $p < 0.05$ in univariable analysis as co-predictors of survival. Variables were removed at $p > 0.1$.

Immunoblotting and RT-PCR results are presented as mean with bars for standard deviation, and were compared by analysis of variance (ANOVA), with Tukey post-hoc analysis performed if $p < 0.05$. BMP-6 and hepcidin absolute values are shown in dot-plots, compared by t-test. All calculations were performed with SPSSv17.0 (SPSS, Inc., Chicago, IL) or GraphPad Prism4 (GraphPad Software Inc, La Jolla, CA).

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Proteomic discovery of altered haptoglobin expression in IPAH

Chapter 3 – Proteomic discovery of altered haptoglobin expression in IPAH

3.1 Introduction

Proteomic approaches to studying diseases, which can lead to the identification and even quantification of hundreds or thousands of proteins in any one sample, have become popular in the hunt for disease biomarkers (Bons et al. 2007; Cutillas & Vanhaesebroeck 2007; Kolker et al. 2006; Li et al. 2007; Mayr et al. 2006; Terrier et al. 2008; Yu et al. 2007; Zhu et al. 2008a; Zhu et al. 2008b). We have utilised an LC-MS/MS-based method to compare the proteomes of lung tissue samples from IPAH patients with those from lobectomy patients (Abdul-Salam et al. 2010). One preliminary result to come from our proteomic studies (see Results section) was the downregulated expression of the protein haptoglobin in the lung tissue of patients with idiopathic pulmonary arterial hypertension in comparison with normal lung tissue taken from lobectomy samples.

The main function of haptoglobin (Hp) is in the regulation of circulating iron levels by the sequestration of cell-free haemoglobin, which is released from erythrocytes during haemolysis. Cell-free haemoglobin scavenges nitric oxide (Azarov et al. 2008; Wang et al. 2004). The acute phase protein Hp (Theilgaard-Monch et al. 2006) prevents the scavenging of iron by infecting bacteria (Francis, Jr. et al. 1985; Zakaria-Meehan et al. 1988), and may have roles in angiogenesis (Cid et al. 1993) and vascular remodelling (Lohr et al. 2005). In humans there exist two alleles of haptoglobin which produce 3 major haptoglobin phenotypes in humans; homozygous Hp1-1 or Hp2-2, and heterozygous Hp2-1. Particular Hp polymorphisms have been linked with a variety of inflammatory, autoimmune and cardiovascular diseases (Carter & Worwood 2007; Langlois & Delanghe 1996). These include conditions associated with PAH (Bons et al. 2007; Delanghe et al. 1998; Pavon et al. 2006; Quero et al. 2004; Rodriguez et al. 2006). Individuals with the Hp2-2 phenotype have the lowest circulating levels of haptoglobin, with Hp2-1 intermediate and Hp1-1 levels the highest (Langlois & Delanghe 1996). The Hp2-2 phenotype is a cardiac risk factor in diabetes mellitus (Levy et al. 2002) and Hp phenotypes predict 30-day mortality and heart failure in patients with diabetes post-myocardial infarction (Suleiman et al. 2005).

When Hp is depleted by excessive haemolysis, Hb-mediated toxicity driven by free radical production and NO scavenging may occur in diseases such as PAH that are linked to oxidative stress (Vallelian et al. 2008). Haemolysis itself is related to PAH (Barnett et al. 2008; Frei et al. 2008; Hsu et al. 2007). Haptoglobin gene expression is not confined to the liver. Significant expression also occurs

in the lung, where haptoglobin mRNA has been localised to airway epithelial cells and alveolar macrophages (Yang et al. 2000; Yang et al. 2003). Haptoglobin is also reported to be expressed in the arterial wall, where it has been implicated in vascular remodelling, adventitial cell migration and angiogenesis (Cid et al. 1993; De Kleijn et al. 2002; Smeets et al. 2003). Hypoxia reduced Hp expression in human adipocytes (Wang et al. 2007) and increased expression in HepG2 cells (Wenger et al. 1995). Thus, it is possible that the hypoxia-sensitive regulation of haptoglobin may vary in different tissues.

I hypothesised that low haptoglobin levels, and the Hp 2-2 phenotype, may contribute to the onset and progression of pulmonary arterial hypertension. Investigation of the relationship between haptoglobin and iron status in patients and animal models of PAH will improve our understanding of the pathophysiology of the disease and could lead to the development of novel therapeutic targets.

I set out to a) validate and expand the observation of downregulated haptoglobin protein expression in lung tissue and plasma from IPAH patients, b) investigate the role of haptoglobin protein levels in the onset, progression and outcome of PAH, c) investigate the role of haptoglobin phenotype in the onset, progression and outcome of PAH, d) further understand the biology and possible mechanisms of downregulation of haptoglobin in the setting of PAH through studies of patient samples and animal models of PAH and e) assess the relationship of haptoglobin levels to systemic iron status in patients with PAH.

3.2 Methods

See Chapter 2 for detailed experimental materials and protocols.

All tissues and blood samples were obtained with written, informed consent and ethical approval from local research ethics committees.

Lung tissue samples from 7 IPAH and 7 lobectomy patients were first compared using a 1D gel electrophoresis, liquid chromatography and tandem-mass spectrometry (LC-MS/MS) proteomic approach, which identifies and quantifies tryptic peptides originating from proteins encoded in the SEQUEST database, based on sequencing of their fragmentation patterns. Western blotting was used to confirm the proteomic result in lung tissue of IPAH patients versus lobectomy. Semi-quantitative RT-PCR and quantitative real time PCR were both employed to analyse mRNA expression. Circulating LDH was measured by a clinical pathology-approved hospital assay.

Immunohistochemistry was used to localise Hp protein expression in lung tissue sections from IPAH patients who underwent lung transplants. The Millipore OxyBlot reagent kit was used to detect protein oxidation in lung tissue homogenates from IPAH and lobectomy patients by western blotting as per manufacturer's guidelines.

Changes in Hp expression were assessed in experimental models of PH. Development of PH in the chronic hypoxia and monocrotaline rat models was assessed by the development of right ventricular hypertrophy, as measured by the weight of the excised right ventricle over the weight of the left ventricle and septum. Mean pulmonary artery pressure and left lobe weights (normalised to body weight) also indicated the development of PH in the chronic hypoxia model. Western blotting and RT-PCR methods were again used to assess Hp, HIF-1 α and HIF-2 α levels in animal tissues.

Circulating Hp levels were analysed in plasma samples from controls (n=73) and patients with IPAH (n=131), connective tissue disease-related PAH (CTD-PH, n=16), other associated PAH (APAH, n=34), chronic thromboembolic PH (CTEPH, n=24) and lung disease-associated PH (n=5) using a Luminex method. Subject characteristics are shown in Table 3.1. Serial Hp levels were measured in a subgroup of IPAH patients (n=71) sampled at standard 3/6-monthly clinic visits over 3-24 month periods, where samples were retrospectively available. Serum iron and transferrin saturations were measured by standard clinical pathology assays.

Diagnosis	Controls		IPAH		CTD-PH		APAH		CTEPH		Lung disease		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Age, y	36.5	9.5	46.0	15.4	57.4	15.1	57.1	15.2	61.8	13.3	55.4	15.0	
Gender	F	33	45%	96	73%	16	100%	27	79%	12	50%	4	80%
	M	40	55%	35	27%	0	0%	7	21%	12	50%	1	20%
WHO	I		1		0		0		0		0		
functional	II		20		0		3		0		1		
class	III		96		12		27		22		4		
	IV		14		4		4		2		0		
6 min walk, m			287.5	151.2	223.2	154.1	294.8	115.6	243.9	117.2	325.8	165.5	
mPAP, mmHg			56.1	11.3	44.1	9.4	39.4	11.9	51.6	21.0	36.5	6.6	
Wedge pressure, mmHg			11.3	4.6	.	.	.	.	.	.	.	.	
mRAP, mmHg			11.2	6.0	8.5	2.8	12.8	9.0	12.7	11.3	5.0	2.0	
Cardiac output L/min			4.1	1.6	.	.	.	.	.	.	.	.	
PVR, Woods			13.4	8.1	6.7	3.7	7.2	5.7	8.3	4.3	8.9	10.3	

Table 3.1 – Subject characteristics. mPAP = mean pulmonary artery pressure. mRAP = mean right atrial pressure.

Hp phenotypes/genotypes were assessed by protein isoform expressions in plasma samples or by PCR. Survival was estimated using the Kaplan-Meier method and compared by log rank tests. Means and standard deviations or medians with IQR are shown for continuous patient data, compared by Mann Whitney U-test. Categorical values are shown as frequencies compared by chi-squared tests.

3.3 Results

Lung tissue samples from 7 lobectomy (control) patients and 7 patients with IPAH were compared by a gel electrophoresis-liquid chromatography-tandem mass spectrometry proteomic approach. Over 300 proteins were detected and 25 were shown to be differentially expressed in IPAH (Abdul-Salam et al. 2010). Two of the proteins successfully identified that displayed differential expression and were of possible pathophysiological relevance in IPAH, versus control tissues, were haptoglobin (Figure 3.1) and hemopexin (Figure 3.2).

Ten unique tryptic peptides originating from the beta-chain of Hp were detected in the analysis and 6 were quantifiable (Figure 3.1A). All 6 demonstrated a similar 80% reduction in expression in lung tissue from IPAH patients. Four of the sequences were specific to Hp versus haptoglobin-related protein (Figure 3.1A). Western blotting of lung tissue homogenates from 14 controls and 14 IPAH patients confirmed the significant reduction in Hp protein expression in IPAH (Figure 3.1B). Quantitative data from the proteomic and immunoblotting studies showed similar reductions in Hp could be detected by these two methods (Figure 3.1C&D, respectively). Four unique peptides originating from hemopexin were detected in the proteomic study (Figure 3.2A); all of these demonstrated a 30-40% reduction in hemopexin expression. Combining these data from the 4 peptides normalised to the control averages confirmed that hemopexin expression was significantly reduced in IPAH tissue samples ($p=0.038$, Figure 3.2B).

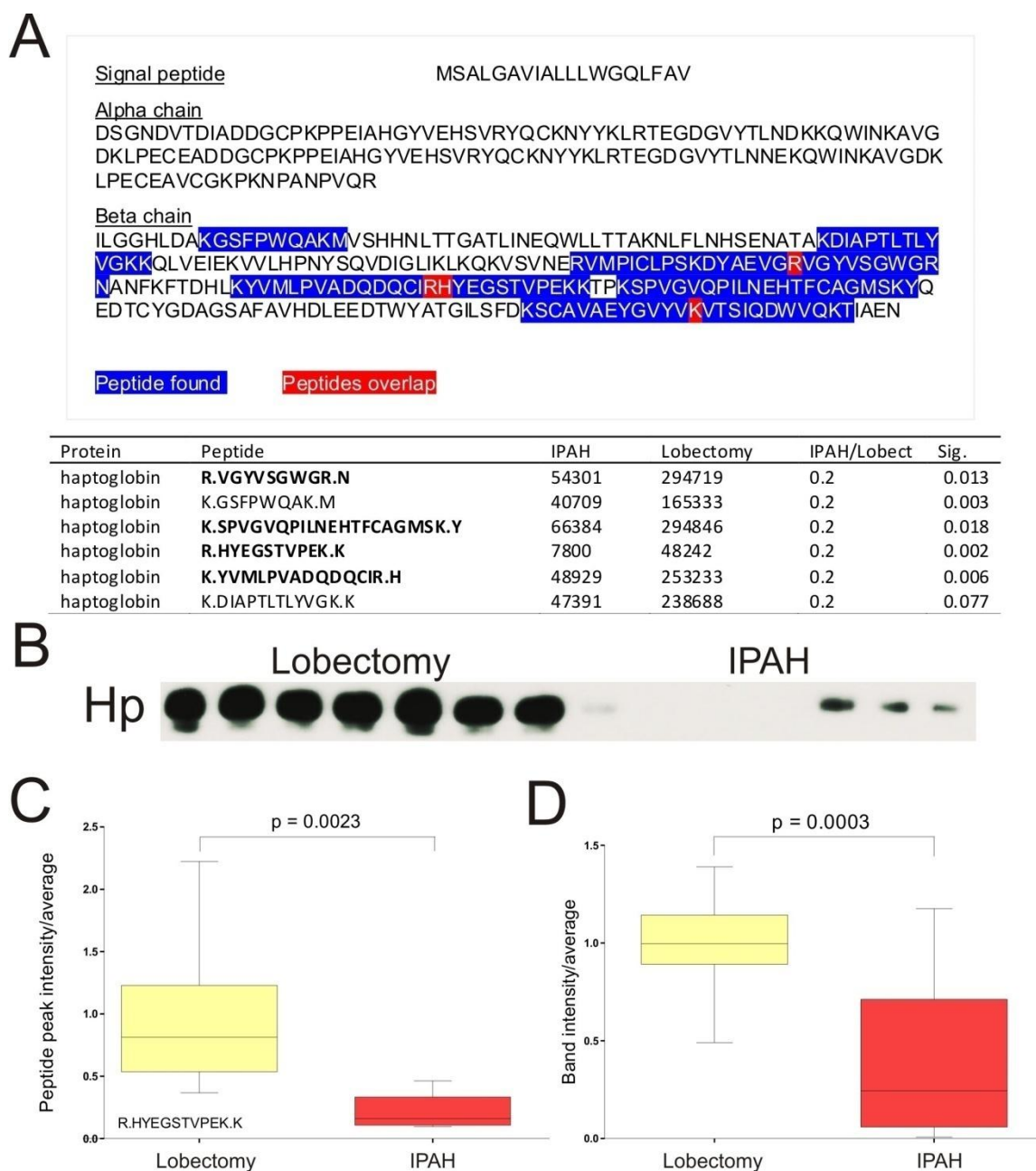


Figure 3.1 - Haptoglobin expression in IPAH lung versus lobectomy tissue. A. 6 tryptic peptides originating from haptoglobin protein were identified by a 1D-gel electrophoresis LC-MS/MS-based proteomic approach and detected peaks quantified in IPAH lung tissue versus lobectomy controls (n = 7 per group). Sequences highlighted in bold in the table cannot be found in haptoglobin-related protein. Values in the table represent peak intensity in arbitrary units. B. Western blotting confirmed reduced lung haptoglobin expression in IPAH (n = 14 per group). C & D display quantitative data from A & B, respectively. Statistics are p-values from t-tests.

A

Hemopexin

MARVLGAPVALGLWSLCWSLAATPLPPTSAHGNVAEGETKDPDPVTERCSDGWSFDATLDDNGTMLFFKGEF
VWKSHKWDRELISERWKNFPSPVDAAFRQGHNSVFLIKGDKVWVYPPEKKEKGYPKLLQDEFPGIPSPLDAAVEC
HRGECQAEGVLFFQGDREWFWDLATGTMKERSWPAVGNCSSALRWLGRYYCFQGNQFLRFDPVRGEVPPRYP
RDVRDYFMPCPGRGHGHRNGTGHGNSTHHGPEYMRCSPHLVLSALTSDNHGATYAFSGTHYWRLDTSRDGWH
SWPIAHQWPQGSAVDAAFSWEEKLYLVQGTQVYVFLTKGGYTLVSGYPKRLEKEVGTPHGIILDSVDAAFCPGS
SRLHIMAGRRLWWLDLKSGAQATWTELPWPHEKV DGALCMEKSLGPNSCSANGPGLYLIHGPNLYCYSDVEKLN
AAKALPQPQNVTSLGCTH

Peptides found

Protein	Peptide	Lobectomy	IPAH	IPAH/Lobect	Sig.
Hemopexin	K.SGAQATWTELPWPHEK.V	147728	104216	0.71	0.30
Hemopexin	K.NFPSPVDAAFR.Q	364608	211937	0.58	0.01
Hemopexin	K.EVGTPHGIILDSVDAAFCPGSSR.L	204209	121694	0.60	0.11
Hemopexin	R.YYCFQGNQFLR.F	330431	215686	0.65	0.07

B

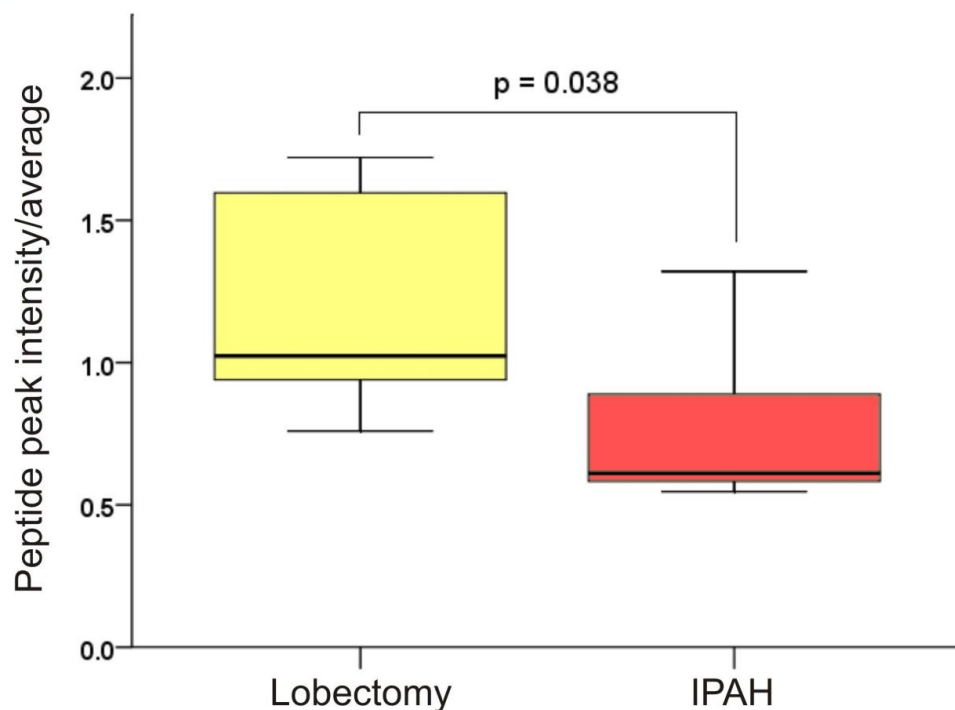


Figure 3.2 - Hemopexin expression in IPAH versus lobectomy. A. 4 peptides originating from hemopexin were detected, and quantified in IPAH versus lobectomy. Values in the table represent peak intensity in arbitrary units. B. Overall average peptide intensities indicated a significant decrease in hemopexin protein levels in IPAH lung samples.

Following confirmation of the downregulation of the two iron homeostasis proteins haptoglobin and hemopexin, further studies were performed to investigate the cause of the significant reduction in haptoglobin. The first mechanism considered was reduced transcription or stability of Hp mRNA. Analysis of Hp mRNA expression in lung tissue samples from the same subjects revealed no significant difference in IPAH versus lobectomy (Figure 3.3). Semi-quantitative data are displayed in Figure 3.3A&B and quantitative PCR confirmed no significant reduction in Hp mRNA expression in IPAH patients whose Hp protein levels were reduced ($p=0.62$, Figure 3.3C). These data suggest that the reduction in Hp protein expression in IPAH lung tissue is due to mechanisms downstream of transcription.

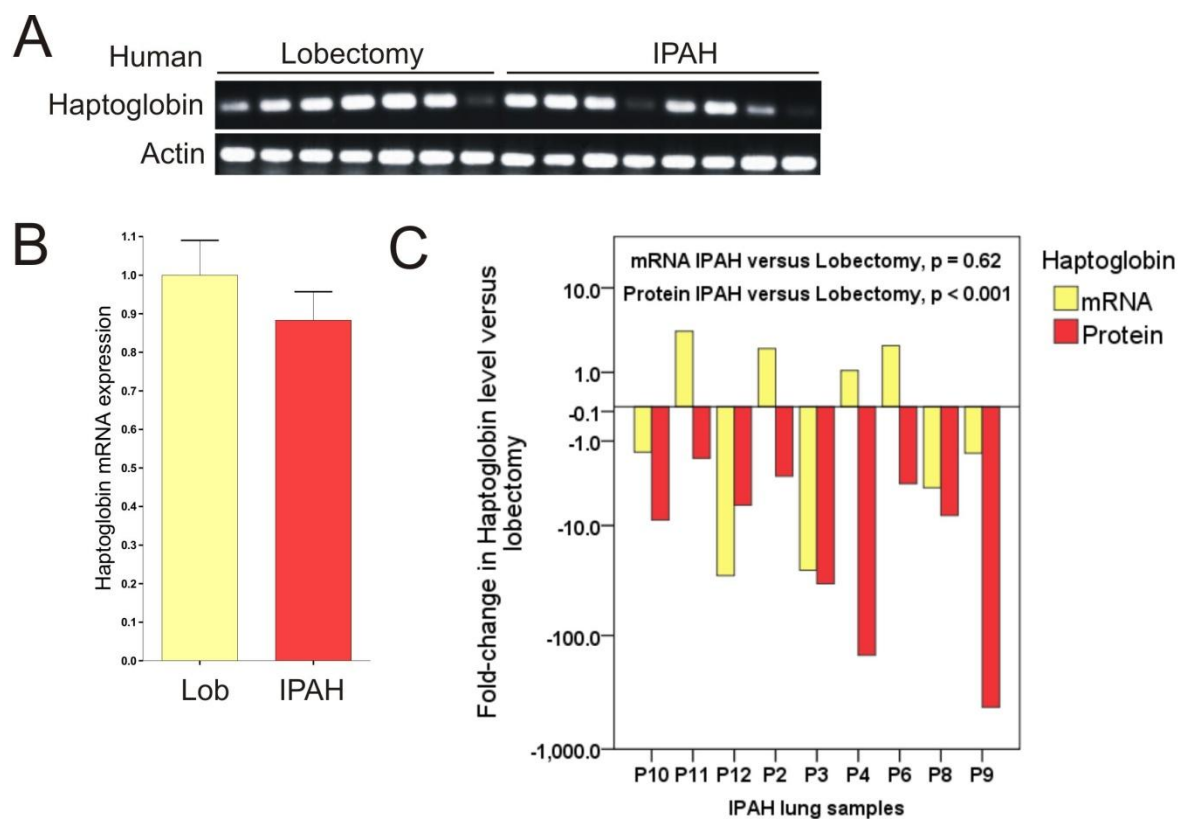


Figure 3.3 – Haptoglobin mRNA in IPAH lung tissue versus lobectomy. A. RT-PCR products from lobectomy and IPAH lung tissue show haptoglobin levels are variable but not significantly different between groups. B. Densitometry of RT-PCR data. C. Quantitative PCR results normalised to tubulin confirmed that haptoglobin mRNA was on average unchanged versus lobectomy, showing a disconnection between mRNA levels and consistent reductions in haptoglobin protein levels.

To explore whether the reduction in Hp expression in IPAH was a local or more systemic effect, and whether it was specific to idiopathic versus associated PAH (APAH), I measured plasma Hp levels in controls (n=73) and patients with IPAH (n=131), connective tissue disease-related PAH (CTD-PH, n=16), other associated PAH (APAH, n=34), chronic thromboembolic PH (CTEPH, n=24) and lung disease-associated PH (n=5). CTD-PAH cases were considered as a separate group due to the likely effects of inflammation on the expression of Hp. Although the spread of values was quite large in each group, the average levels of haptoglobin were significantly reduced versus controls in IPAH ($p=0.02$) where 55/131 (42%) were below normal. 19/34 (56%) of the APAH patients and 6/24 (25%) of chronic thromboembolic patients had reduced Hp levels (Figure 3.4), whereas only 1/16 (6%) with connective tissue disease-PAH and none of the 5 lung disease PH patients had low Hp. This showed Hp levels were reduced at the systemic level in several aetiologies of PAH. The lack of specificity of low Hp levels meant that Hp could not be considered as a diagnostic biomarker in distinguishing IPAH either from controls or from other PAH aetiologies.

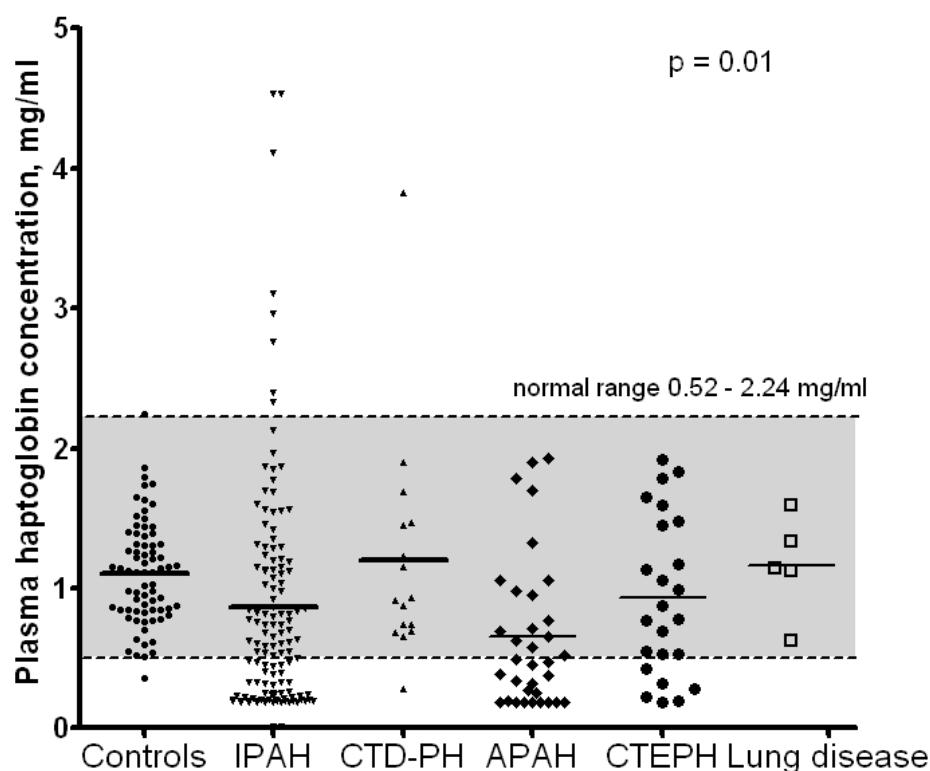


Figure 3.4 – Haptoglobin protein in the plasma of patients with IPAH or APAH. Haptoglobin levels were quantified in plasma from controls (n=73) and patients with IPAH (n=131) connective tissue disease-related PAH (CTD-PH, n=16), other associated PAH (APAH, n=34), chronic thromboembolic PH (CTEPH, n=24) and lung disease-associated PH (n=5) using Luminex beads. Statistic shown is p-value from one-way ANOVA.

A mechanism that must be considered when investigating reduced plasma haptoglobin expression is haemolysis. When red blood cells lyse they release haemoglobin and LDH; haptoglobin is degraded in the process of scavenging the haemoglobin as part of the iron recycling process. As a surrogate of haemolysis, plasma LDH levels were measured in a subset of the IPAH cohort used above. Although LDH levels were in general towards the upper end of the normal range (normal: 125-243 IU/L), there was no significant correlation between LDH and Hp levels ($Rho=-0.1$, $p=0.39$, Figure 3.5A); LDH levels were not higher in those patients with low Hp ($p=0.10$, Figure 3.5B).

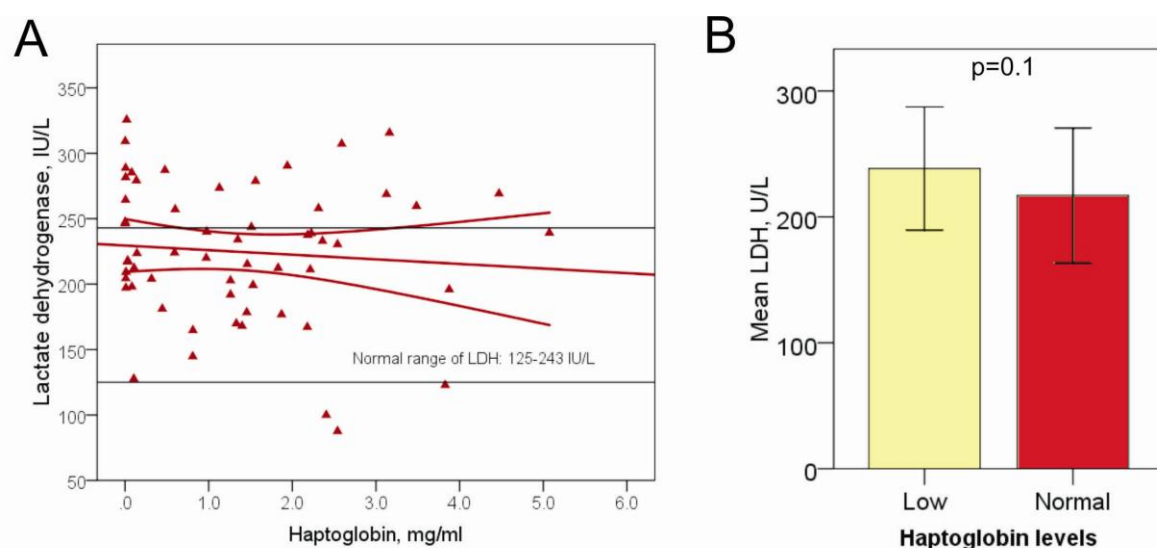


Figure 3.5 – Lactate dehydrogenase and haptoglobin levels in IPAH patients. A. Scatter-plot of LDH and Hp levels. Grey lines indicate normal range of LDH. Curved red lines indicate 95% confidence intervals for line of best fit. B. LDH levels between patients with low (n=23) or normal (n=34) Hp levels. Statistic shown is p-value for t-test.

To better understand the possible mechanism of Hp regulation in the IPAH lung, immunohistochemistry of lung tissue sections from IPAH and lobectomy samples was performed to localise Hp expression (Figure 3.6). Little specific staining was observed in bronchiolar cells (Figure 3.6A) whereas some staining could be observed in the media and adventitia of pulmonary arteries (Figure 3.6B). The strongest specific staining was observed in macrophages seen in the airways (Figure 3.6C) and alveoli (Figure 3.6D). These data suggested that while the lung tissue itself might represent a source of Hp, the most abundant pulmonary expression of Hp is in macrophages.

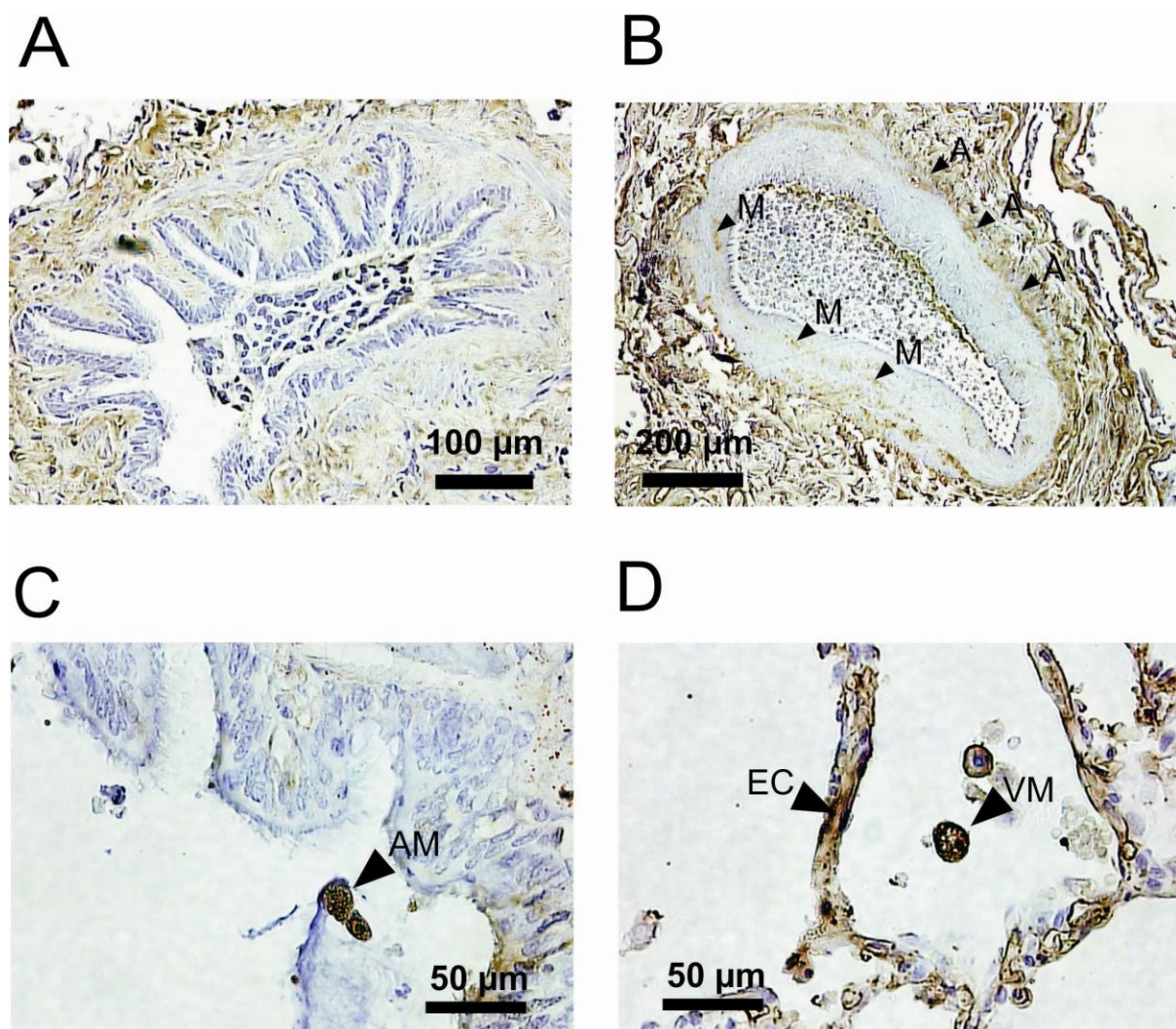


Figure 3.6 – Haptoglobin immunohistochemistry staining in lung tissue. A. Bronchiole. B. Pulmonary artery. (A=adventitial staining M=medial staining.) C. Airway macrophages (AM). D. Alveolar macrophages (VM) and alveolar epithelial cell staining (EC).

Introduction of carbonyl groups to lysine, arginine, proline or threonine residues (Stadtman 1993) can increase protein susceptibility to degradation (Davies 1987). To investigate the likelihood of this mechanism being overactive in IPAH OxyBlot detection of protein oxidation levels in lung tissue homogenates from IPAH and lobectomy was performed. No significant difference in total protein oxidation levels was observed in IPAH lung (Figure 3.7). This result suggested that this mechanism was not responsible for reduced Hp levels in IPAH.

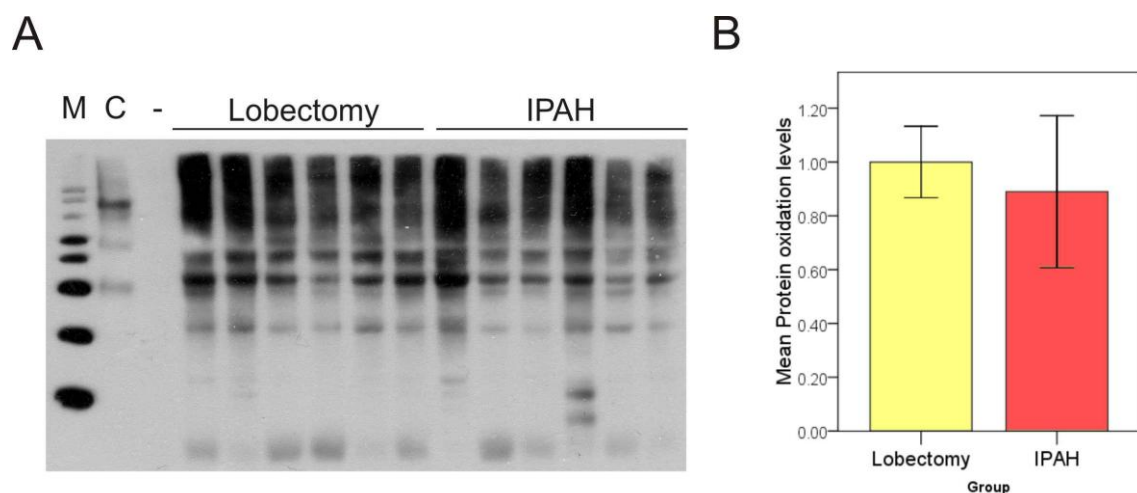


Figure 3.7 - Oxyblot detection of protein oxidation levels in IPAH versus lobectomy. A. Oxyblot image of oxidised proteins in lobectomy versus IPAH lung tissue samples. M= Marker, C = positive control, - = negative control. B. Densitometry quantification of entire lanes by group shown in A. Error bars show standard deviation.

Further understanding of pathophysiological mechanisms in PAH can be obtained from studying the animal models of PH – the chronically hypoxic rat or mouse models, or the monocrotaline-injected rat model. Following 3 weeks at 10% oxygen (Figure 3.8A) or 3 weeks after monocrotaline injection (Figure 3.8B), rats develop significant right ventricular hypertrophy, as demonstrated by an increased weight ratio of the right ventricle to the left ventricle+septum (RV/LV+S). A progressive phenotype can be observed in hypoxia, with RV hypertrophy apparent at 1 and 2 weeks, as well as raised mean pulmonary artery pressures which both indicate pulmonary hypertension.

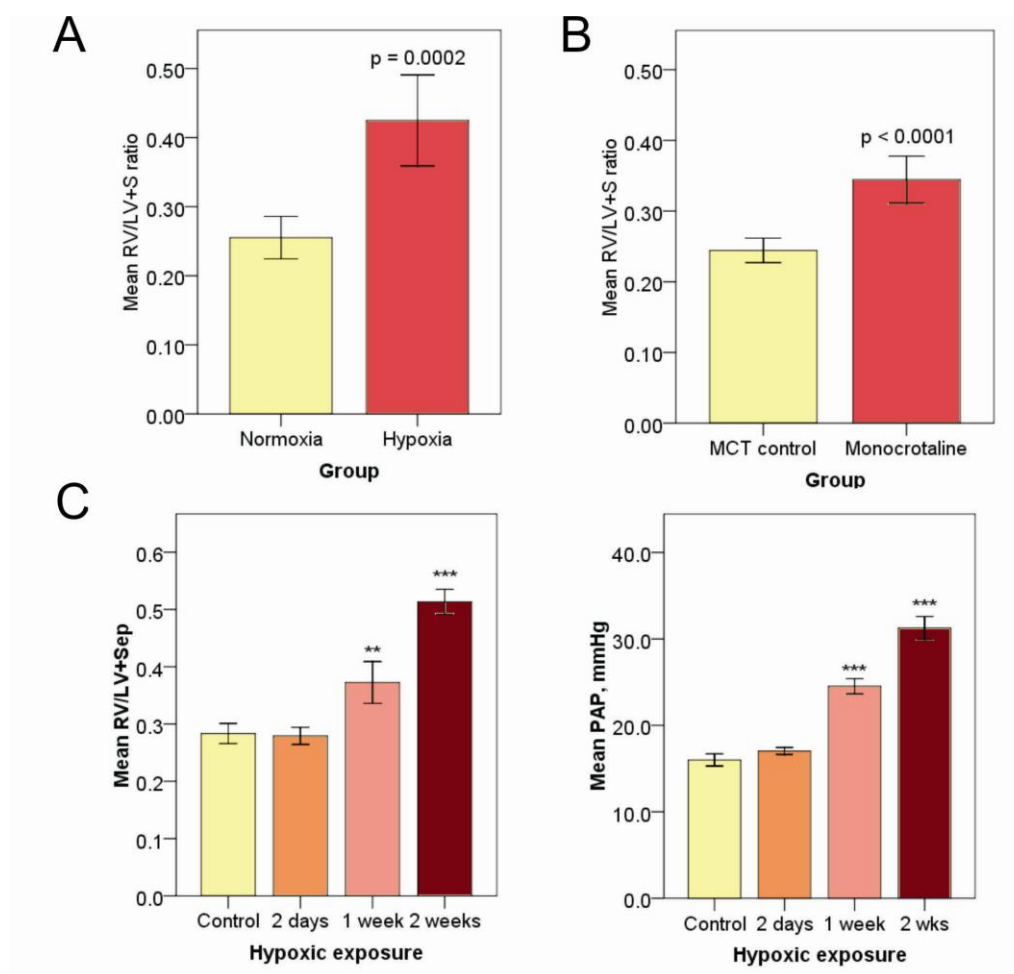


Figure 3.8 - Development of pulmonary hypertension in animal models. A. RV hypertrophy following 3 weeks normoxia or hypoxia in 6 versus 6 male Sprague-Dawley rats. B. Monocrotaline or vehicle effects on RV hypertrophy at 4 weeks in 6 versus 6 male Sprague-Dawley rats. C. Hypoxia time course effects in male Dark Agouti rats (n=3 per time point) on RV hypertrophy and PAP. **=p<0.01 versus controls, ***=p<0.001 versus controls. Error bars shown indicate standard deviation.

Hypoxic exposure for 2 days led to significantly increased protein expression of Hp in the lung, kidney and plasma of rats. After 1 and 2 weeks chronic exposure, Hp protein expression was almost completely obliterated (Figure 3.9A&C). This was mirrored by increased expression of HIF-2-alpha protein in lung tissue following 1 and 2 weeks exposure to hypoxia (Figure 3.9A&D). HIF-1alpha protein expression could not be detected in any of the tissues in preliminary studies. Conversely, in the inflammatory monocrotaline model of PH, Hp protein was increased in the lungs of rats 3 weeks following monocrotaline injection (Figure 3.9B&E). These data implicate a role for hypoxia in the regulation of haptoglobin, and illustrate the role of haptoglobin as an acute phase reactant, being induced in the more inflammatory MCT model.

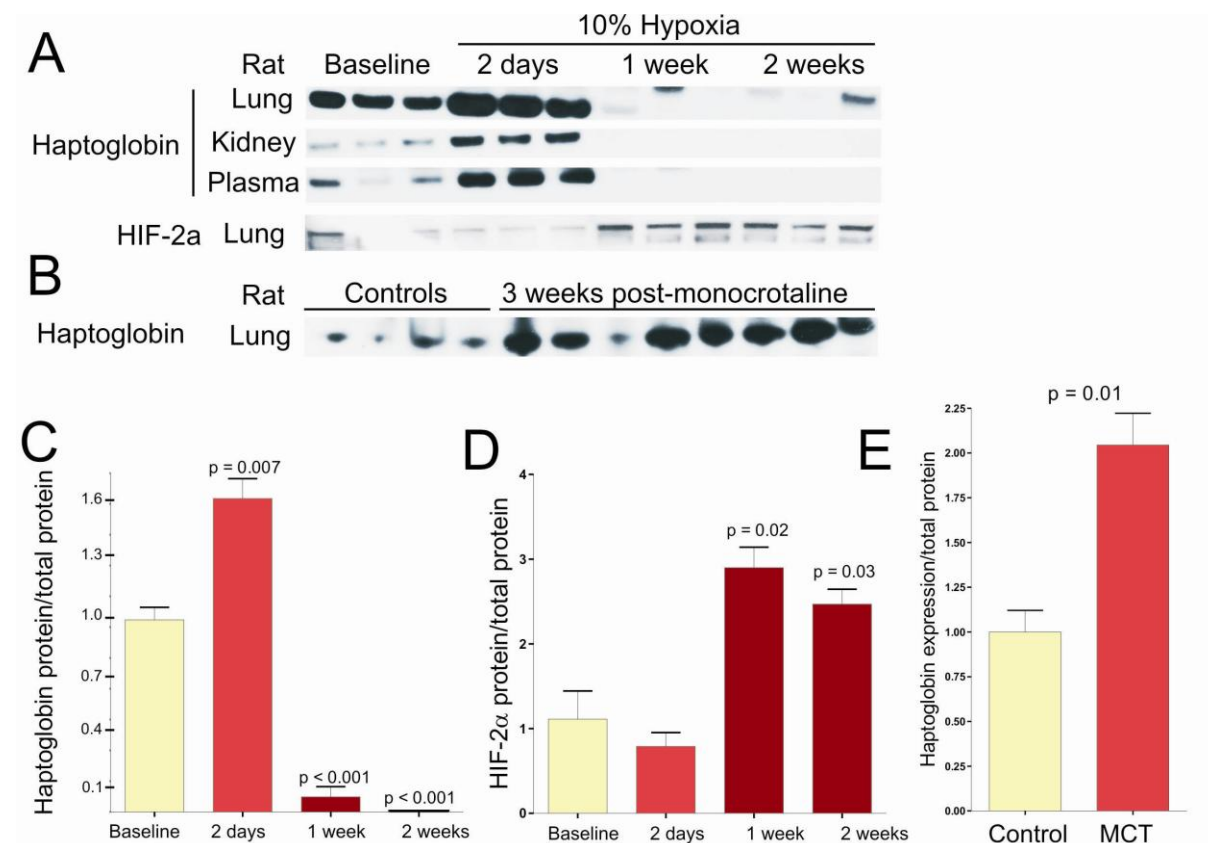


Figure 3.9 – Haptoglobin protein levels in several tissues of hypoxic rats and in rats following monocrotaline injection. A. Western blot results of tissues from hypoxic animals showing haptoglobin and HIF-2alpha expression as an indicator of tissue hypoxia. B. Haptoglobin protein levels in lung tissue from rats 3 weeks following monocrotaline injection. C-E densitometry data from A and B. Statistics in C&D are from Tukey post-hoc test following one way ANOVA and in E from t-test.

To elucidate the mechanism of Hp downregulation in hypoxia, Hp mRNA levels were analysed in lung and liver samples from rats exposed to chronic hypoxia for 3 weeks (Figure 3.10). Neither tissue displayed a significant alteration of Hp mRNA when compared to control animals kept at normoxia for the same time. This suggests that in both the hypoxic rat model and human tissues changes in Hp protein levels occur in the absence of mRNA changes. A similar mechanism may be involved in controlling Hp expression in hypoxia and IPAH and further investigations could use the hypoxic rat model to better understand this phenomenon.

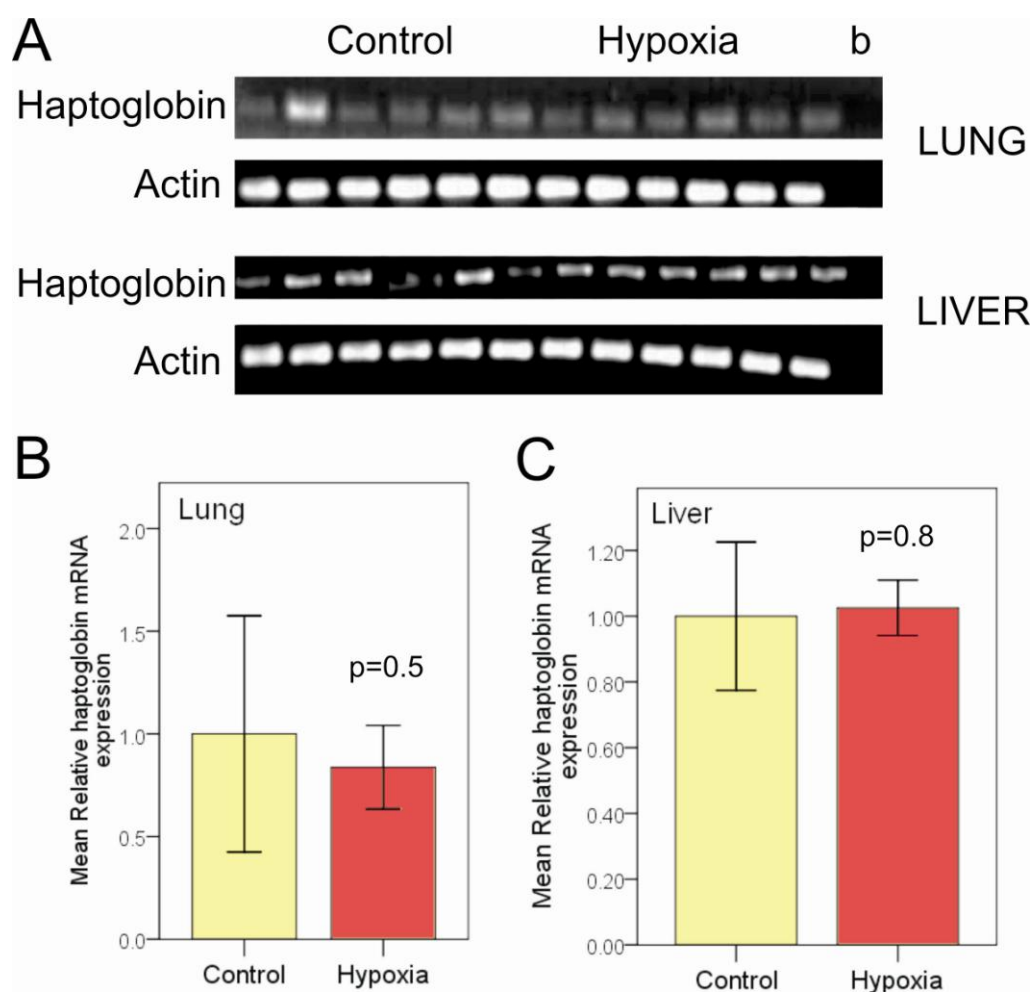


Figure 3.10 - Haptoglobin mRNA in hypoxic rat lung and liver tissues. A. RT-PCR gels showing haptoglobin and actin products from 3 weeks chronically hypoxic rat lung and liver extracts. (n=6 for each group). b = blank. B&C. Densitometry data from A of Lung and Liver, respectively. Statistics shown are from t-tests.

To verify that the source of the Hp protein expression in the lungs was not due to blood contamination, the pulmonary vasculature of control rats was flushed with PBS *ex-vivo*. Hp protein expression was then analysed by immunoblotting and significant expression of Hp was maintained in the flushed lungs (Figure 3.11). This confirmed that Hp protein expression observed in control rat lung tissues was not due to blood contamination.



Figure 3.11 – Haptoglobin protein in rat lungs flushed with PBS to remove blood contamination. Haptoglobin detected in rat lung tissue homogenates by Western blotting.

While the animal models of PH appear to suggest a role for hypoxia in regulating Hp levels, the relevance of this to humans is unclear. One factor known to influence Hp levels in humans is genetics. The haptoglobin gene (HP) has two alleles characterised by two isoforms of the alpha chain coded 1 and 2. Three possible phenotypes arise from this HP1-1 (homozygous for alpha-1), HP2-1 (heterozygous) and HP2-2 (homozygous for alpha-2). HP2-2 subjects have, on average, lower levels of circulating Hp protein. The HP phenotypes of the cohort studied thus far were determined and the relationship between the HP phenotype and circulating Hp levels were analysed (Figure 3.12). The distribution of HP phenotypes in the IPAH population reflected that seen in previously studied healthy populations. Significant differences in Hp levels between phenotypes were observed ($p=0.02$, Figure 3.12B) with HP2-2 individuals having on average lower levels of Hp. This suggests a genetic component contributes to the reduced Hp levels measured in some IPAH patients, but low Hp levels were seen across all 3 phenotypes indicate that other mechanisms are also important.

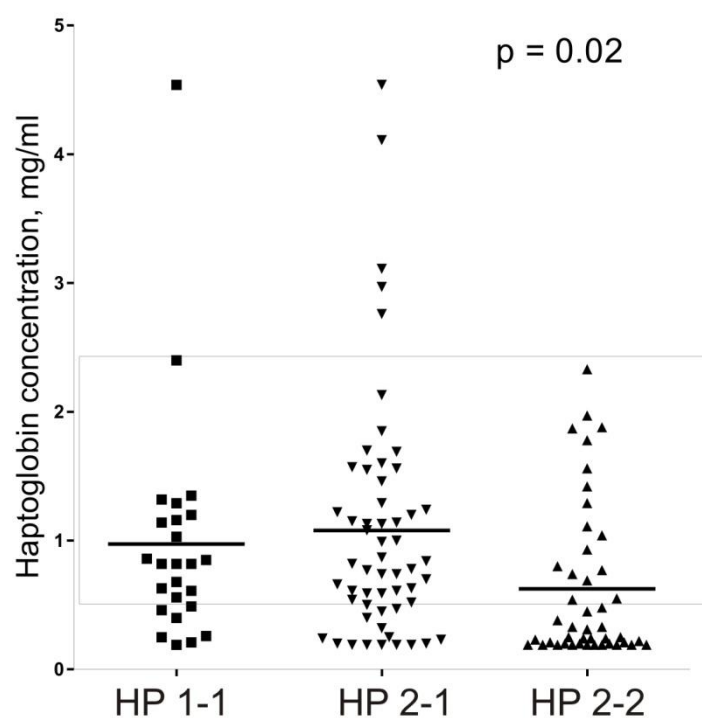


Figure 3.12 – Circulating haptoglobin relationships with HP phenotypes in 127 patients with IPAH. Plasma haptoglobin levels stratified by HP phenotypes (determined by Western blotting and PCR). Box indicates normal range of Hp in healthy populations. Statistic shown is for one-way ANOVA.

HP genotypes have been shown to affect cardiovascular disease occurrence and progression. This, combined with the observation of altered Hp expression in IPAH, raised the possibility that Hp may be important in the severity and progression of IPAH. Kaplan-Meier survival estimates were used to analyse the survival of patients with differing Hp levels or phenotypes (Figure 3.13). Patients with low or normal levels of Hp did not exhibit differing survival, with 5-year survival at around 75% in both groups ($p=0.87$, Figure 3.13A). Hp phenotype also appeared to have no effect on survival in IPAH ($p=0.87$, Figure 3.13B). Circulating Hp levels or phenotypes do not appear to have clinical utility in predicting survival in IPAH, and Hp itself does not affect mortality in these patients.

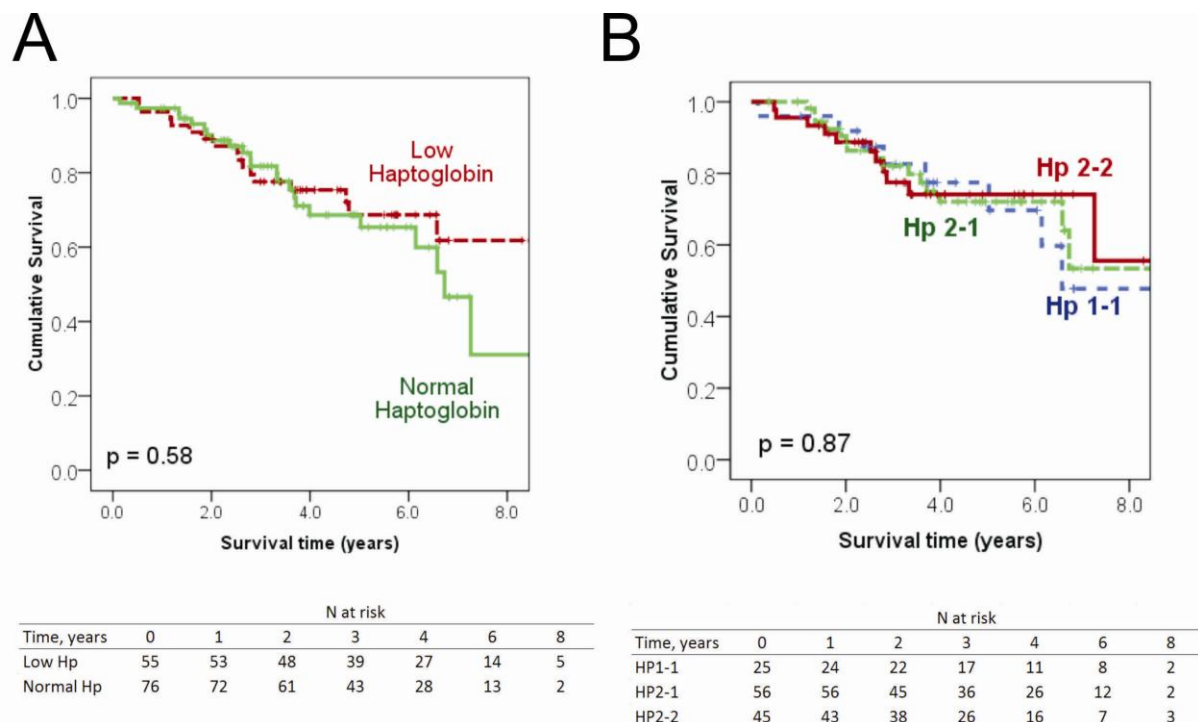


Figure 3.13 – Haptoglobin levels and phenotype do not affect survival in IPAH. Kaplan-Meier survival curves stratified by: A serial haptoglobin levels and B haptoglobin genotype. Statistics shown are p-values from log rank tests. Tables indicate numbers of patients at risk at each time point by group.

While Hp might not affect mortality in IPAH, it remained to be established whether this protein could affect the pathophysiology or morbidity seen in these patients. To obtain more selective patient phenotypes, Hp was measured in serial plasma samples for a subset of the IPAH cohort from the Imperial College PH Biorepository. Hp levels remained stably above or below the lower end of the normal range (0.75 mg/ml) over 6 months to 2 years in 53 (73%) of the 71 patients with available samples. The clinical, haemodynamic and biochemical characteristics of these patients are shown in Table 3.2. The levels of Hp measured are shown in Figure 3.14A. There was little difference in the clinical status (including 6 minute walk distance, WHO class, cardiac index and pulmonary vascular resistance) of the patients with consistently low and those with normal Hp levels, but it is notable that the patients with consistently low Hp levels were significantly younger, on average ~10 years younger at diagnosis and sampling ($p=0.01$ for both).

		Low haptoglobin (n=18)		Normal haptoglobin (n=35)		Sig.
		Value	Variation (SD or IQR)	Value	Variation (SD or IQR)	
Age at sampling		38.7	10.6	48.4	13.4	0.01
Age at diagnosis, y		37.6	9.8	49.2	13.0	0.01
Age at death, y		47.3	17.5	56.5	13.7	0.31
Sex	F	14		27		0.92
	M	4		8		-
WHO functional class	I	0		1		0.30
	II	2		6		-
	III	16		23		-
	IV	0		5		-
Died?	No	14		25		0.87
	Yes	4		10		-
6 minute walk distance, m		265.9	156.9	300.8	157.8	0.65
O2 partial pressure, kPa		8.1	3.0	8.4	1.9	0.44
Mean pulmonary artery pressure, mmHg		59.3	7.2	54.3	12.9	0.19
Mean right atrial pressure, mmHg		7.9	4.7	10.8	4.8	0.13
Right ventricular systolic pressure, mmHg		102.4	16.1	87.3	19.4	0.03
RV end diastolic pressure, mmHg		14.5	8.6	15.9	4.5	0.73
LV end diastolic pressure, mmHg		12.8	3.4	10.3	4.1	0.20
Wedge pressure, mmHg		11.0	1.4	11.6	5.9	0.92
Cardiac output, l/min		4.60	3.95 - 6.72	4.03	3.19 - 4.30	0.11
Cardiac index, l/min/kg/m2		2.62	2.00 - 3.50	2.19	1.72 - 2.55	0.19
Pulmonary arterial O2 saturation, %		70.0	11.0	64.1	12.3	0.22
Aortic O2 saturation, %		93.0	91.0 - 95.3	92.0	89.1 - 95.0	0.24
Pulmonary vascular resistance, Woods units		10.0	5.1	11.7	7.5	0.73
Peripheral O2 saturations, %		97.0	91.0 - 98.0	95.0	91.5 - 97.0	0.64
Peripheral O2 saturations after 6MW, %		87.0	72.0 - 97.0	95.0	83.0 - 97.0	0.43
Serum iron, umol/L		19.8	8.7	13.3	8.6	0.023
Ferritin, µg/L		104.0	37.0 - 154.0	67.0	33.0 - 135.0	0.74
Transferrin Sat, %		24.0	15.0 - 45.0	12.5	9.0 - 24.0	0.048
Total iron-binding capacity, umol/L		70.2	57.0 - 80.9	72.0	53.5 - 89.4	0.89
Red blood cells, 10 ⁸ /ml		5.4	0.9	5.2	0.7	0.53
Haemoglobin, g/dL		16.6	3.0	15.0	2.1	0.06
Haematocrit, ratio		0.49	0.08	0.46	0.06	0.39
Mean cell volume, fL		91.1	7.1	88.2	7.2	0.11
Mean cell haemoglobin, pg/cell		30.7	2.8	29.2	2.7	0.03
Mean corpuscular haemoglobin conc., g/dL		33.9	1.4	33.0	1.4	0.06

Table 3.2 – Clinical, haemodynamic and biochemical characteristics of IPAH patients with consistently low or normal circulating haptoglobin levels. Values shown for continuous variables are means or median when not normally distributed, and for categorical variables, frequencies. P-values shown indicate results of t-tests or chi-squared, respectively. SD= Standard deviation. IQR=interquartile ranges, given for variables not normally distributed.

As the key molecule responsible for the scavenging cell-free haemoglobin, Hp is important in iron homeostasis. Conversely, iron status has been shown to regulate Hp expression *in vivo* (Faye et al. 2007). The iron status of the IPAH patients with consistently low and normal Hp levels was compared using clinical measurements of serum iron, ferritin and transferrin saturation (Table 3.2 and Figure 3.14B). Both serum iron and transferrin saturations were significantly higher in the consistently low-Hp group ($p < 0.05$ for both, Table 3.2). Ferritin levels did not significantly differ between the two groups.

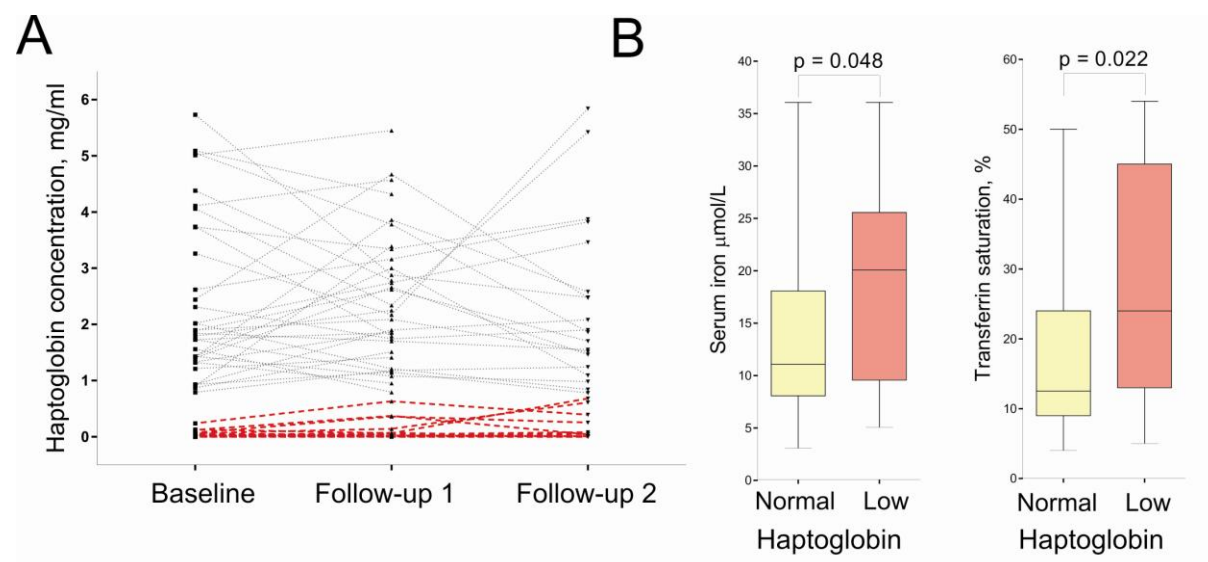


Figure 3.14 – Serial haptoglobin concentrations in IPAH patients and the relationship to serum iron and transferrin saturations. A. Serial measurements of haptoglobin in a subgroup of 53 IPAH patients. Samples were collected at routine clinical outpatient appointments over 3-month to 2-year periods. B. Serum iron and transferrin saturation levels in IPAH patients with consistently normal ($n=35$) or low ($n=18$) haptoglobin levels. Statistics shown are p-values from t-tests.

3.4 Discussion

In this study I have successfully employed a proteomic analysis of lung tissue from patients with idiopathic pulmonary arterial hypertension and from lobectomy samples to identify two functionally-related proteins with decreased expression in the hypertensive lung, haptoglobin and haemopexin. The detection of 10 unique peptides originating from haptoglobin (Hp), giving good coverage of the beta-chain sequence, and quantification of 6 of these peptides in each sample, giving strong correlations between peptides, suggested this result was robust. The result was validated by immunoblotting and decreased circulating haptoglobin levels were shown in two-fifths of patients with IPAH measured, and over half of patients with non-CTD APAH displayed similarly reduced levels. A considerable proportion of the patients analysed maintained consistently low Hp levels over months to years. Hp mRNA expression in lung tissue, from the same patients who displayed reduced Hp protein expression, showed similar levels between IPAH and lobectomy samples, suggesting that reduced Hp levels are caused by actions downstream from transcription. Other clinical indices provided no conclusive evidence of haemolysis and although lower levels were associated with a specific genotype, Hp2-2, this did not fully explain the Hp reduction observed. Pulmonary Hp expression was localised primarily to inflammatory cells and animal models of PH suggested a role for hypoxia in regulating Hp. While Hp phenotype or level did not directly relate to patient clinical indices or survival, a relationship to patient iron status was found. These data suggested a broader dysregulation of iron homeostasis might be present in IPAH.

The presence of different haptoglobin phenotypes opens up further avenues for investigation and has proved of interest in diabetes especially, where the Hp2-2 phenotype is a cardiovascular risk factor (Levy et al. 2002). The distribution of haptoglobin phenotypes was similar to normal European populations in the IPAH and other PH patients, but the Hp2-2 phenotype appears to predispose to low levels of circulating haptoglobin. The Hp2-2 phenotype is, in healthy populations, associated with lower circulating levels, but the levels measured in the IPAH patients are significantly lower still, with many below the limit of detection of the standard hospital assay. Low levels of haptoglobin are normally associated with haemolytic anaemia (Dash & Das 1978). In these patients indicators of haemolysis do not suggest that it is occurring significantly. Reticulocyte profiles and bilirubin levels are within normal ranges, although levels of circulating LDH, which is also released along with haem from red blood cells when they lyse (Laessig et al. 1976), are raised above normal values in some patients and close to the upper limit of normal on average (average 220 ± 60 , normal range 125-243 IU/L). However, LDH levels do not differ significantly between the low/normal Hp patient populations or between the different phenotypes. This suggests consumption is not the mechanism

by which these patients' Hp levels are reduced, although chronic low-grade haemolysis may still be occurring.

To address the issue of possible haemolysis in patients with low circulating Hp levels, I could have studied the survival of red blood cells in the circulation of 6 IPAH and 6 CTEPH patients, using ⁵¹chromium-labelling. This is a standard clinical assay (International Committee for Standardization in Haematology 1980) and involves removing 15ml of blood, loading with ⁵¹Cr and slowly returning it to the patient by slow iv injection. Measurements of circulating ⁵¹Cr are then made to determine the survival half-time of ⁵¹Cr-labelled cells, which have a mean lifespan of 120 days in healthy individuals. Ethical approval was obtained from Brent research ethics committee for this study (application number: 08/H0717/58). Based on a standard deviation of 1% (normal red cell survival is very tightly regulated), to detect a 10% reduction in survival from normal (which would explain the marked reduction in haptoglobin), two subjects per group would provide 80% power at p=0.05. Six subjects per group will allow us to detect whether any change is disease specific (e.g. found in IPAH but not CTEPH) and associated with low Hp levels. However, since there is no convincing clinical data indicating that haemolysis is occurring in the low Hp patients, as well as the high cost and poor patient acceptability of such involved investigations, I decided not to proceed with this study.

Pulmonary Hp expression was localised primarily to inflammatory cells, and lower expression could be observed in alveolar epithelial cells and the media and adventitia of pulmonary arteries. This emphasised Hp's role as an acute phase reactant protein but also suggested there could be some vascular involvement in the dysregulation of Hp. Introduction of carbonyl groups to lysine, arginine, proline or threonine residues (Stadtman 1993) can increase protein susceptibility to degradation (Davies 1987). Increased oxidative stress in these tissues, leading to increased protein oxidation and therefore targeting for degradation, could be a mechanism by which proteins such as Hp are downregulated. To investigate this possibility, I analysed the levels of protein oxidation in IPAH lung versus lobectomy samples. No difference in protein oxidation was observed, suggesting this pathway was not overactive in IPAH, and therefore couldn't explain the reduction in Hp.

Translation of these findings to animal models was investigated in the two main models of PAH, hypoxia- and monocrotaline-induced PAH. In rat, hypoxia led to a marked downregulation of Hp protein expression in the lung tissue, mirroring the results seen in patient samples. In the monocrotaline model, where pulmonary hypertension leads on from the damage to the pulmonary vascular endothelium caused by this plant alkaloid (Tanino 2001), Hp levels were actually increased after three weeks, and this may reflect the degree of inflammation that occurs in this model, with Hp

acting out its role as an acute phase protein. The specific downregulation in the hypoxic animals suggests a primary effect on Hp levels by hypoxia, rather than a secondary mechanism triggered by increased pulmonary arterial pressure. To assess the tissue distribution of Hp downregulation by hypoxia, levels of Hp in plasma and kidney as well as lung tissue were measured by immunoblotting. Hp protein levels were significantly lower in plasma, kidney, and lung tissue. Expression levels of Hp between lung, plasma and kidney showed a similar time course of changes, which suggests a shared hypoxia response mechanism which itself varies between animals, mirroring the differences seen in human tissue samples which may be regulated in a similar fashion.

Analysis of Hp mRNA levels in the lungs and liver by RT-PCR shows that mRNA expression of Hp is not affected by hypoxia even in the animals where Hp was barely detectable at the protein level. This suggests that hypoxia is acting downstream of transcription to downregulate Hp, but by what mechanism if not haemolysis? If translation of the mRNA to protein were being inhibited, the normal turnover of red blood cells and concomitant release of haemoglobin could consume the circulating Hp. But what could be inhibiting translation of Hp in hypoxia? Iron response element (IRE) binding proteins (IRPs) are one candidate. As mentioned above, hypoxia regulates many proteins involved in iron homeostasis. Alterations in intracellular iron status alter the activity of IRPs, which are deactivated by conformational changes triggered upon iron binding (Eisenstein 2000). IRPs bind IREs on mRNA sequences and alter translation and/or stability of the molecule. If IRPs affect Hp mRNA translation then this could be one mechanism by which Hp is regulated by hypoxia without affecting mRNA levels or red cell turnover.

The regulation of translation in hypoxia has been actively investigated in models of ischaemia (Burda et al. 1994; Martin, V et al. 2001), stroke (Ratan et al. 2004) and in cancer cells (Bi et al. 2005; Koritzinsky et al. 2007; Liu et al. 2006), where hypoxia drives the selection of a proliferative, apoptosis-resistant phenotype believed to explain the poor prognosis of hypoxic cancers. Hypoxia appears to primarily regulate translation at the initiation step (Simon et al. 2008), which begins with the assembly of the m7GTP cap-binding eukaryotic initiation factor eIF4F complex (made up from eIF4E, eIF4G and eIF4A), and the ternary complex containing eIF2 α (with GTP bound) and the initiator methionine transfer RNA (which binds the start codon AUG). Regulation of these two complexes can be affected by hypoxia (Figure 1.4.1); in the short term (30 minutes <0.05% O₂) protein kinase R-like endoplasmic reticulum kinase (PERK) is activated, which phosphorylates eIF2 α (Harding et al. 1999), reducing its ability to replace guanosine diphosphate with guanosine triphosphate and thus its activity (Koumenis & Wouters 2006). This leads to global downregulation of translation initiation.

However, some proteins are preferentially expressed in hypoxia upon eIF2 α phosphorylation and this includes activating transcription factor 4 (ATF4) (Blais et al. 2004). ATF4 then stimulates gene expression of C/EBP-homologous protein (CHOP) and growth arrest and DNA damage-inducible (GADD)-34. GADD34 is a regulatory subunit of protein phosphatase-1, which can dephosphorylate eIF2 α , completing a negative feedback cycle that prevents the long-term phosphorylation of eIF2 α . This pathway is part of the unfolded protein response (UPR), which is triggered by the ER when improperly-folded proteins are detected, for example when cells are under oxidative stress (Koumenis et al. 2002). Another protein activated by the UPR is inositol-requiring protein 1, which leads to splicing of X-box binding protein-1 mRNA, necessary for its activation. X-box binding protein-1 activates expression of ER chaperone genes (including CHOP), which enable correct folding or degradation of misfolded proteins. If the UPR is unsuccessful in correcting protein folding, apoptosis is stimulated by CHOP and PERK via caspase-12 and Jun N-terminal kinase activation, reduction of survival factors and stimulation of pro-apoptotic factors (Schroder & Kaufman 2005).

The importance of this pathway in regulation of Hp synthesis has been investigated previously in mice (Faye et al. 2007). Mice were fed on an iron-rich diet and this caused dramatic post-transcriptional decreases in serum and liver Hp levels. mRNA and protein levels of an endoplasmic reticulum chaperone were decreased in iron-fed mice, strengthening the author's hypothesis of iron-induced oxidative stress altering endoplasmic reticulum conditions and causing misfolding of Hp, leading to its degradation. In this study I found that low Hp levels were associated with higher, but not abnormal, iron levels, against a background of low serum iron in the normal Hp group. Whether the circulating iron in these patients is sufficient to suppress expression of chaperone proteins and activate the unfolded protein response is unclear.

Longer term regulation (>16-24h) of translation initiation by hypoxia can be achieved via the regulation of eIF4E-binding protein-1 by mammalian target of rapamycin (mTOR). Hypoxia inhibits mTOR activity in several tissue types (Arsham et al. 2003; Brugarolas et al. 2004) although in tissues of the cardiovascular system, including pulmonary artery adventitial fibroblasts (Gerasimovskaya et al. 2005), mTOR can actually be activated by hypoxia, as part of the proliferative response, and this may be due to upregulated protein kinase B activity (Pore et al. 2006). As the name suggests, eIF4E-binding protein-1 binds eIF4E, preventing formation of the eIF4F complex, inhibiting cap-dependent translation initiation (Simon et al. 2008). The difference in time scale of these two different pathways emphasises the importance of elucidating the time frames over which regulation of Hp levels occur.

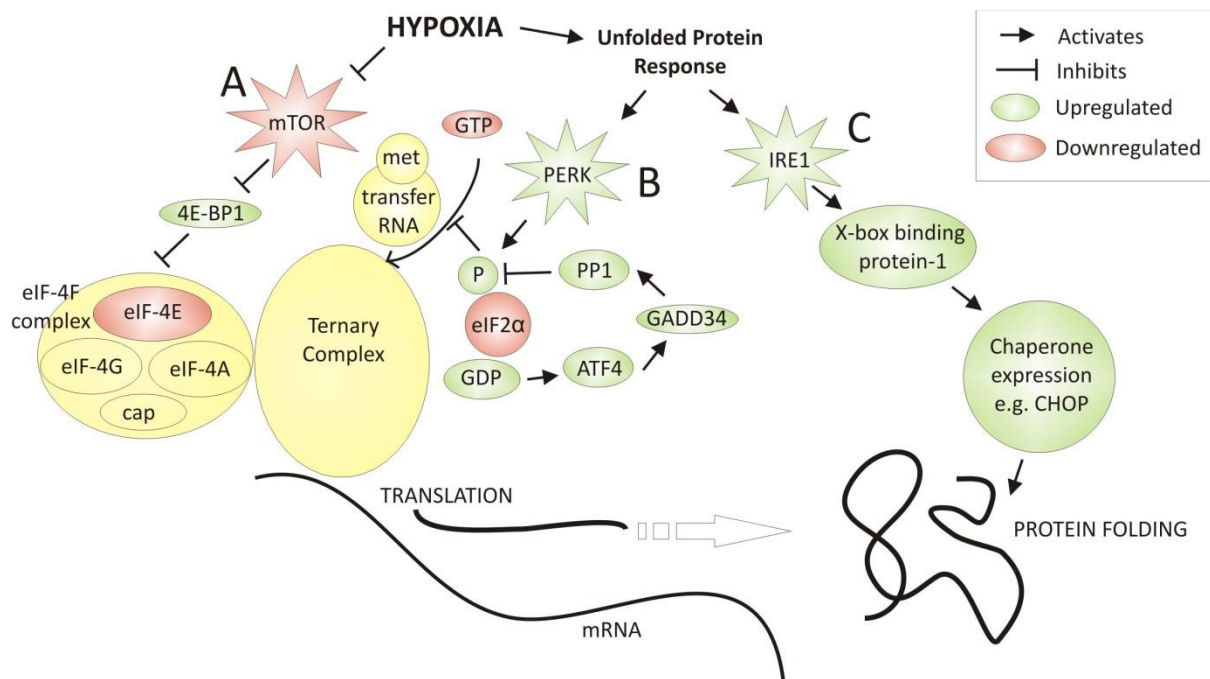


Figure 3.15 - Regulation of translation by hypoxia is mediated by multiple pathways including that of the unfolded protein response and mammalian target of rapamycin (mTOR) regulation of the eIF-4F complex. A. Hypoxia regulates mTOR activity, which regulates eIF-4F complex formation by inhibiting eIF-4E-binding protein (4E-BP1). Inhibition of mTOR leads to inhibition of eIF-4F complex formation and hence translation. B. Hypoxia activates protein kinase R-like ER kinase (PERK), causing phosphorylation of eIF2 α . Phosphorylated eIF2 α cannot bind GTP and therefore does not recruit methionine transfer RNA to the ternary complex, inhibiting translation initiation. C. Hypoxia upregulates inositol-requiring protein 1 (IRE1), which activates X-box binding protein-1 by mRNA splicing. X-box binding protein-1 increases expression of ER chaperones, which attempt refolding of misfolded proteins.

Kaplan-Meier survival estimates show no significant difference in survival between patients with different haptoglobin phenotypes or circulating levels. One mechanism by which the Hp2-2 phenotype might be conferring increased cardiovascular risk in diabetes that has been proposed is diabetes-specific dysfunction of the Hp2-2 protein in preventing the oxidation of low density lipoprotein (Asleh et al. 2008). This may be due to the inability of Hp to prevent the oxidative activity of glycosylated haemoglobin (Asleh et al. 2005), whose levels are elevated in diabetes. This effect would not be seen in IPAH and may explain why the Hp2-2 phenotype does not appear to be associated with decreased survival in IPAH.

To further assess the importance of Hp levels and phenotype in pulmonary hypertension, analysis of clinical and haemodynamic data from Hammersmith Hospital for the patients whose samples were measured and/or phenotyped was carried out. No significant differences in haemodynamics such as pulmonary vascular resistance, mean right atrial pressure, mean pulmonary artery pressure or cardiac output were found between the different phenotypes. One finding in this analysis was that on average patients with low haptoglobin levels were 10 years younger when first seen in the clinic and when undergoing cardiac catheterisation. Age at death was also lower on average but did not reach significance. What this suggests is that patients with low Hp levels are presenting earlier and hence being diagnosed earlier in their lifetimes. Whether Hp itself could be playing an active role in the earlier development of PAH or if it is a secondary product of the underlying processes which could be causing this difference is unclear. Further investigation of this could be performed in animal models, for example using Hp-knockout mice (Lim et al. 1998) versus wild-type to compare the development and severity of pulmonary hypertension over time in hypoxia.

The most interesting finding of this analysis was that patients with consistently low Hp levels had significantly higher serum iron and transferrin saturation measurements. While this was consistent with the hypothesis that downregulation of Hp (and haemopexin) in IPAH indicated changes in iron homeostasis in these patients, the absolute values were mostly within the normal ranges seen in healthy populations. Values of transferrin saturation below 20%, which are normally associated with iron deficiency, were common in the group with normal Hp levels. This result suggested that within IPAH a broader dysregulation of iron homeostasis was present, which required further investigation.

The questions I set out to address in Chapter 4 were: ***Is iron status abnormal in the IPAH population? Is this clinically relevant? What could be the mechanism(s) behind dysregulation of iron homeostasis in IPAH?***



Investigation of iron status and its clinical relevance in IPAH

Chapter 4 – Investigation of iron status and its clinical relevance in IPAH

4.1 Introduction

In chapter 3, I identified alterations in haptoglobin expression and iron homeostasis in patients with IPAH in both lung tissue and at the systemic level. There is increasing interest in the role of iron status in PAH. Robbins and colleagues, who have conducted physiology studies manipulating iron status in human subjects, hypothesise that iron deficiency mimics hypoxia in inducing stabilisation of HIF proteins due to the requirement for iron and oxygen as cofactors of the HIF degrading prolyl hydroxylases (Ivan et al. 2001; Jaakkola et al. 2001). They have demonstrated that alterations in iron around the physiological range alter pulmonary vascular resistance, basal pulmonary artery pressure and the pulmonary vasoconstrictor response to hypoxia in humans (Balanos et al. 2002; Smith et al. 2008; Smith et al. 2009).

Intravenous infusion of the iron chelator desferrioxamine increased basal PVR in healthy volunteers over a similar time course and with similar individual variability as seen in response to hypoxia, supporting the hypothesis that iron deprivation might lead to activation of HIF signalling (Balanos et al. 2002). Exacerbation of the hypoxic pulmonary vasoconstrictor response by pre-exposure to 8 hours of hypoxia is completely prevented by pre-infusions of intravenous iron and reproduced by infusion of desferrioxamine (Smith et al. 2008). Ascent to high altitude is accompanied with increased PVR due to hypoxic pulmonary vasoconstriction occurring throughout the lung. This effect can be partially reversed by treatment with intravenous iron and exacerbated by iron depleting venesections (Smith et al. 2009). In the latter case iron replacement failed to significantly reverse the effects seen.

Another clue to the potential importance of iron status in PAH can be found in studies of prognostic biomarkers for this disease, and this will be covered in greater detail in Chapter 5. Red cell distribution width is a measure of the variation in red blood cell size, and is used clinically to diagnose iron deficiency anaemias. Growth differentiation factor-15 is a member of the TGF- β superfamily that can be induced in many cell types *in vitro* by iron deprivation and measured easily in plasma samples (Lakhal et al. 2009). Measurements of both these biomarkers have been shown to predict survival in patients with PAH, adding value even to the most established blood biomarker in PAH, NT-proBNP (Hampole et al. 2009; Nickel et al. 2008). Indeed, anaemia itself has been shown to predict survival in patients with mixed aetiology PH, irrespective of diagnosis (Krasuski et al. 2010). These studies suggest iron status may be of clinical relevance in PAH.

Iron homeostasis

The control of circulating and intracellular iron concentrations is important to both enable effective erythropoiesis and the activity of iron-dependent biochemical reactions, iron being essential for normal cellular metabolism and respiration. Iron overload is also detrimental, leading to toxicity and cell death, primarily due to formation of free radicals via the Fenton or Haber-Weiss reactions (Halliwell & Gutteridge 1990; Papanikolaou & Pantopoulos 2005). Thus, iron homeostasis is tightly controlled. Around two-thirds of the body's iron is found in haemoglobin in erythrocytes and approximately 10% in muscle myoglobin or enzymes and cytochromes in other tissues. Under normal iron-replete conditions, the majority of the remaining iron is stored intracellularly, bound to ferritin or hemosiderin molecules, in the reticuloendothelial system and bone marrow (Finch et al. 1970). Erythrocyte iron is effectively recycled by macrophages of the reticuloendothelial system, with cell-free haemoglobin and haem being scavenged by haptoglobin and haemopexin molecules respectively. There is no mechanism for iron excretion and the small daily loss of iron (1-3 mg), due to sloughing of intestinal mucous cells, menstruation and other blood loss, is balanced by dietary iron absorption.

The master regulator of iron homeostasis is hepcidin, which acts to inhibit the efflux of iron into the blood from enterocytes and iron storing cells by binding to ferroportin and inducing its degradation in lysosomes (Nemeth & Ganz 2009). Hepcidin is mainly expressed in the liver and its production is inhibited by iron deficiency and increased erythropoiesis and induced by iron overload and inflammation. Chronically raised hepcidin levels cause systemic iron deficiency and underlie the anaemia of chronic disease (Weiss & Goodnough 2005).

Historically iron status has been most commonly defined by circulating levels of the iron storage molecule ferritin, serum iron itself and saturation of the iron transport molecule transferrin. Measurements of these factors are affected significantly by inflammation, which may render them inappropriate for estimating iron status in a disease with an inflammatory component, such as IPAH. Soluble transferrin receptor (sTfR) levels offer an alternative quantification of tissue iron availability that is unaffected by inflammation (Cook et al. 1990; Huebers et al. 1990) and therefore may represent the best measure of iron status in IPAH.

I hypothesised that iron deficiency is prevalent and clinically important in IPAH and that increased hepcidin expression might contribute to this. I defined iron status by circulating sTfR levels. I investigated the relationship of iron deficiency to circulating hepcidin levels and survival in IPAH.

4.2 Methods

See Chapter 2 for detailed experimental materials and protocols.

Plasma samples with sufficient volume for the required analyses were obtained from the Imperial College Pulmonary Hypertension biorepository. This repository comprises blood samples from consenting patients with IPAH attending Hammersmith Hospital, London, between 2002 and 2009. Haemodynamic data were acquired at catheterization. Patients with co-morbidities that might affect iron homeostasis including haemolytic anaemias, genetic disorders of haemoglobin, diabetes and systemic cardiovascular disease, were excluded. Healthy controls were recruited from Imperial College London, UK. Blood samples were coincident with measurements of WHO functional class, six minute walk distance and oxygen saturation. Subject characteristics are detailed in Table 4.1.

Human liver tissue samples (n=5) were used to demonstrate hepatic BMPR2 expression. Samples were obtained from unused transplant donor tissues.

Circulating iron parameters

sTfR, GDF-15 and IL-6 levels were determined by ELISA (R&D Systems Europe, Abingdon, Oxfordshire, UK). Hepcidin levels were determined by a competitive radioimmunoassay, as previously described (Busbridge et al. 2009). EPO was measured using an Immulite-2000 analyser (Siemens, Frimley, Surrey, UK). All other measures were determined by standard clinical pathology accredited hospital assays. Iron deficiency was defined by high levels of sTfR (normal range 8.7-28.1 nmol/L). Serum iron (normal range: Male: 9-29; Female 7-27 $\mu\text{mol/L}$), transferrin saturation (<20%), and ferritin (<100 $\mu\text{g/L}$) were also measured. The normal range of hepcidin was previously determined at 2-55 ng/mL and independently validated in a second group of healthy volunteers (Ashby et al. 2009). EPO had a normal range of 3.3-16.6 mIU/mL. Anaemia was assessed by haemoglobin measurements (lower limits: Females: 12 g/dl, males: 13 g/dl). RDW indices were measured on the Sysmex-XE-2100 auto-analyser (Sysmex, Wymbush, Milton Keynes, UK); a method based upon electrical impedance using hydrodynamic focusing. NT-proBNP levels were measured using a non-competitive assay as previously described (Omland et al. 2002).

Data presentation and statistical analysis

Protein measurements are displayed as dot-plots, bars indicate medians. Clinical and haemodynamic data are presented as group means, with standard deviations, or as median values with IQR. Several variables were not normally distributed, as shown by the Kolmogorov-Smirnov test, and were therefore transformed to their natural logarithm or square-root, as appropriate to best normalize the data, before further analysis. Continuous data across categorical variables were compared by Kruskal-Wallis ANOVA. Correlations were assessed by Spearman's Rank test. Cox regression analyses identified prognostic predictors. Significant predictors from univariate analysis ($p < 0.05$) were then entered into a multivariable model. These variables were compared by stepwise backward hazard ratios Cox analysis; variables removed at $p > 0.10$. RT-PCR results were compared by ANOVA, with Tukey post-hoc analysis performed if $p < 0.05$. All calculations were performed with SPSSv17.0 (SPSS, Inc., Chicago, IL) or GraphPad Prism4 (GraphPad Software Inc, La Jolla, CA).

4.3 Results

98 patients with IPAH and 106 healthy volunteers were analysed in this study. Subject characteristics are detailed in Table 4.1.

Subject Characteristics		IPAH (n = 98)	Controls (n = 106)
Age		47.1 (±15.5)	34.2 (±9.9)
Gender	Female	69 (70%)	52 (49%)
	Male	29 (30%)	54 (51%)
Baseline mean Pulmonary Artery Pressure, mmHg		56.1 (±12.7)	-
Right atrial pressure, mmHg		11.4 (±5.9)	-
Pulmonary capillary wedge pressure, mmHg		12.1 (±5.3)	-
Cardiac index, L/min/m ²		2.01 (1.65–2.78)†	-
Pulmonary artery O ₂ saturation, %		63.9 (±12.6)	-
Pulmonary vascular resistance, Woods units		14.1 (±9.4)	-
WHO	I	5	-
	II	20	-
	III	54	-
	IV	19	-
6 minute walk, m		282 (±161)	-
Treatment	Naive	32	-
	Warfarin	81	-
	Bosentan	39	-
	Sildenafil	17	-
	Treprostinil	10	-

Table 4.1 – Baseline characteristics of healthy volunteers and IPAH patients used in assessing iron status in IPAH. Data shown as means ± standard deviations. † indicates variable is not normally distributed and therefore given as median (interquartile range).

Iron deficiency is prevalent in IPAH.

Circulating sTfR levels were raised (median 33.0, IQR:23.0-43.0 nmol/L) in IPAH, with 62/98 (63%) patients having levels above the normal range (>28.1 nmol/L, Figure 4.1) compared to 16.7% of controls. Low serum iron (8.7, IQR: 5.0 - 14.0 μ mol/L) and ferritin (50.0, IQR: 27.5 – 142.0 μ g/L) levels were present in the IPAH patients and transferrin saturations (19.0, IQR: 12.0 – 29.0 %) were <20% in over half of the patients assessed (Figure 4.1). Although a large proportion of the IPAH patients displayed features of iron deficiency, they were not overtly anaemic, with mean haematocrit and haemoglobin levels of 0.47 ± 0.06 and 14.7 ± 2.5 g/dL (Figure 4.1), and mean cell volume/haemoglobin values of 88.9 ± 9.1 fL and 29.8 ± 3.1 pg, respectively. Median red cell distribution width was 14.9% (IQR: 13.7 – 16.4 %), towards the upper end of the normal range (8.3-17.5%).

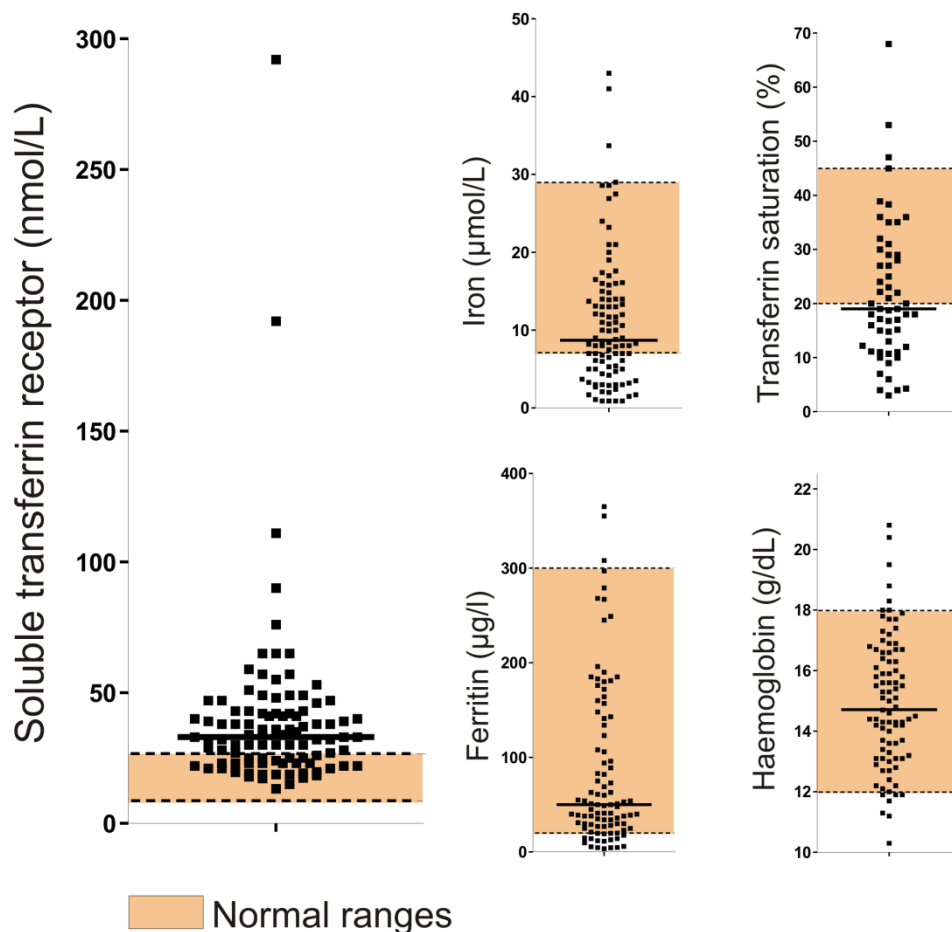


Figure 4.1 – Characterisation of iron status in 98 patients with idiopathic pulmonary arterial hypertension. Dot-plots of soluble transferrin receptor, iron, transferrin saturation, ferritin and haemoglobin levels were all measured in plasma samples by standardised clinical pathology accredited assays. Shaded areas delineated by dashed lines indicate normal ranges in healthy populations.

Bleeding, which might be exacerbated by warfarin therapy in patients with IPAH, is a potential cause of iron deficiency. sTfR levels were raised irrespective of warfarin therapy (Figure 4.2 and Table 4.2).

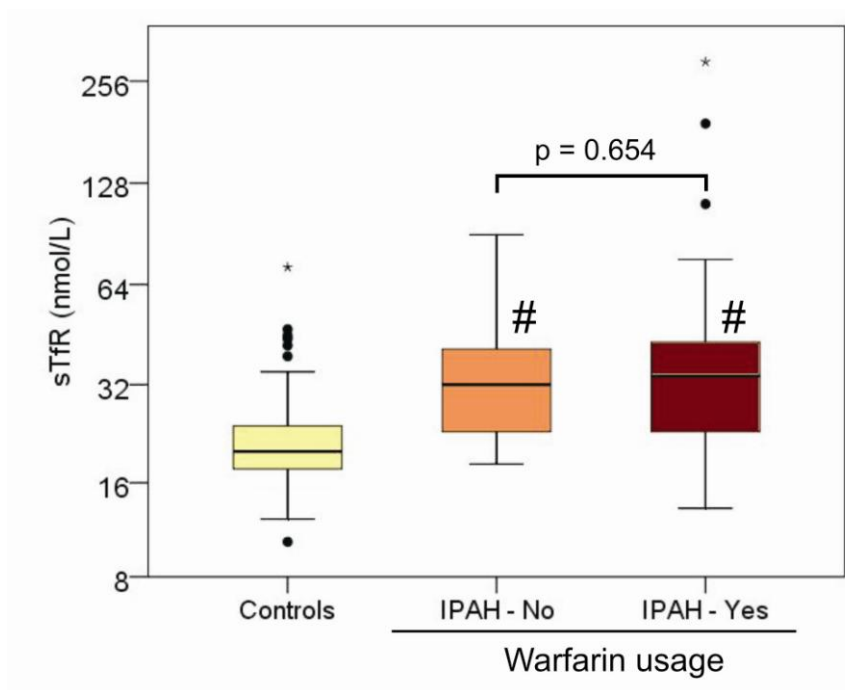


Figure 4.2 - Warfarin usage and soluble transferrin receptor (sTfR) levels. sTfR levels were measured in plasma samples from healthy volunteers (Controls) and IPAH patients not on warfarin (IPAH-No) or following warfarin initiation (IPAH-Yes). sTfR levels were equally raised in both groups of IPAH patients indicating that iron deficiency was similarly present in those patients not taking warfarin. #, control group vs. both IPAH groups $p < 0.01$. Within IPAH, warfarin vs. no warfarin $p = 0.654$. Lines indicate median and dots indicate outliers ($> 2SD$ from mean). Star indicates extreme outlier ($> 4SD$ from mean).

	Warfarin usage					
	Control, n=60		No, n=17		Warfarin, n=81	
	Mean	SD	Mean	SD	Mean	SD
sTfR (nmol/L)	23.0	10.3	34.1	17.2	40.9	36.7

Table 4.2 - Warfarin usage and soluble transferrin receptor (sTfR) levels. SD = Standard deviation.

Higher sTfR levels were observed in IPAH versus controls across all age groups (Figure 4.3A) and irrespective of gender (Figure 4.3B). Seventy of the patients were British but elevated levels could also be observed in some patients in other ethnic groups (Figure 4.4).

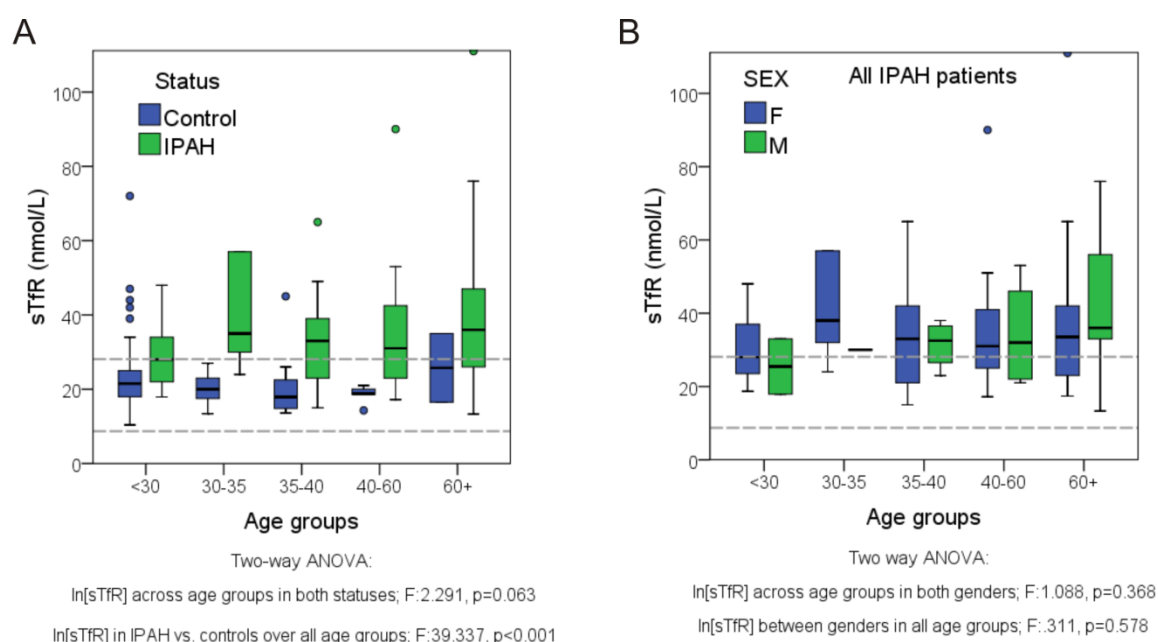


Figure 4.3 – Soluble transferrin receptor levels stratified by age and gender. A. Comparison of sTfR levels between IPAH and controls across different age subgroups. B. Comparison of sTfR levels between genders in IPAH patients across different age subgroups.

Racial Origin	Count
Patient refused	6
British	70
Any other White background	4
Indian	1
Pakistani	1
Bangladeshi	1
Any other Asian background	2
Caribbean	2
African	3
Any other Black background	1
Chinese	1
Any other Ethnic group	6

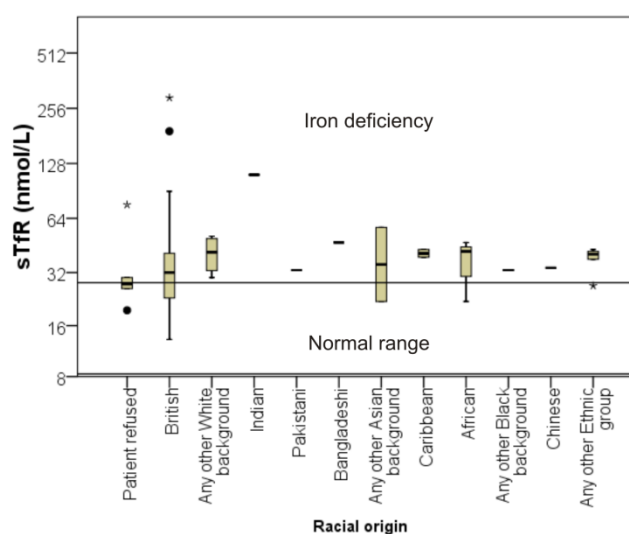


Figure 4.4 – Soluble transferrin receptor levels by racial origin in IPAH. Table shows racial origins of all 98 IPAH patients analysed. sTfR levels are shown by racial origin in box-plot.

Levels in healthy volunteers measured in this study, which agreed with the established normal ranges for these assays, are detailed in Table 4.3. sTfR and ferritin levels did not differ between patients on different PAH therapies (Table 4.4).

	Value
Hepcidin (ng/mL)	18.5 (9.9-48.0)†
EPO (mIU/mL)	10.3 (±4.2)
Iron (umol/L)	8.7 (4.9-14.8)†
%TSAT	27.77 (±14.34)
Ferritin (µg/L)	55 (29-118)†
sTfR (nmol/L)	20 (18-24)†

Table 4.3 – Iron status of healthy volunteers measured in this study. † indicates variable is not normally distributed and therefore given as median (interquartile range).

	Naive	Bosentan	Sildenafil	Treprostinil	Sig.
Haemoglobin (g/dL)	15.4 (±2.0)	14.7 (±2.4)	13.9 (±2.8)	14.3 (±2.0)	0.20
Hepcidin (ng/mL)	39.5 (6.8-98.0)†	22.0 (0.9-45.0)†	51.0 (3.6-102.0)†	37.0 (14.8-42.0)†	0.34
EPO (mIU/mL)	14.6 (10.5-19.6)†	33.0 (17.2-52.0)†	14.2 (8.8-33.7)†	49.5 (14.8-71.0)†	0.002
Iron (umol/L)	10.9 (6.4-16.3)†	8.4 (4.7-13.7)†	10.0 (3.3-13.3)†	7.0 (3.2-13.9)†	0.47
%TSAT	20.4 (13.6-29.5)†	18.5 (11.0-29.0)†	19.0 (17.0-27.0)†	18.0 (13.0-29.0)†	0.98
Ferritin (µg/L)	57 (33-183)†	39 (21-83)†	46 (23-143)†	64 (29-172)†	0.33
sTfR (nmol/L)	33 (25-39)†	38 (23-49)†	40 (24-49)†	31 (22-36)†	0.41

Table 4.4 – Iron parameters in different PAH treatment groups. P-values shown are for Kruskal-Wallis ANOVA. † indicates variable is not normally distributed and therefore given as median (interquartile range).

Hepcidin levels are abnormal in IPAH and relate to iron status.

Hepcidin levels were significantly higher in patients with IPAH, compared with healthy controls (34.5, IQR: 3.6 – 83.0 ng/ml; n=98 versus 25.5, IQR: 11.7 – 50.0 ng/ml; n=106; $p=0.001$), and 33 (34%) patients had concentrations above the previously defined upper limit of 55 ng/ml (Figure 4.5A) (Ashby et al. 2009). Hepcidin levels did not correlate with IL-6 values before (Figure 4.6) or after adjusting for iron status (Table 4.5), suggesting raised hepcidin levels were not due to inflammation. In agreement with this, no correlation was observed with C-reactive protein (CRP) in a subgroup of patients where CRP measurements were available ($\rho=0.02$, $p=0.92$, $n=45$).

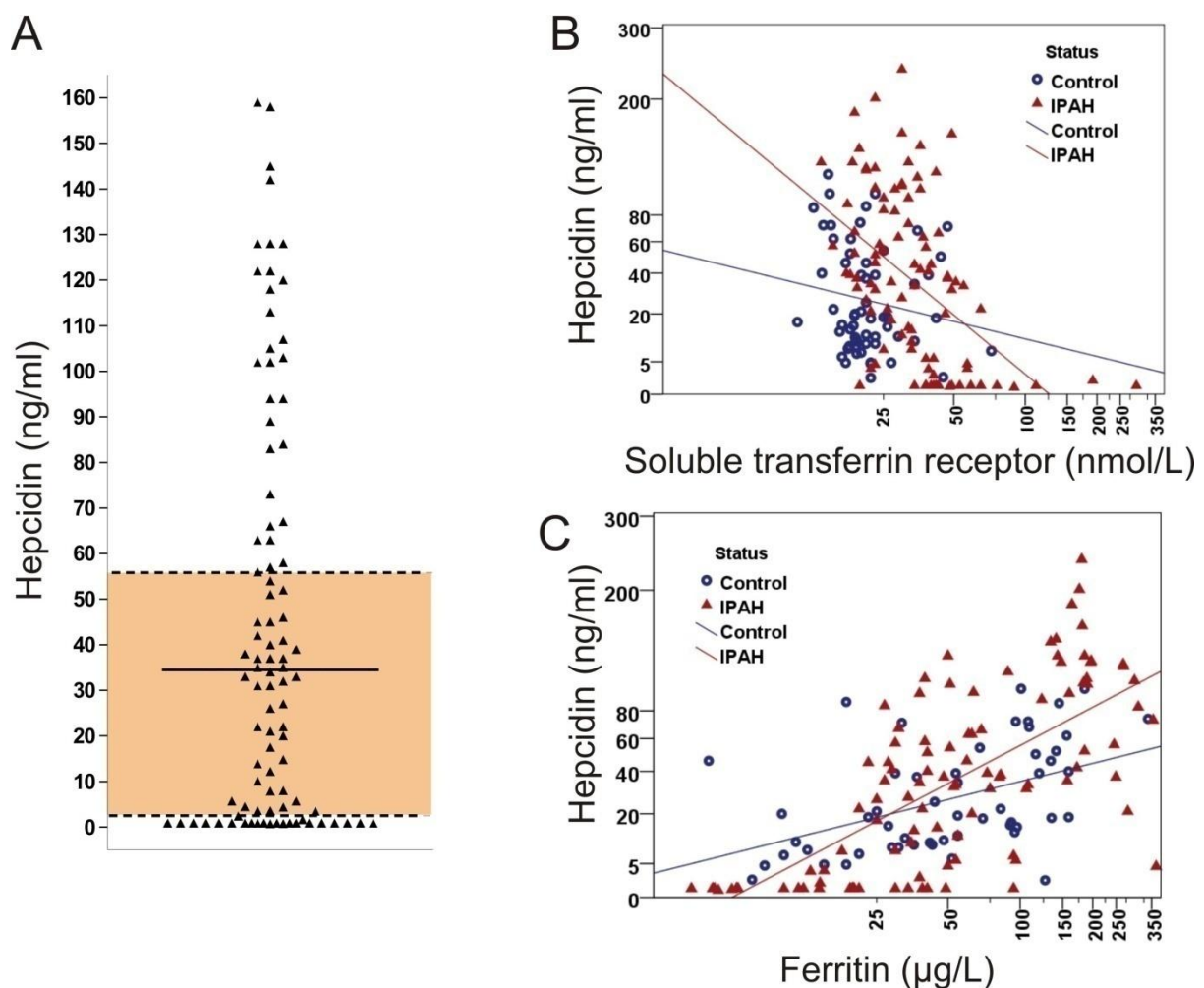


Figure 4.5 – Hepcidin levels in IPAH. A. Circulating hepcidin levels in patients with IPAH. Shaded area represents normal range. B-C. Scatter-plots of square root scaled-hepcidin versus ln scaled-soluble transferrin receptor and ln scaled-ferritin levels in plasma from patients with IPAH and healthy controls.

In contrast to healthy controls, where all hepcidin levels were >2 ng/ml, there was also a cohort of 21 IPAH patients with hepcidin levels <2 ng/ml (Figure 4.5A), who exhibited marked iron deficiency (Figure 4.5B&C). While hepcidin levels were lowest in these patients, correlating inversely with sTfR (Figure 4.5B) and positively with increasing ferritin levels (Figure 4.5C), a significant proportion of patients with iron deficiency displayed inappropriately normal or even high hepcidin levels.

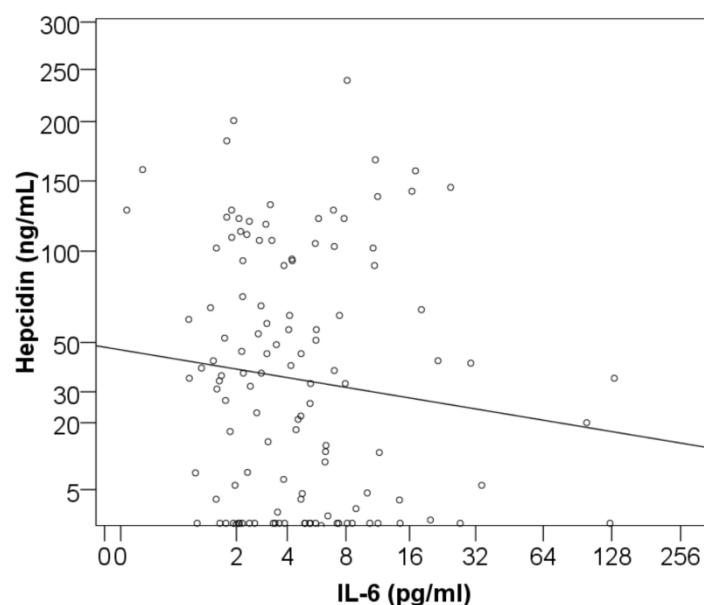


Figure 4.6 – Scatter-plot of hepcidin vs. IL-6 in IPAH. Square-root hepcidin against logged IL-6.

Linear regression modelling confirmed that IPAH patients had significantly higher hepcidin levels than controls after adjusting for soluble transferrin receptor levels or ferritin and EPO levels (Table 4.5). Hepcidin levels did not differ between subgroups on different PAH-targeted therapies (Table 4.4).

Linear regression modelling			
Dependent Variable: square root Hepcidin			
Parameter	B	Std. Error	Sig.
<i>Model 1 – Just sTfR</i>			
Intercept	17.254	2.134	<0.001
ln[IL-6]	0.016	0.281	0.953
ln[soluble transferrin receptor]	-3.308	0.614	<0.001
IPAH vs. Control	2.000	0.683	0.004

<i>Model 2 – sTfR, Ferritin & EPO</i>			
Intercept	4.663	2.798	0.099
ln[IL-6]	-0.269	0.269	0.320
ln[sTfR]	-0.952	0.661	0.153
ln[Ferritin]	1.971	0.254	<0.001
ln[EPO]	-0.883	0.429	0.042
IPAH vs. Control	2.350	0.674	0.001

Table 4.5 – Linear regression modelling of plasma hepcidin values from patients with IPAH and healthy controls. Variables were square root- or ln-transformed to normalise where needed.

Erythropoietin is raised in IPAH and relates to lower hepcidin levels.

EPO levels were raised in the IPAH population (17.3, IQR: 12.1 – 43.0 IU/L versus 9.7, IQR: 7.4 – 12.6 IU/L in controls, $p < 0.001$) and correlated inversely with hepcidin (Figure 2.7A-B).

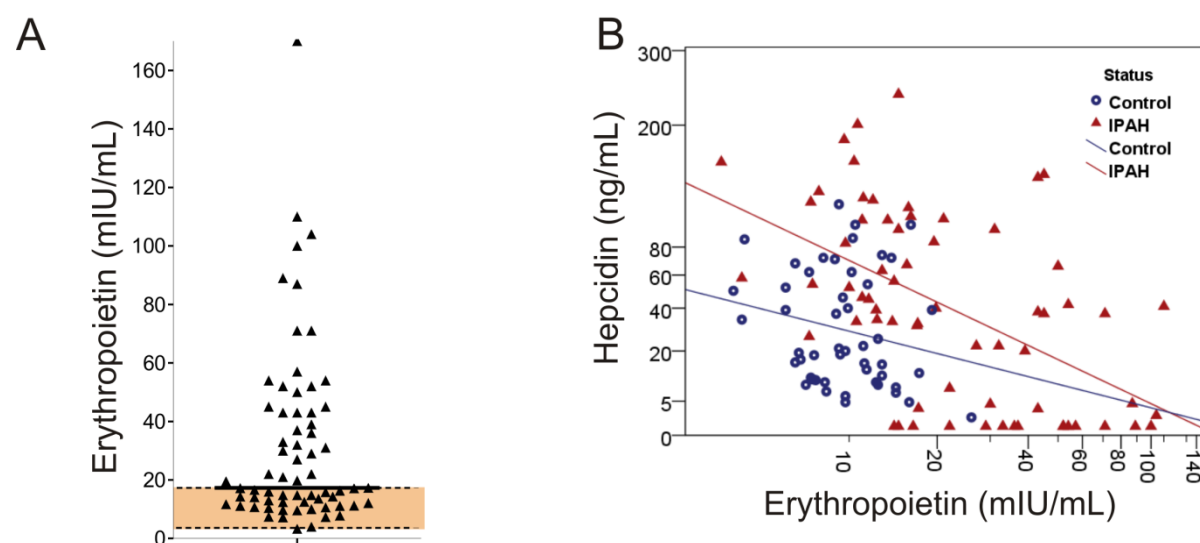


Figure 4.7 – Erythropoietin (EPO) levels in IPAH. A. Dot-plot of erythropoietin levels in patients with IPAH. B. Scatter-plot of square root scaled-hepcidin versus ln scaled-EPO levels in plasma from patients with IPAH and healthy controls.

EPO levels did not correlate with red blood cell counts ($Rho = -0.034$, $p = 0.90$), haematocrit ($Rho = -0.356$, $p = 0.15$) but did correlate with haemoglobin levels ($Rho = -0.277$, $p = 0.028$) and RDW ($\rho = 0.527$, $p < 0.001$). EPO levels did not correlate with peripheral oxygen saturations ($Rho = -0.150$, $p = 0.25$), but did correlate inversely with the product of this measure and haemoglobin ($Rho = -$

0.357, $p=0.006$). We found raised GDF-15 levels in patients with high sTfR (Table 4.2) which also correlated with increased RDW ($\rho=0.332$, $p<0.001$). GDF-15 did not correlate with EPO ($\rho = 0.223$, $p=0.10$) or hepcidin ($\rho = -0.084$, $p=0.37$).

sTfR levels relate to functional class, exercise capacity and survival in IPAH.

sTfR levels were associated with other markers of iron status (Table 4.6), increased with WHO functional class ($p<0.0001$) (Figure 4.8A) and correlated negatively with 6 minute walk distance (Table 4.6).

	soluble Transferrin receptor levels		Spearman's	
	Normal (8.7 - 28.1 nmol/L)	High (>28.1 nmol/L)	Rank test	
	n = 36	n = 62	Rho	Sig.
Age, y	45.5 (± 16.0)	48.1 (± 15.7)	0.11	0.37
6 minute walk, m	340 (± 156)	247 (± 154)	-0.39	0.03
WHO			N/A	<0.05
I/II	12	13		
III	19	35		
IV	5	14		
Peripheral O ₂ sat, %	95.0 (93.0-98.0)†	93.5 (88.0-95.5)†	-0.33	0.01
O ₂ sat. post-6MW, %	95.0 (88.0-97.0)†	88.5 (80.0-95.0)†	-0.34	0.03
Iron, $\mu\text{mol/l}$	13.0 (7.4-16.8)†	7.7 (3.3-12.1)†	-0.41	0.003
TIBC, $\mu\text{mol/l}$	59.0 (55.0-67.0)†	61.9 (47.3-68.0)†	0.09	0.53
Transferrin saturation, %	27.0 (18.8-35.1)†	15.6 (10.7-22.0)†	-0.46	<0.001
Ferritin, $\mu\text{g/l}$	66.5 (38.0-170.0)†	43.0 (20.5-91.5)†	-0.33	<0.001
sTfR/logFerritin	0.8 (0.7-1.0)†	1.9 (1.4-2.8)†	0.88	<0.001
Hepcidin, ng/ml	53.0 (31.5-102.5)†	14.4 (0.9-56.0)†	-0.49	<0.001
EPO, mIU/ml	12.5 (10.0-19.5)†	32.5 (14.8-52.0)†	-0.50	<0.001
RBC, $\times 10^{12}/\text{L}$	4.8 (± 0.6)	5.3 (± 0.6)	0.23	0.07
Haemoglobin, g/dl	15.3 (± 1.7)	14.4 (± 2.8)	-0.28	0.008
Haematocrit	0.43 (± 0.05)	0.45 (± 0.04)	0.06	0.50
Red cell distribution width, %	13.9 (13.5-14.9)†	15.7 (14.4-17.6)†	0.57	<0.001
IL-6, pg/ml	2.1 (1.5-4.7)†	5.6 (2.9-10.8)†	0.46	<0.001
GDF-15, pg/ml	549 (350-875)†	915 (359-1860)†	0.26	0.01
NT-proBNP, ng/l	532 (186-1031)†	838 (410-1448)†	0.13	0.04

Table 4.6 – Patient characteristics stratified by normal/high soluble transferrin receptor levels.

Clinical and biochemical measures stratified by the upper limit of the normal range (28.1 nmol/L). Data are presented as mean $\pm$ standard deviation or as frequencies. † indicates variable is not normally distributed and therefore given as median (interquartile range). Statistics shown are from Kruskal-Wallis ANOVA for WHO class and Spearman's rank correlations for all other parameters.

During the follow-up period, 39 patients died. Patients with raised sTfR (>28.1 nmol/L) had higher mortality ($p=0.022$, Figure 4.5B). sTfR levels predicted mortality in IPAH and this was independent of WHO functional class, 6 minute walk distance and age ($p=0.011$, Table 4.7). Whether patients were sampled before or after treatment was commenced did not predict survival in this group ($p=0.09$, Table 4.7).

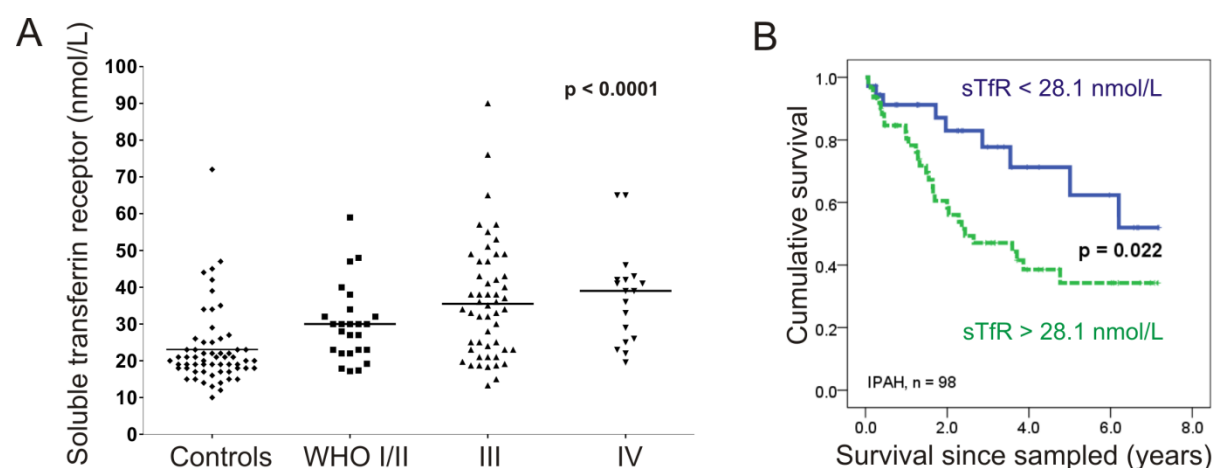


Figure 4.8 – Soluble transferrin receptor in IPAH. **A.** sTfR levels according to WHO class in patients with IPAH and healthy controls. P value relates to Kruskal-Wallis ANOVA. **B.** Kaplan-Meier survival estimates stratified by sTfR level below (blue solid line) or above (green dashed line) the pre-defined upper limit of the normal range, 28.1 nmol/L.

Variable	Simple model		Multiple covariate model	
	Hazard ratio (95% CI)	Sig.	Hazard ratio (95% CI)	Sig.
Age (per 10 years)	1.387 (1.128-1.707)	0.002		0.493
WHO functional class I/II (vs. WHO IV)	0.133 (0.038-0.468)	0.005		0.105
class III (vs. WHO IV)	0.535 (0.277-1.033)			
6 minute walk distance (per 100 metres)	0.618 (0.499-0.764)	<0.001	0.653 (0.515-0.828)	<0.001
ln[Soluble transferrin receptor]	2.449 (1.292-4.642)	0.006	2.569 (1.240-5.320)	0.011
Peripheral artery O ₂ saturation, %	0.955 (0.901-1.012)	0.118		0.529
Treated versus treatment-naïve	1.289 (0.961-1.728)	0.090		0.115

Table 4.7 – Cox regression survival analysis shows soluble transferrin receptor as an independent predictor of survival in IPAH. Simple model indicates predictive power of individual variables. Significant predictors from univariate analysis were then entered into a multiple covariate model, which was developed by the backwards stepwise hazard ratios analysis method. Soluble transferrin receptor was normalised by ln-transformation before entry into the model.

4.4 Discussion

The importance of iron regulation in cardiovascular disease (Anker et al. 2009) and in the control of pulmonary vascular tone is now recognised (Balanos et al. 2002; Smith et al. 2008; Smith et al. 2009). I have identified iron deficiency without overt anaemia in a large proportion (approximately 2/3) of patients with IPAH, characterised primarily by raised plasma sTfR, but also by reduced serum iron, transferrin saturation and ferritin levels and increased RDW.

Recently iron deficiency, defined by reduced serum iron and transferrin saturations, was reported in 30/70 of patients with idiopathic PAH (IPAH), which correlated negatively with exercise capacity, a surrogate of prognosis. Interestingly, oral iron therapy was ineffective at restoring normal ferritin levels suggesting a problem with iron absorption (Ruiter et al. 2010). Soon *et al.* have also observed a high prevalence of iron deficiency, defined by very low ferritin levels, in patients with IPAH, but not in patients with CTEPH (Soon et al. 2011).

Circulating soluble transferrin receptor levels are a sensitive marker of tissue iron availability, which, unlike ferritin and serum iron measurements, are unaffected by inflammation (Cook et al. 1990). When iron deficiency is present, sTfR levels are increased disproportionately to erythropoietic activity (Huebers et al. 1990), as the bone marrow attempts to maximise iron uptake. For these reasons, sTfR is the best available circulating indicator of iron deficiency in diseases with an inflammatory component, such as IPAH.

The cause of iron deficiency in IPAH is not immediately clear. Three possibilities to be considered are chronic blood loss, polycythaemia due to the development of hypoxaemia and insufficient iron absorption from the gut. The majority of IPAH patients receive warfarin and are female, many of whom are pre-menopausal, and this might implicate blood loss. However, this is not a complete explanation. sTfR levels were also raised in patients not taking warfarin and did not significantly differ with gender nor age; women above 60 and men with IPAH also exhibited raised sTfR levels.

This agrees with the data of Ruiter *et al.* (Ruiter et al. 2010) who observed an equal prevalence of iron deficiency in pre- and post-menopausal women with IPAH and no reported abnormal gastrointestinal or gynaecological blood loss. Soon *et al.* also demonstrated that CTEPH patients, who all receive warfarin anticoagulation therapy, do not demonstrate a high prevalence of iron deficiency (Soon et al. 2011), indicating that warfarin therapy itself does not lead to significant iron deficiency. Furthermore, the distribution of plasma hepcidin levels in the IPAH patient population is not consistent with blood loss as a single cause; iron deficiency from chronic blood loss would normally be associated with reduced circulating hepcidin (Zimmermann et al. 2009). The iron

deficient patients had lower peripheral oxygen saturations, but in the absence of an associated increase in haemoglobin or haematocrit it is unlikely that reactive polycythaemia could explain the deficiency seen.

Hepcidin inhibits dietary uptake and mobilisation of iron via degradation of the unique iron exporter, ferroportin, on enterocytes and reticuloendothelial cells, respectively (Hentze et al. 2010). The low or undetectable hepcidin levels observed in some of the profoundly iron-deficient IPAH patients may represent an attempt to increase dietary iron absorption. Hepcidin levels raised or maintained at normal levels in the presence of raised sTfR in a significant proportion of IPAH patients suggests that inappropriate hepcidin production may contribute to the iron deficiency by inhibiting dietary iron uptake. This hypothesis is supported by the observation that only a small proportion of IPAH patients respond to oral iron therapy (Ruiter et al. 2010).

Regulation of hepcidin production in IPAH

In the initial investigation of the factors underlying elevated hepcidin levels in IPAH, two mechanisms were considered – inflammation and erythropoietic activity.

Inflammation can raise hepcidin levels, primarily through IL-6 induction (Nemeth et al. 2004) and raised hepcidin production underlies anaemia of chronic disease (Weiss & Goodnough 2005). IL-6 levels were raised in IPAH versus controls and despite low serum iron and transferrin saturations, most patients had ferritin levels within the normal range indicating that inflammation was present in these patients to a certain degree. However, sTfR levels were raised and plasma hepcidin did not correlate with IL-6 in IPAH patients whether or not iron status was taken into account by considering sTfR or ferritin levels in parallel. This is inconsistent with anaemia of chronic disease, where ferritin levels are normal or increased and sTfR levels repressed (Weiss & Goodnough 2005). Indeed, IL-6 levels in IPAH are no higher than in CTEPH patients who are not generally iron deficient (Soon et al. 2011). These data suggest that inflammation and IL-6 stimulation, though present, are not the main cause of raised hepcidin levels in IPAH.

Erythropoietic activity inhibits hepcidin expression under anaemic conditions (Pak et al. 2006) and is impaired in anaemia of chronic disease (Weiss & Goodnough 2005). Serum from patients with overactive erythropoiesis inhibits hepcidin expression *in vitro* (Tanno et al. 2007) and recombinant human EPO administration lowers hepcidin levels in healthy volunteers (Ashby et al. 2010). Dysfunctional erythropoiesis or erythropoietic signalling could contribute to inappropriately increased hepcidin levels. EPO levels correlated inversely with hepcidin in IPAH patients, consistent

with a model where EPO directly or indirectly inhibits hepcidin production. EPO does not suppress hepatocyte hepcidin levels *in vitro* (Lee & Beutler 2009) and other circulating factors implicated as possible mediators include GDF-15 (Tanno et al. 2007), a TGF- β superfamily member that is elevated in IPAH (Nickel et al. 2008). GDF-15 was raised in iron-deficient patients, but did not correlate with EPO or hepcidin levels, suggesting GDF-15 is not important in regulating hepcidin in IPAH.

Iron status and pulmonary hypertension

sTfR levels increased significantly with disease severity, correlated inversely with exercise capacity, and independently predicted survival after adjustments for standard clinical measures including 6 minute walk distance and WHO class. These data, together with the recent observation that RDW and anaemia predict mortality in pulmonary hypertension (Hampole et al. 2009; Krasuski et al. 2010); suggest that iron deficiency is clinically relevant in IPAH.

Iron status influences pulmonary vascular responses to hypoxia. Iron chelation elevates pulmonary vascular resistance in humans (Balanos et al. 2002) and increases sensitivity to acute hypoxic exposure (Smith et al. 2008). Iron deficiency induced by venesection also exacerbates pulmonary vasoconstriction at high altitude (Smith et al. 2009) whereas iron infusion reduces these pulmonary vasopressor responses (Smith et al. 2008; Smith et al. 2009). The underlying mechanism may involve normoxic stabilisation of hypoxia inducible factors (HIF) as HIF hydroxylases require iron as a co-factor (Ivan et al. 2001; Jaakkola et al. 2001). Mice deficient in either HIF-1 α or HIF-2 α develop less severe pulmonary hypertension and vascular remodelling when exposed to chronic hypoxia (Brusselmans et al. 2003; Yu et al. 1999) and pulmonary endothelial HIF-1 α expression is increased in PAH (Fijalkowska et al. 2010). By preventing HIF activation, iron replacement therapy may potentially reduce pulmonary endothelial dysfunction, HIF-dependent gene activation and vascular remodelling in pulmonary hypertension.

Although the observation of elevated levels of EPO is of interest in other respects, it appears abnormal erythropoietic signalling is not responsible for hepcidin dysregulation in IPAH. Increased circulating EPO levels in patients with PAH have been reported by another study (Farha et al. 2011). The authors of the study hypothesised that iron deficiency or anaemia in patients with PAH, causing activation of HIF-inducible factors including EPO, could lead to clinical worsening through induction of myeloid abnormalities and increased recruitment of highly proliferative progenitor cells to the pulmonary vasculature. In support of this they demonstrated increased numbers of hemangioblast (CD34⁺CD133⁺) cells in the walls of the pulmonary arteries of patients with PAH. Elevated levels of

HIF-inducible myeloid-activating factors (stromal-derived factor-1 α , stem cell factor and hepatocyte growth factor) were also demonstrated in plasma from patients with PAH. Interestingly, myeloid abnormalities were also demonstrated in unaffected relations of HPAH patients, suggesting these mechanisms may contribute to the pathology of PAH prior to clinical presentation. The relative importance of iron deficiency in regulating these abnormalities was not established and should be explored in future studies, but this work suggests another potential mechanism by which iron status might affect the pathogenesis of PAH.

Iron repletion increases exercise capacity and reduces New York Heart Association functional class in left heart failure (Anker et al. 2009; Okonko et al. 2008; Toblli et al. 2007). Contrary to previous observations (Okonko et al. 2008), improvements were similar in patients irrespective of anaemia (Anker et al. 2009). Coupled with our findings, this suggests a study investigating the role of iron-replacement therapy in IPAH is needed.

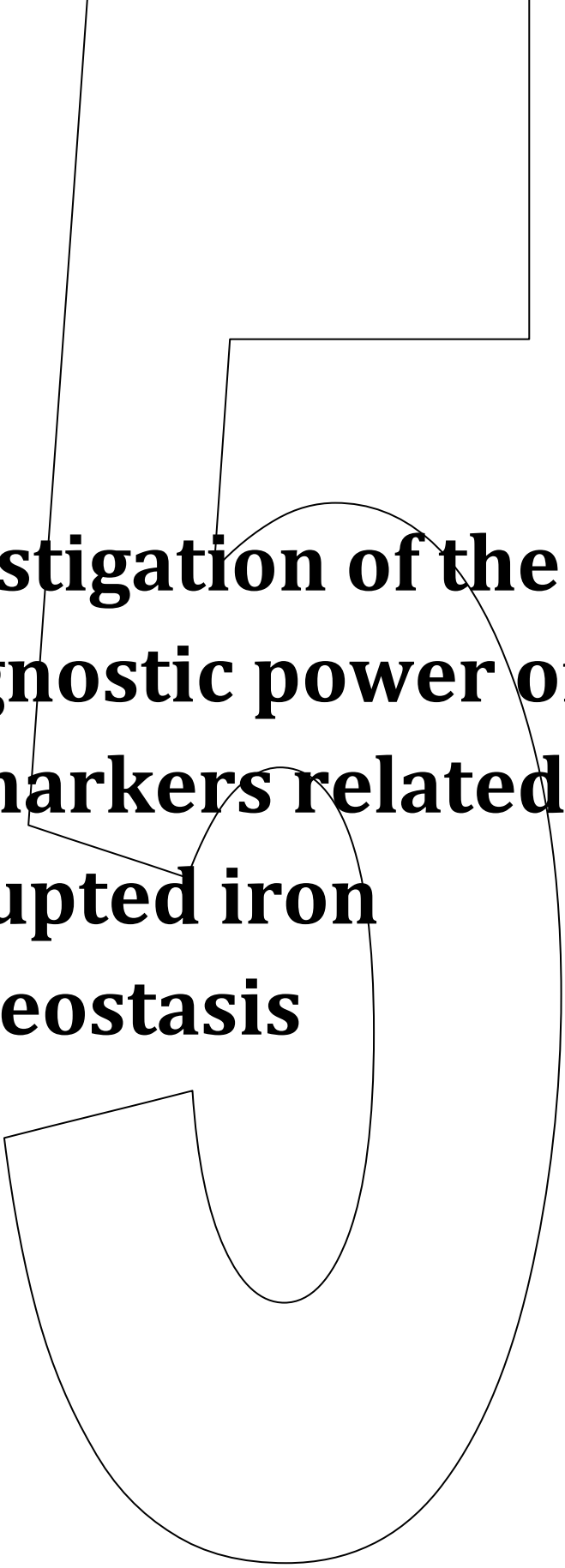
Limitations and summary

One limitation of this study is the lack of longitudinal data; it would be of interest to investigate the onset and time course of iron deficiency in IPAH. The control population in this study was younger and contained a more equal distribution of sexes; however gender differences in hepcidin are related to lower ferritin levels in younger women hence no gender-dependent differences in iron parameters were observed. The lack of occult blood stool measurements and gynaecological questionnaire limits our ability to rule out bleeding as a cause of iron deficiency in IPAH. There is no reason to believe that our patients were receiving a poor diet. Furthermore, Ruiter *et al.* (Ruiter et al. 2010) found that more than half of IPAH patients who received oral iron therapy were unresponsive, suggesting that absorption, rather than dietary content, is a significant problem in IPAH. The survival analysis included patients who had commenced therapy. When this was included in the model as a factor, survival did not significantly differ between treated and treatment-naïve groups. While I elected to study only those patients with idiopathic PAH, it would be of interest for further studies to investigate the prevalence and clinical significance of iron deficiency in other forms of PAH to determine the potential for therapeutic intervention.

In summary, I have characterised iron deficiency in a significant proportion of patients with IPAH and demonstrate a relationship between sTfR levels and disease severity and outcome. Inappropriately raised hepcidin levels may contribute to the development of iron deficiency in some patients with IPAH. Importantly, iron status represents an accessible target for novel therapeutic intervention.

Three main questions arise from this chapter that I will explore in chapters 5 - 7:

1. Iron deficiency measured by sTfR level predicts survival in IPAH irrespective of age, WHO functional class and 6 minute walk distance. ***Could circulating markers related to iron status improve prognostication in IPAH?***
2. Hepcidin is raised in IPAH despite the presence of iron deficiency and could therefore be a driver of the iron deficiency itself. This does not appear to be related to inflammation or altered erythropoietic regulation. BMP signalling is important in the regulation of hepcidin. ***What is the mechanism behind hepcidin dysregulation in IPAH; could it be related to BMPR2 dysfunction?***
3. Iron status is important in regulating pulmonary vascular tone and hypoxic pulmonary vasoconstriction. Iron deficiency measured by sTfR level predicts survival in IPAH and is related to worse WHO functional class and lower exercise capacity. Increased hepcidin in IPAH suggests that oral iron therapies would have low efficacy, as seen by Ruiter *et al.* Intravenous iron replacement therapy has shown impressive results in left heart failure, improving 6 minute walk distances, quality of life and functional class in clinical trials. ***Does intravenous iron therapy improve pulmonary haemodynamics, exercise capacity and/or symptoms in patients with IPAH and iron deficiency?***



Investigation of the prognostic power of biomarkers related to disrupted iron homeostasis

Chapter 5 – Investigation of the prognostic power of biomarkers related to disrupted iron homeostasis

5.1 Introduction

In chapter 4, I identified a high prevalence of iron deficiency in patients with IPAH, which was related to symptoms and exercise capacity, but also most remarkably, survival. In this chapter, I will explore the potential of circulating biomarkers related to iron (namely RDW and GDF-15) to improve prognostication alongside other putative biomarkers (creatinine, interleukin-6 and NT-proBNP) and current standard clinical measures.

Circulating biomarkers in PAH

As discussed, circulating proteins have considerable potential to inform the clinical management of patients with pulmonary arterial hypertension (Badesch et al. 2009; McLaughlin et al. 2009b). Currently, only BNP-32 and NT-proBNP are recognised as clinically useful in idiopathic PAH (Badesch et al. 2009). Other biomarkers that report different pathogenic mechanisms or involvement of different tissues may add utility, particularly when determined and interpreted in combination with established clinical measures and predictors of mortality. Recently identified biomarkers that I have chosen to focus on and that show promise include red cell distribution width (Hampole et al. 2009), growth differentiation factor-15 (Nickel et al. 2008), interleukin-6 (Soon et al. 2010) and creatinine (Shah et al. 2008).

RDW is measured as part of the full blood count in standard hospital analyses and quantifies the variability in red blood cell size. Anisocytosis, indicated by increased RDW, is seen in iron-deficiency anaemia, thalassemias, and myelodysplastic syndromes. RDW levels correlated significantly with sTfR levels in the IPAH cohort (Figure 5.1). RDW predicts survival in left heart failure (Felker et al. 2007), coronary disease (Tonelli et al. 2008) and in older adults (Patel et al. 2010) with hazard ratios of 1.14-1.17 per % increase. RDW has also recently been reported to predict survival in patients with pulmonary hypertension of mixed aetiologies, where it out-performed NT-proBNP (Hampole et al. 2009).

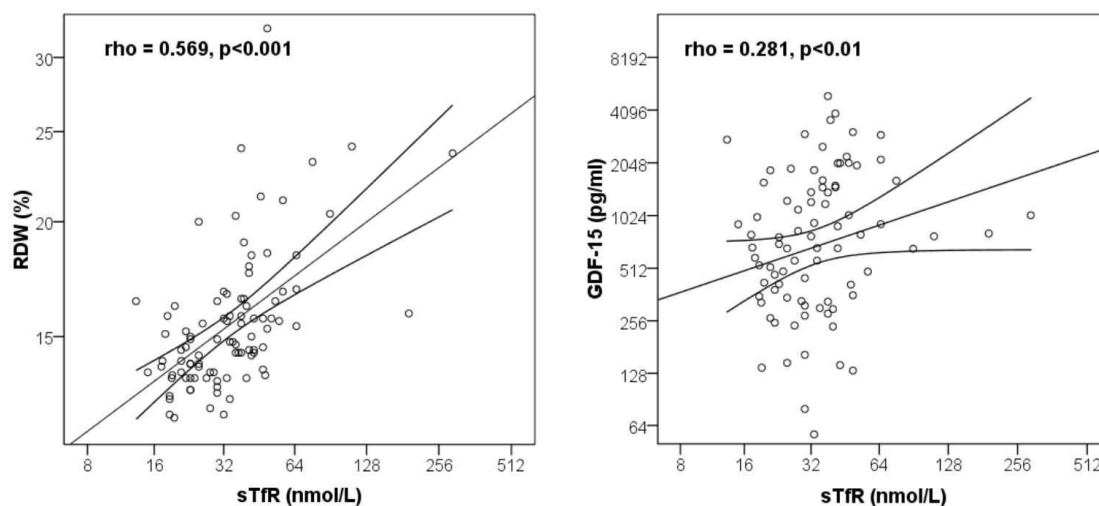


Figure 5.1 – Association of RDW and GDF-15 to iron status in IPAH. Scatter plots of RDW and GDF-15 versus soluble transferrin receptor (sTfR) levels. N=94 for RDW vs. sTfR and n=85 for GDF-15 vs. sTfR.

GDF-15 is a member of the TGF- β superfamily (Bootcov et al. 1997) responsive to various stimuli, including inflammation and chemical, mechanical and hypoxic stress (Albertoni et al. 2002; Frank et al. 2008; Zimmers et al. 2005). Iron chelation has been shown to elevate GDF-15 expression in a variety of cell lines (Lakhal et al. 2009). Indeed GDF-15 levels correlated significantly with sTfR in the IPAH cohort (Figure 5.1) suggesting a possible relationship to iron status, although GDF-15 is released from erythroid progenitors and therefore it is possible this correlation may be confounded by changes in erythropoietic activity. Circulating levels are raised in patients with ischemic heart disease and chronic heart failure (Kempf et al. 2007; Wollert et al. 2007). Increased GDF-15 levels have been observed in patients with acute pulmonary embolism, possibly reflecting changes in right ventricular (RV) function (Lankeit et al. 2008), and in treatment-naïve IPAH patients (Nickel et al. 2008). It has also been shown recently to be raised in patients with systemic sclerosis-associated pulmonary hypertension and predicted survival in this population (Meadows et al. 2010). GDF-15 is sensitive to hypoxia, anoxia and iron depletion (Albertoni et al. 2002; Lakhal et al. 2009), and thus GDF-15 levels may report on two signalling pathways implicated in PAH; TGF- β and hypoxia-iron sensing.

Inflammation has long been known to be a facet of IPAH (Hassoun et al. 2009) and several data have implicated a role for IL-6 in the development of PH in *in vivo* models (Miyata et al. 1995; Savale et al. 2009; Steiner et al. 2009). Patients with pulmonary hypertension secondary to connective tissue disorders generally exhibit a poorer prognosis (McLaughlin et al. 2004). Recently it has been shown that levels of several inflammatory molecules are raised and predict survival in IPAH, including IL-6

(Soon et al. 2010), which performed strongly, predicting survival independently of haemodynamic measurements. However, the usefulness of IL-6 measurements in combination with other PAH biomarkers such as NT-proBNP has not been assessed.

Renal dysfunction is linked to mortality in cardiovascular diseases and specifically in patients with pulmonary hypertension (Leuchte et al. 2007). Serum creatinine is a relatively crude but practical measure of kidney function and a large study of 500 PAH patients has previously demonstrated its use as a prognostic biomarker (Shah et al. 2008). It may also affect other biomarkers; impaired renal clearance of NT-proBNP could lead to accumulation unrelated to ventricular dysfunction, RDW is inversely related to kidney function (Lippi et al. 2008) and IL-6 levels are raised and predict survival in renal failure (Simmons et al. 2004).

These biomarkers have so far been analysed primarily in incident PAH populations in single separate studies. I set out to examine and directly compare the performance of RDW and GDF-15, as well as IL-6, NT-proBNP and creatinine as putative markers of prognosis in a “real life” clinical setting including both incident and prevalent IPAH patients and to explore the value of adding these biomarkers to established clinical measures in predicting survival of patients with IPAH.

5.2 Methods

Study subjects

Plasma samples from the pulmonary hypertension biorepository, Imperial College London were used in this study. This biorepository includes samples from 139 patients with IPAH attending Hammersmith Hospital, London, between 2002 and 2009, who consented to providing a blood sample for research. Ethical approval was received via the National Research Ethics Service (reference numbers 2001/6157 and 09/H0711/4). The diagnosis of IPAH was based on established criteria with confirmation by right heart catheterization and exclusion of other forms of pulmonary hypertension (Galie et al. 2009b). WHO functional class and six minute walk distance (6MWD) were determined at the same day as blood samples were collected. Blood samples were obtained from IPAH patients upon enrollment either at diagnosis or at outpatient appointments during the course of the disease. Survival status was censored on July 26, 2010. Death was defined as the primary endpoint, and no patient was lost to follow-up. Samples were also analysed from healthy volunteers from Imperial College London, UK.

Methods

All EDTA blood samples were immediately cooled (4°C) and centrifuged at 1000 rpm for 5 minutes, the plasma removed and frozen (-80°C). Plasma GDF-15 and IL-6 levels were measured using ELISA kits (R&D Systems, Abingdon, UK) as per the manufacturer's instructions. Creatinine was measured by standard Clinical Pathology Accreditation-approved hospital assay. RDW was measured on the Sysmex XE-2100 automated analyser (Sysmex, UK); a method based upon electrical impedance using hydrodynamic focusing. NT-proBNP levels were measured using a non-competitive assay as previously described (Omland et al. 2002).

Statistical analysis

Data are presented as absolute numbers, percentages, mean ($\pm$ standard deviation, SD), or median and interquartile range. The relationship between patient characteristics and biomarkers were assessed by Spearman's rank test or Mann-Whitney U and Kruskal-Wallis tests for categorical variables. Receiver Operating Characteristic (ROC) curves assessing all-cause mortality and all possible biomarker cut-offs and their area-under-the-curve were used to compare prognostic values

of biomarkers. 2-years was selected as the time point for ROC analysis as this provided a good event rate (29 deaths) without excluding too many patients (n=101). Highly sensitive (>80%) cut-offs were selected by the maximum combined sensitivity and specificity. Kaplan-Meier plots illustrated events during follow-up in relation to biomarker levels, assessed by the log-rank test. Several parameters were not normally distributed, as shown by the Kolmogorov-Smirnov test, and were therefore respectively transformed to their natural logarithm (ln) and square root, as appropriate to best normalize the data, before Cox analysis (Altman 1991). Cox regression analyses identified prognostic predictors. All variables with $p < 0.05$ were tested by proportional hazard ratios Cox regression analysis; variables were removed at $p > 0.10$. All calculations were performed with SPSS 17.0 (SPSS, Inc., Chicago, IL).

5.3 Results

Biomarker levels and clinical and haemodynamic measurements in IPAH

Baseline patient characteristics for all 139 patients recruited are detailed in Table 5.1. 49 patients died during follow-up. Median time since diagnosis at sampling was 1.17 years; (interquartile range 0.2-2.5 years). Samples from 40 sex- and aged-matched healthy controls were also collected (Table 5.1).

Clinical variables	Controls	IPAH	Biomarker relationships with clinical variables (Rho & p values)				
			RDW	GDF-15	IL-6	Creatinine	NT-proBNP
Age, years	39.7 (±9.3)	47.6 (±15.8)	0.258, p=0.004	0.437, p<0.001	0.289, p=0.002	0.335, p<0.001	0.078, p=0.4
Gender - F	25	98	0.8	0.2	0.6	0.015	0.4
M	15	41					
mPAP, mmHg	-	55.6 (±12.4)	-0.004, p=1.0	-0.146, p=0.2	-0.129, p=0.3	0.018, p=0.9	0.132, p=0.2
PCWP, mmHg	-	11.7 (±5.4)	0.255, p=0.1	-0.035, p=0.8	0.000, p=1.0	-0.271, p=0.1	-0.027, p=0.9
mRAP, mmHg	-	11.4 (±5.9)	0.191, p=0.07	0.234, p=0.03	0.154, p=0.2	0.253, p=0.02	0.283, p=0.008
CI, L/min/m ²	-	2.38 (±1.08)	-0.115, p=0.3	-0.229, p=0.039	-0.017, p=0.9	-0.276, p=0.01	-0.378, p<0.001
PVR, Woods units	-	13.2 (±8.4)	0.152, p=0.2	-0.038, p=0.8	-0.105, p=0.4	0.224, p=0.054	0.321, p=0.006
<i>WHO class</i>							
I	-	7	<0.001	<0.001	<0.001	0.004	0.1
II	-	31					
III	-	80					
IV	-	21					
6MWD, m	-	302 (±154)	-0.250, p=0.008	-0.480, p<0.001	-0.434, p<0.001	-0.158, p=0.1	-0.217, p=0.028
<i>PAH therapies</i>							
Warfarin	-	114	0.5	0.6	0.8	0.5	0.2
Bosentan	-	64	0.2	0.4	0.4	0.6	0.5
Sildenafil	-	22					
Treprostinil	-	12					
Diltiazem	-	1					
None	-	40					
RDW, %	-	15.5 (±2.9)	-	0.332, p<0.001	0.250, p=0.006	0.146, p=0.1	0.199, p=0.033
GDF-15, pg/ml	315 (±131)	1015 (±933)	0.332, p<0.001	-	0.534, p<0.001	0.468, p<0.001	0.460, p<0.001
Interleukin-6, pg/ml	1.4 (±1.4)	8.6 (±18.7)	0.250, p=0.006	0.534, p<0.001	-	0.376, p<0.001	0.283, p=0.003
Creatinine, µmol/L	-	96 (±30)	0.146, p=0.1	0.468, p<0.001	0.376, p<0.001	-	0.373, p<0.001
NT-proBNP, fmol/ml	-	909 (±849)	0.199, p=0.033	0.460, p<0.001	0.283, p=0.003	0.373, p<0.001	-

Table 5.1 – Relationships between biomarkers and patient clinical and haemodynamic data.

Significant relationships are indicated by bold text.

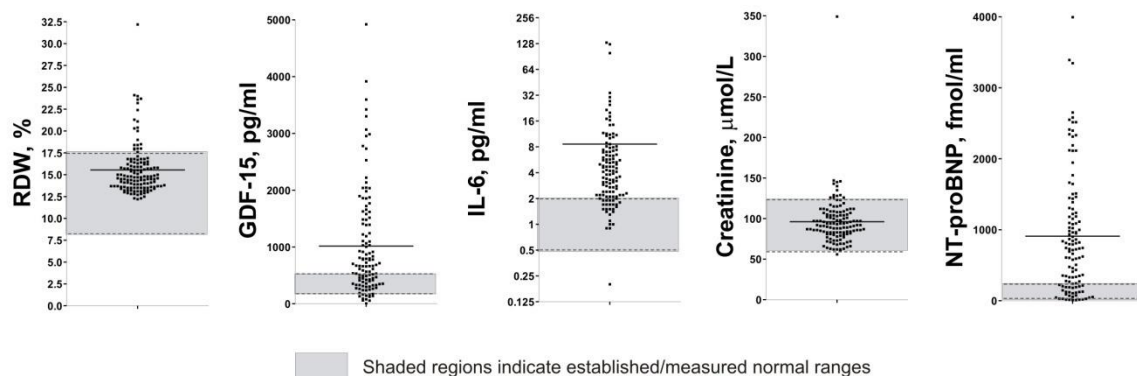


Figure 5.2 – Biomarker levels in patients with IPAH. RDW and creatinine were measured with clinical hospital assays. GDF-15 and IL-6 were determined by ELISA. NT-proBNP was measured using a non-competitive assay as previously described (Omland et al. 2002).

RDW levels were on average towards the upper end of the normal range (15.54 ± 2.92 %) and GDF-15 (1015 ± 933 pg/ml), IL-6 (8.6 ± 18.7 pg/ml) and NT-proBNP (909 ± 849 fmol/ml) levels were all raised in patients with IPAH, whereas creatinine levels were mostly within the normal range (96 ± 30 μ mol/L) (Figure 5.2). The normal ranges for RDW and creatinine are established for the hospital assays. Levels of GDF-15 and IL-6 in healthy volunteers were used to estimate the normal ranges for these ELISA assays. The normal range for the NT-proBNP assay was based on levels measured in a total of 37 healthy volunteers in previously published studies (Hughes et al. 1999; Talwar et al. 2000; Talwar et al. 2001).

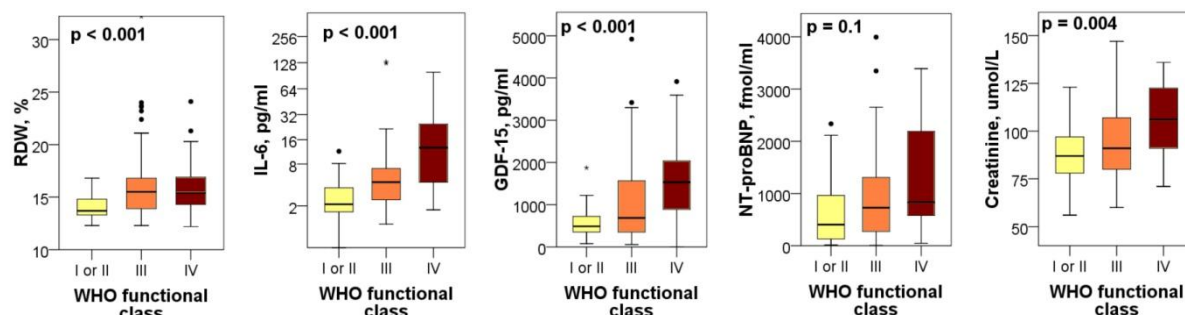


Figure 5.3 – Biomarker levels in IPAH patients stratified by WHO functional class. Classes I and II were pooled due to limited numbers in functional class I. Statistics shown are one-way ANOVA significance values. Dots indicate outliers and stars extreme outliers.

Biomarker relationships with coincident clinical/biochemical (6MWD, WHO, age, gender, treatment, biomarkers) and baseline haemodynamic data were analysed; p-values are shown in Table 5.1. Cardiac index and mean right atrial pressures correlated with GDF-15 ($Rho=-0.229$, $p=0.039$ and 0.234 , $p=0.030$), creatinine ($Rho=-0.276$, $p=0.012$ and 0.253 , $p=0.018$) and NT-proBNP levels ($Rho=-0.378$, $p<0.001$ and 0.2863 , $p=0.008$). NT-proBNP levels correlated significantly with PVR ($Rho=0.321$, $p=0.006$). RDW, GDF-15, IL-6 and creatinine levels all increased significantly with WHO functional class ($p<0.01$, Figure 5.3) whereas NT-proBNP did not ($p=0.1$). All biomarkers except creatinine correlated significantly with 6 minute walk distances (RDW: $Rho=-0.250$, $p=0.008$; GDF-15: $Rho=-0.480$, $p<0.001$; IL-6: $Rho=-0.434$, $p<0.001$; NT-proBNP: $Rho=-0.217$, $p=0.028$) and with each other except RDW and creatinine (all others $p<0.05$). GDF-15 in particular correlated with all 4 other biomarkers with very high significance (all $p<0.001$).

Biomarkers and outcomes in IPAH

Receiver operating characteristic (ROC) curve analysis of 2-year all-cause mortality confirmed that all biomarkers measured except creatinine were significantly powerful predictors of survival (Figure 5.4 and Table 5.2). RDW was the strongest performer in this analysis, with an area under the curve of 0.82, followed by GDF-15 and 6 minute walk distance (0.76/0.75) which both outperformed NT-proBNP and IL-6 (0.67/0.66).

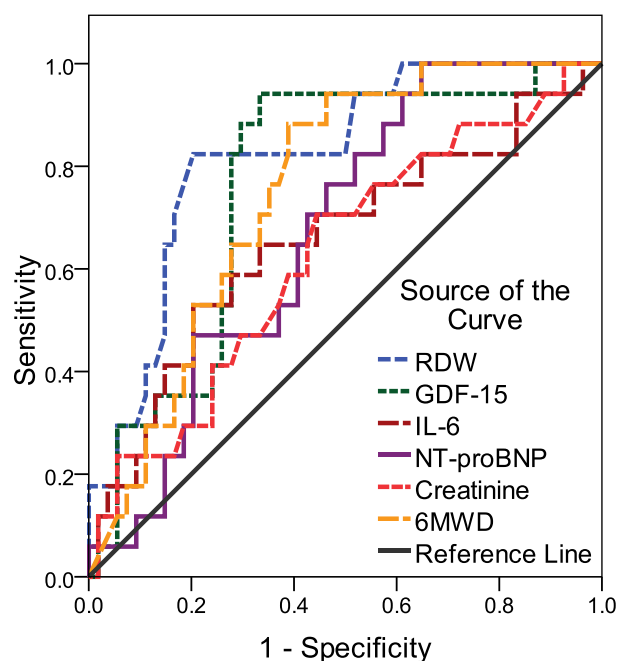


Figure 5.4 – ROC curve showing power of biomarkers and 6MWD to predict 2-year all-cause mortality

From the ROC analysis, highly sensitive cut-off values were derived (Table 5.3) to predict mortality for RDW (15.7%, 82.4% sensitivity), GDF-15 (779 pg/ml, 88.2% sensitivity), IL-6 (2.5 pg/ml, 82.4% sensitivity), creatinine (80.5 μ mol/L, 85.7% sensitivity), NT-proBNP (491 fmol/ml, 82.4% sensitivity) and 6MWD (322 m, 88.2% sensitivity).

Variable	Area Under the Curve			95% CI	
	Area	Std. Error	Sig.	Lower	Upper
RDW	0.820	0.055	<0.001	0.712	0.927
GDF-15	0.764	0.062	0.001	0.641	0.886
IL-6	0.657	0.081	0.052	0.498	0.816
Creatinine	0.626	0.078	0.118	0.474	0.779
NT-proBNP	0.670	0.066	0.036	0.541	0.799
6MWD	0.752	0.058	0.002	0.638	0.866

Table 5.2 – ROC area under the curve values for biomarkers and 6MWD in 2-year mortality analysis

Variables	Cut-off	Sensitivity	Specificity
RDW (%)	15.7	82.4%	78.6%
GDF-15 (pg/ml)	779	88.2%	69.6%
IL-6 (pg/ml)	2.5	82.4%	35.7%
Creatinine	80.5	85.7%	34.6%
NT-proBNP (fmol/ml)	491	82.4%	48.2%
6MWD (m)	322	88.2%	60.7%

Table 5.3 – Cut-offs derived from ROC curve survival analysis. Cut-offs were selected by a) maximum combined sensitivity and specificity and b) sensitivity > 80%

All five circulating biomarker cut-offs significantly predicted survival over time (Figure 5.5) stratifying low and high risk patient groups (all $p < 0.05$).

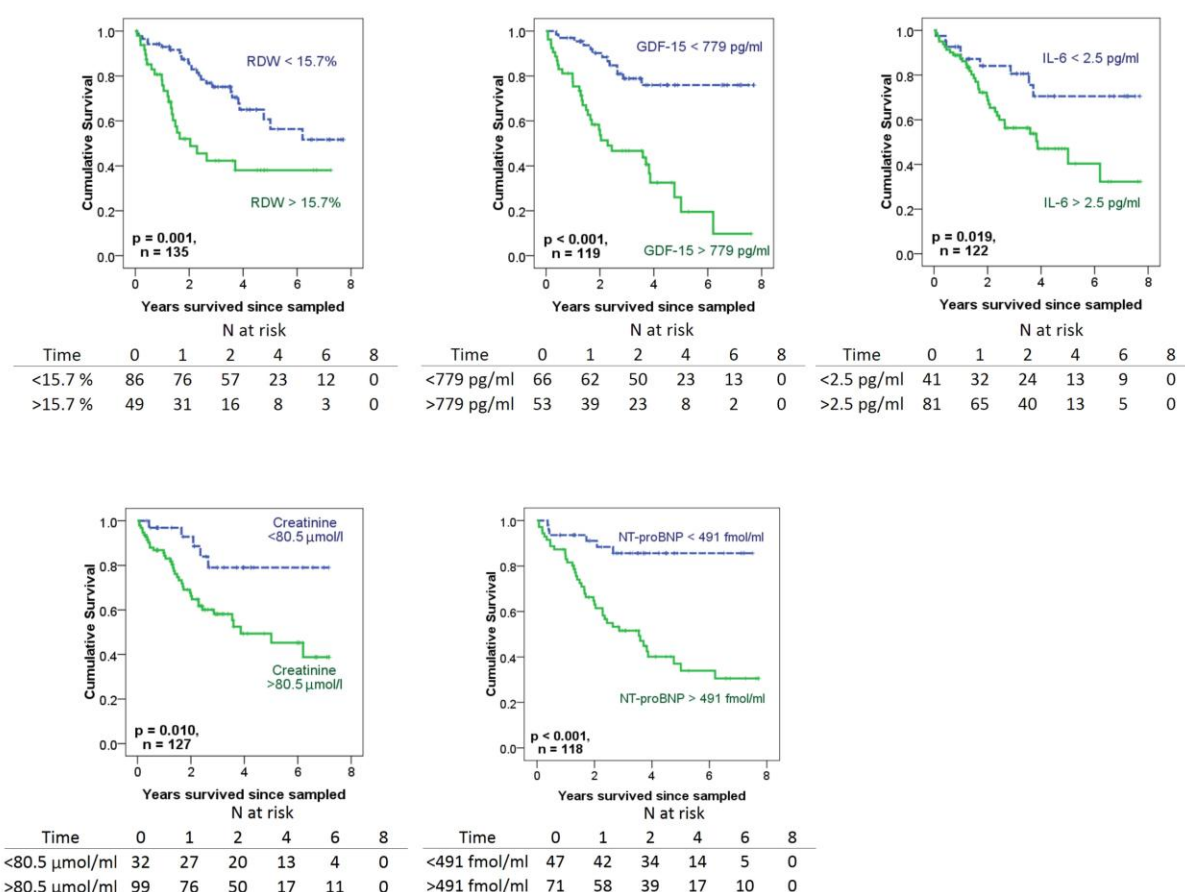


Figure 5.5 – Kaplan-Meier survival curves displaying survival estimates in patients with IPAH, stratified by biomarker levels above and below ROC-derived values. Tables indicate patient numbers at risk in subgroups.

Clinical and haemodynamic parameters and outcomes in IPAH

ROC curve analysis of baseline haemodynamic data, along with functional class and 6MWD data coincident with blood sampling performed in the same manner as for the biomarkers above. Cut-off values were derived for haemodynamic measurements including mean pulmonary artery pressure (mPAP, 57.5 mmHg), pulmonary vascular resistance (PVR, 10.9 Woods units) and cardiac index (1.87 l/min/m²). These cut-off values significantly predicted survival over time in patients with IPAH (all p<0.05, Figure 5.6). A cut-off of 322 m for 6MWD also significantly predicted survival over time (p<0.0001) and the two arms further separated into quartiles of 6MWD were also analysed to illustrate the powerful prognostic information provided by this simple clinical measure (Figure 5.6). The four WHO functional classes also significantly distinguished higher mortality risks in patients with IPAH as functional class deteriorated (both p<0.01, Figure 5.6).

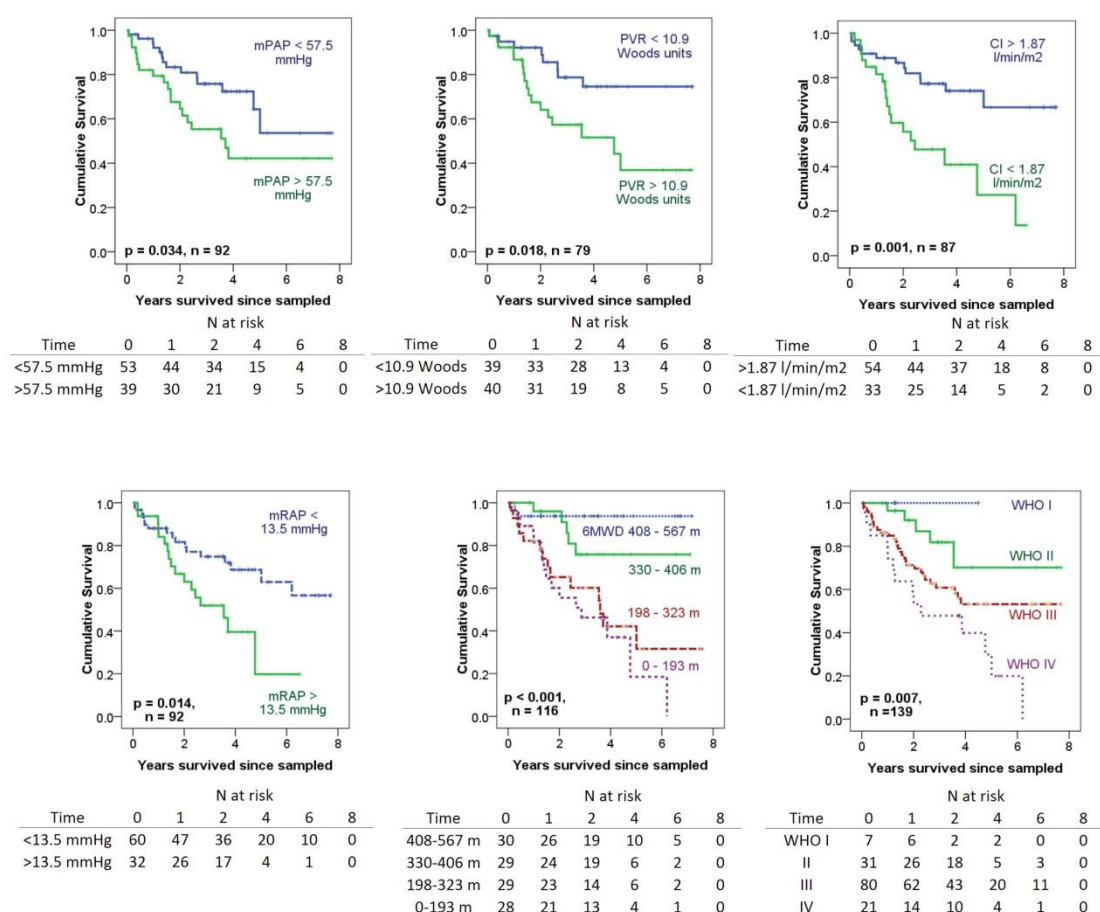


Figure 5.6 – Kaplan-Meier survival curves displaying survival estimates in patients with IPAH, stratified by haemodynamic and clinical parameters. Cut-offs were ROC-derived, and 6MWD is represented by quartiles. Tables indicate patient numbers at risk in subgroups.

Identification of independent prognostic markers in IPAH

Cox regression survival analysis was performed to identify prognostic markers from the variables measured in this study (Table 5.4). Time since diagnosis and the use of PAH-targeted therapies and therapy type were included as parameters to capture the effects of therapy and disease progression. Age, WHO class, 6MWD, time since diagnosis and all five biomarkers measured were identified as significant prognostic markers in IPAH (all $p < 0.05$). All parameters with $p < 0.1$ were entered into multivariable modelling for completeness. RDW, NT-proBNP and 6MWD were identified as significant, independent prognostic markers in IPAH (all $p < 0.01$, Table 5.4). No other measurements could add significant prognostic information to this model. The prognostic value of RDW was still evident in combination with either haemoglobin or mean cell volume and both RDW and IL-6 were independently prognostic when combined with creatinine (Table 5.5).

Variables	Single variable models		Multivariable model	
	Hazard ratio (95% CI)	Sig.	Hazard ratio (95% CI)	Sig.
Age, 10y	1.345 (1.106 - 1.635)	0.003		0.8
Gender	1.245 (0.677 - 2.288)	0.48		
Mean pulmonary artery pressure, mmHg	1.015 (0.984 - 1.046)	0.35		
Pulmonary capillary wedge pressure, mmHg	0.931 (0.813 - 1.065)	0.30		
Mean right atrial pressure, mmHg	1.058 (1.001 - 1.117)	0.045		0.4
ln[Cardiac index, l/min/m ²]	0.377 (0.142 - 1.006)	0.051		0.7
Pulmonary vascular resistance, Woods units	1.041 (1.000-1.085)	0.053		0.4
WHO at sampling	2.143 (1.383 - 3.320)	0.001		0.2
Six minute walk, 100m	0.637 (0.519 - 0.782)	<0.001	0.714 (0.569 - 0.896)	0.004
Therapies	0.919 (0.649 - 1.301)	0.63		
Treated vs. untreated	0.590 (0.328 - 1.062)	0.078		0.1
Years since diagnosis sampled	1.039 (1.016 - 1.062)	0.001		0.1
Red cell distribution width, %	1.142 (1.054 - 1.238)	0.001	1.274 (1.147 - 1.416)	<0.001
ln[Growth differentiation factor, pg/ml]	1.961 (1.401 - 2.743)	<0.001		0.3
ln[Interleukin-6, pg/ml]	1.652 (1.286 - 2.123)	<0.001		0.3
ln[Creatinine, µmol/L]	14.52 (4.256 - 49.52)	<0.001		0.4
square root[N-terminal pro-brain natriuretic peptide, fmol/ml]	1.038 (1.018 - 1.058)	<0.001	1.044 (1.020 - 1.069)	<0.001

Table 5.4 – Cox regression survival analysis of all biomarkers measured in IPAH patients.

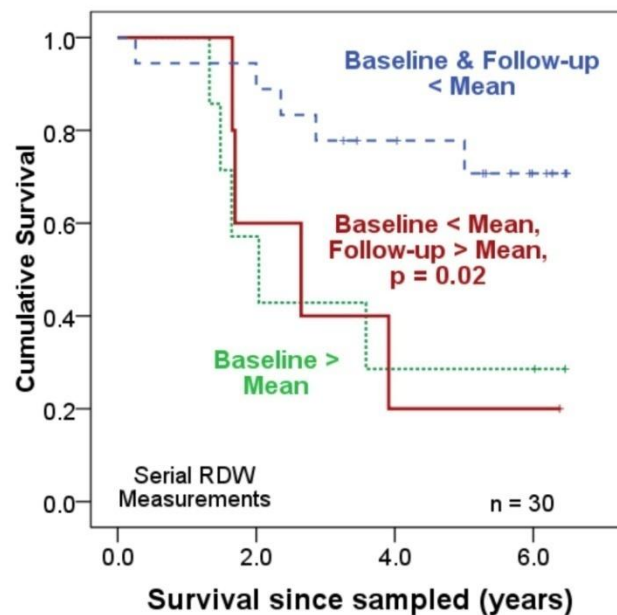
Individual variables associations with survival were first analysed. Variables with p<0.1 were then further analysed in a multivariable approach to identify independent prognostic variables.

Variables	Hazard ratio	95.0% CI for HR		Sig.
		Lower	Upper	
<i>RDW versus haemoglobin</i>				
RDW	1.146	1.051	1.250	0.002
Hb	0.901	0.796	1.020	0.100
<i>RDW versus mean red cell volume</i>				
RDW	1.278	1.146	1.424	0.00001
MCV	1.087	1.023	1.154	0.007
<i>RDW versus creatinine</i>				
RDW	1.140	1.047	1.241	0.002
ln[Creatinine]	13.35	3.855	46.22	<0.001
<i>IL-6 versus creatinine</i>				
ln[IL6]	1.498	1.147	1.956	0.003
ln[Creatinine]	6.304	1.560	25.47	0.010

Table 5.5 – Cox regression analyses showing biomarker independence from potential confounders

Serial RDW measurements improve risk stratification

RDW values were determined for a subset of the IPAH patients who consented for follow-up studies at standard clinical appointments over 3 month - 2 year periods. Patients whose RDW levels were below the mean (15.15%) of this subgroup at baseline and follow-up had a relatively good prognosis for this condition. Patients whose levels were high (above the mean) at baseline had a poor prognosis, irrespective of the follow-up measurement. Patients whose levels started “low” (i.e. below the mean) but then rose over time to become “high” (i.e. above the mean) had, as a group, significantly poorer survival than those who stayed low (Figure 5.7).



	N at risk				
Time	0	2	4	6	8
Both < mean	18	16	12	5	0
Baseline < mean, follow-up > mean	5	3	1	1	0
Baseline > mean	7	4	2	2	0

Figure 5.7 – Serial RDW measurements and risk stratification in patients with IPAH. A subset of patients (n=30) had serial measurements of RDW. Statistic shown is log rank test versus baseline & follow-up < mean. Mean = 15.15%.

5.4 Discussion

Several novel biomarkers have been identified as prognostic in IPAH (Hampole et al. 2009; Kumpers et al. 2010; Nickel et al. 2008; Soon et al. 2010) but rarely validated in a second cohort or directly compared in a multiple biomarker analysis. I have measured and compared levels of RDW, GDF-15 and IL-6 as well as NT-proBNP and creatinine with established clinical and baseline haemodynamic data. RDW was the strongest performer in stringent comparative statistical interrogations (ROC and Cox regression survival analyses), leading to its identification as an independent predictor of survival, even when measured in combination with 6MWD, NT-proBNP and other clinical indices.

RDW is routinely measured as part of a full blood count but has only recently emerged as a predictor of cardiovascular disease and mortality (Felker et al. 2007; Hampole et al. 2009; Patel et al. 2010; Tonelli et al. 2008). It is used to distinguish anaemias, and is raised in iron-deficiency anaemia. Iron status is thought to be important in the regulation of pulmonary vascular tone (Smith et al. 2009), and significant iron deficiency has been demonstrated in IPAH (Ruiter et al. 2010). RDW may also be raised due to haemolysis, but contemporaneous haemoglobin and haptoglobin (low levels indicate haemolysis) measurements did not correlate with RDW in this study and the prognostic value of RDW was not lost when combined with measurements of haemoglobin or mean cell volume. In heart failure, RDW levels are thought to represent a combination of chronic inflammation, dysfunctional erythropoiesis, kidney dysfunction, oxidative stress and nutritional state (Forhecz et al. 2009; Zalawadiya et al. 2010). For these reasons, RDW may reflect many factors relevant to the prognosis of PAH and hence provide additional prognostic information to NT-proBNP, while outperforming more specific markers such as IL-6, creatinine and GDF-15.

A variety of stimuli can induce GDF-15 in several different tissues (Clerk et al. 2007; Frank et al. 2008; Hsiao et al. 2000; Zimmers et al. 2005). Circulating levels may reflect the function of several organs and complement NT-proBNP measurements in predicting survival in IPAH (Nickel et al. 2008). Conversely, the prognostic utility of GDF-15 in a variety of cardiovascular diseases which stress the myocardium (Kempf et al. 2007; Khan et al. 2009; Lankeit et al. 2008; Wollert et al. 2007) suggests cardiomyocytes could be the predominant source of the protein. The fact that GDF-15 levels in IPAH patients correlated with cardiac index and mean right atrial pressure supports this argument, though other tissue sources cannot be excluded. GDF-15 is also sensitive to iron status (Lakhal et al. 2009) and this may explain in part its correlation with RDW values. The potential importance of the right ventricle and iron status in determining GDF-15 levels in these patients may partially explain why

this biomarker did not emerge as an independent predictor of survival after adjustments for other factors, including NT-proBNP and RDW.

Inflammation is a component of pulmonary hypertension and IL-6 in particular has been linked to the development of pulmonary hypertension in experimental models (Miyata et al. 1995; Savale et al. 2009; Steiner et al. 2009). Injections of IL-6 lead to development of PH in rats, which was reduced by anti-IL-6 antibodies (Miyata et al. 1995). IL-6 knockout mice develop less pulmonary vascular remodelling in response to 2 weeks chronic hypoxia (Savale et al. 2009), whereas IL-6 overexpression increases muscularisation of the proximal arterial tree, an effect which can be enhanced by chronic hypoxic exposure (Steiner et al. 2009). Levels of cytokines predict survival in IPAH (Soon et al. 2010). In both that study and in this chapter, levels of IL-6 above the normal range for the assay were associated with mortality, indicating that the presence of an inflammatory component in IPAH can be indicative of poor survival. However, levels do not predict survival once exercise capacity and NT-proBNP/RDW measurements have been taken into consideration. This suggests that while IL-6 measurements may be of future use in determining whether anti-inflammatory treatment strategies are appropriate in individual patients, they have limited additional clinical utility in stratifying patient risk over and above currently-accepted markers.

Renal failure is an indicator of a poor prognosis in pulmonary hypertension (Leuchte et al. 2007) and serum creatinine measurements have shown that even mild renal dysfunction is associated with poorer survival in this disease (Shah et al. 2008). Renal function is also related to haemodynamics as decreased arterial perfusion (i.e. cardiac output) in the kidneys and increased renal vein pressure (i.e. right atrial pressure) lead to decreased glomerular filtration rates in PAH. Indeed, significant correlations were found between serum creatinine levels and both right atrial pressures and cardiac index as well as NT-proBNP. Increased creatinine levels were also associated with a significantly poorer survival. However, it did not predict survival independently of RDW, NT-proBNP and 6MWD, and this may reflect the close relationship between renal and cardiac function, captured by NT-proBNP measurements.

In a recent study of cytokines in PAH (Soon et al. 2010), haemodynamic measurements performed poorly as prognostic markers. Consistent with other studies (McLaughlin et al. 2002; Sitbon et al. 2002), I found significant prognostic utility in these measures, though none was independent in the final modelling. This also agrees with the NIH registry data that showed prognostic power of mean pulmonary artery and right atrial pressures and cardiac index (D'Alonzo et al. 1991).

Biomarkers with different tissue origins that inform on different signalling pathways may give a more complete insight into disease pathology and perform better in stratifying disease when used in combination than when used alone (Nozaki et al. 2009; van Kimmenade et al. 2006). Hampole *et al.* (Hampole et al. 2009) found that NT-proBNP added little to RDW in a population of patients with pulmonary hypertension of mixed aetiology, whereas I found the combination gave higher predictive value. The relatively long follow-up period in this study, mean 4.0 ± 2.4 years versus 2.1 ± 0.8 years in the study by Hampole *et al.*, and the fact that only patients with IPAH were studied may provide more power to detect contributions by different covariates. The inclusion of clinical measures such as 6MWD and WHO class may have also altered the relative contributions of these markers in our model. The power of RDW to improve survival predictions in combination with established measures, along with the fact RDW is already established as a standard clinical assay, suggests it may be the easiest and most useful test to add to clinical practice in the management of IPAH.

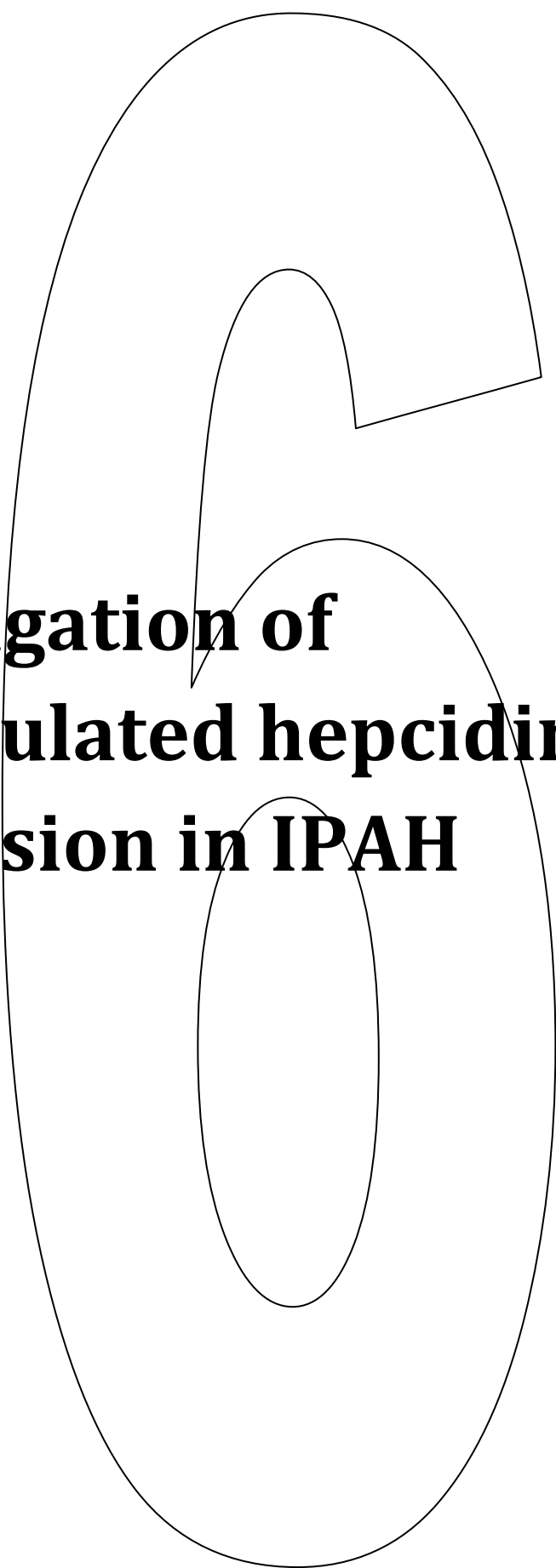
Serial BNP measurements have previously been shown to add prognostic value in cohort studies (Nagaya et al. 2000), but there is considerable inter-individual variation in response, which compromises the use of serial BNP measurements to monitor individual patient response to treatment. RDW predicted survival independently of NT-proBNP, suggesting these markers could be used in combination with NT-proBNP to improve risk estimates, thus improving the detection of high risk cases over that seen with NT-proBNP alone. Serial measurements of all three biomarkers could provide further information as to their value in monitoring response to therapy.

A strength of this study is that it is hypothesis-driven and independently validates several of the novel biomarkers currently of interest in IPAH, comparing them not only with each other but also the more established NT-proBNP, and clinical and haemodynamic parameters. Future studies assessing the usefulness of novel prognostic biomarkers should compare their value against other potential candidates as well as established measures. The number of subjects (139) and events (49) analysed in this study compares favourably with previously published biomarker studies in this condition (Hampole et al. 2009; Kumpers et al. 2010; Nickel et al. 2008; Soon et al. 2010). To maximise homogeneity of data, only IPAH patients were selected as other presentations of pulmonary hypertension are associated with differing prognoses (McLaughlin et al. 2004). Neither treatment status nor time since diagnosis proved to be independently related to survival. This emphasises that it is the response to therapy, which can be captured through clinical observations and biochemical measurements, rather than the presence of therapy itself which is important in determining patient prognosis. The majority of patients in this study were WHO class III or above and therefore these results are most relevant to severe disease. A larger patient population with

multiple repeated sampling would be required to assess the potential influence of different drug classes on biomarker levels. It would also provide a stronger foundation for the use of these markers in the day-to-day management of individual patients.

Conclusions

RDW, GDF-15, IL-6 and NT-proBNP are all related to disease severity and may be used to predict survival in patients with IPAH. RDW added significant prognostic value to measurements of NT-proBNP and exercise capacity and therefore could be adopted in a multiple biomarker approach to disease monitoring.



Investigation of dysregulated hepcidin expression in IPAH

Chapter 6 – Investigation of dysregulated hepcidin expression in IPAH

6.1 Introduction

In chapter 4, I identified increased levels of hepcidin expression both overall and in relation to iron status in IPAH versus healthy controls. Increased hepcidin expression underlies the anaemia of chronic disease (Weiss & Goodnough 2005), emphasising the importance of hepcidin in regulating not just local but systemic iron status. Therefore the high hepcidin levels in IPAH may be in part responsible for the iron deficiency seen in these patients. In this chapter, I will explore the regulation of hepcidin in IPAH using *in vitro* studies, plasma samples from PAH patients and animal models of PH.

Bone morphogenetic protein signalling and hepcidin

The mechanisms of iron sensing are not well understood, but it has become apparent that BMP signalling has a critical role in controlling hepcidin expression. The BMPs are a multifunctional family of proteins which act through binding to heteromeric combinations of type 1 and type 2 receptors, leading to the activation (phosphorylation) and nuclear translocation of receptor-activated Smads (Smad1/5/8) and the common Smad4 (Goumans et al. 2009). BMP-6 has emerged as the principal BMP regulating hepatic hepcidin expression *in vivo*. BMP-6 knockout mice display severe iron overload and reduced hepcidin expression (Andriopoulos, Jr. et al. 2009; Meynard et al. 2009; Niederkofler et al. 2005; Wang et al. 2005). This pathway is of particular interest in PAH as BMPR2 expression is reportedly reduced in IPAH patients and loss of function BMPR2 mutations have been linked to >70% of heritable and ~20% of idiopathic cases of PAH (Atkinson et al. 2002; Machado et al. 2009). Levels of BMP-6 itself also track dietary iron availability in mice and BMP-6 binds the BMP co-receptor hemojuvelin, mutations in which cause iron overload due to decreased hepcidin expression in humans (Niederkofler et al. 2005). The BMP signalling inhibitor dorsomorphin prevents hepcidin induction by iron *in vivo*, as does soluble hemojuvelin (Yu et al. 2008). In human liver, BMP-6 signalling is postulated to involve ActRIIA rather than BMPR2 receptors (Xia et al. 2008b), but downregulation of BMPR2 can increase BMP-6 and -7 signalling through ActRIIA (Yu et al. 2005). Therefore, I hypothesised that dysfunctional BMPR2 signalling could lead to increased expression of hepcidin, as seen in patients with IPAH (Figure 6.1).

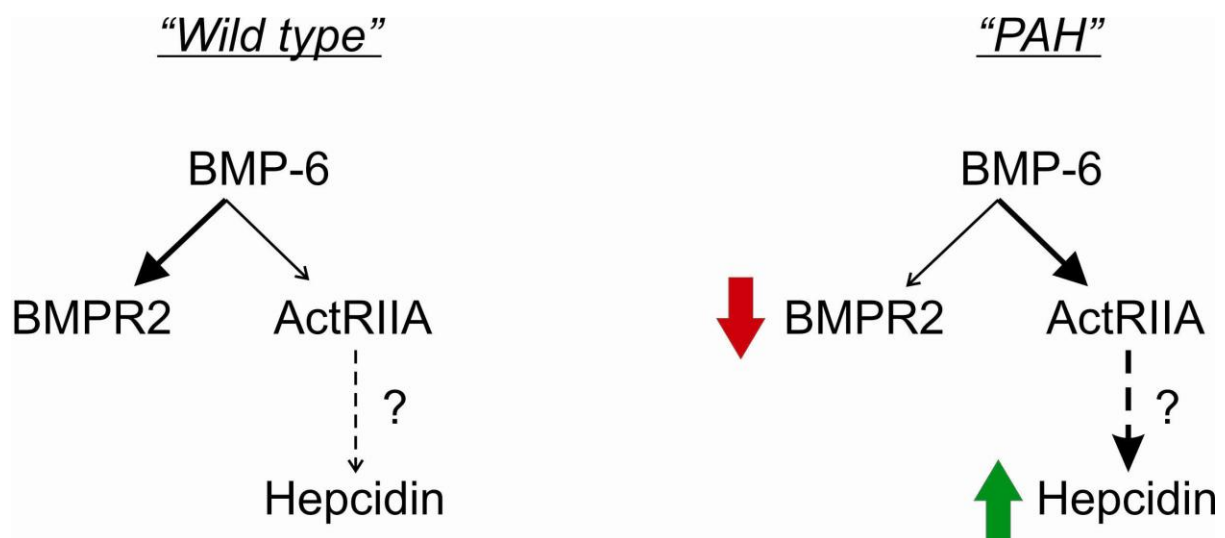


Figure 6.1 – Working hypothesis for link between dysregulation of BMPR2 and increased hepcidin expression. BMP-6 is thought to be important in regulating hepcidin levels. BMP-6 acts through the type II BMP receptors BMPR2, ActRIIA and ActRIIB. Downregulation of pulmonary BMPR2 expression is found in IPAH and in pulmonary vascular cells leads to increased ActRIIA activity in response to BMP-6. One study has suggested ActRIIA is the most important in the regulation of hepcidin and therefore increased activity through this receptor might be expected to lead to increased hepcidin expression.

BMPR2 is highly expressed in lung and heart tissue but its detection in liver tissue has been inconsistent (Kawabata et al. 1995; Nohno et al. 1995; Rosenzweig et al. 1995; Xia et al. 2008b). The potential importance of disrupted BMPR2 signalling on hepatic activity is unclear without evidence of BMPR2 protein expression in liver tissue. Other tissues may also represent a significant source of hepcidin under pathological conditions. For example, bacterial lipopolysaccharide treatment induces significant hepcidin expression in airway macrophages (Nguyen et al. 2006) and chronic hypoxia leads to increased hepcidin production in cardiac tissue whereas hepatic expression was reduced (Merle et al. 2007). Local as well as systemic hepcidin production and iron regulation may be important in PAH.

I set out to investigate further the possible mechanisms and tissue involvements in the dysregulation of hepcidin in IPAH. To establish the importance of BMP signalling in hepcidin regulation, I performed experiments using siRNA-mediated knockdown of type II BMP receptors in a human hepatocellular carcinoma cell line (HepG2). I also analysed circulating levels of BMP-6 which is directly implicated in hepcidin regulation and assessed iron status in a small number of heritable PAH cases. I then analysed the mRNA expression of two iron-regulatory proteins (hepcidin and

ferroportin) in tissues from animal models of PH, lung tissue from IPAH and lobectomy patients and human pulmonary vascular cells. Finally, to determine whether results from HepG2 experiments could be replicated *in vivo*, I analysed hepcidin expression in liver tissue from BMPR2 heterozygous mice.

6.2 Methods

Patient samples

Plasma samples from 97 IPAH patients and 71 healthy volunteers as controls were used to determine BMP-6 levels in this chapter. Subject characteristics are shown in Table 6.1.

Treatment	Controls (n=71)		Naive (n=40)		Treated (n=57)	
	Value	Variation (SD or IQR)	Value	Variation (SD or IQR)	Value	Variation (SD or IQR)
Age at sampling	35.6	10.1	51.5	16.1	44.5	16.5
Sex - F	31		28		40	
M	40		12		17	
WHO at sampling - I			3		2	
II			6		12	
III			24		32	
IV			7		11	
6 minute walk at sampling, m			309.5	154.7	294.6	169.4
Days since first seen sampled			83†	8-755	608†	324-1208
<i>PAH-targeted therapies</i>						
Bosentan			-		33	
Sildenafil			-		15	
Treprostinil			-		7	
Diltiazem			-		2	
<i>Baseline haemodynamic data from cardiac catheter</i>						
Mean pulmonary artery pressure, mmHg			53.2	11.9	55.6	13.2
Right atrial pressure, mmHg			9.2	4.7	11.0	6.4
Pulmonary capillary wedge pressure, mmHg			13.6	6.2	8.0	3.3
Cardiac index, L/min/m ²			2.39†	1.87-3.27	2.07†	1.59-2.65
Pulmonary artery O ₂ saturation, %			69.3	12.6	62.0	10.8
Pulmonary vascular resistance, Woods units			10.5†	5.2-14.0	10.8†	7.3-18.4

Table 6.1 – Subject characteristics of IPAH cases and controls. † indicates variable is not normally distributed and therefore is presented as median with IQR.

Five heritable PAH cases used to investigate iron status in HPAH are described in Table 6.2.

Sample	Age	Sex	mPAP, mmHg	CO, L/min	PVR, Woods	PAH Treatment	6 min walk, m	WHO class
HPAH1	35	F	na	na	na	Sildenafil	na	na
HPAH2	37	F	79	na	31.4	Treprostinil	na	IV
HPAH3	36	F	52	2.36	17.4	Sildenafil	372	na
HPAH4	20	M	na	na	6.0	Nicardipine + Sildenafil	528	II
HPAH5	35	M	na	na	na	-	na	na

Table 6.2 – Subject characteristics of heritable PAH cases. mPAP, mean pulmonary artery pressure; CO, cardiac output; PVR, pulmonary vascular resistance. na=not available

Animals

The expression of hepcidin and ferroportin was analysed in tissues from rats exposed to chronic hypoxia as described in Chapter 2. A time course of baseline, 2 days, 1 week, 2 weeks and 3 weeks hypoxia was used to determine the changes in levels of these molecules over time. 9 animals were used for normoxia controls, 6 for 3 weeks hypoxia and 3 at all other time points.

Isolation of primary hepatocytes

The primary hepatocytes isolation protocol was developed from published reports (Berry & Friend 1969; Truksa et al. 2006). The hepatocytes were isolated by perfusion of the liver with 0.05% collagenase (Sigma) in Hank's Buffered Saline solution (Sigma), single cells isolated with a 70-µm nylon filter (VWR) and allowed to attach to collagen-coated (5 µg/cm², BD Biosciences) plates overnight. Cells were seeded at 6x10⁵ to 6x10⁶ per well in 6-well plates. DMEM medium (containing 10% FBS) was then refreshed at 24h and 48h. At 48h BMP-6 (0-100 ng/ml) or PBS was added and mRNA was extracted after 16 h incubation.

Cell culture

HepG2 (human hepatocellular carcinoma) cells (LGC Standards, Teddington, Middlesex, UK) were seeded at 5×10^5 cells per well in 6-well plates in minimum essential medium (MEM, Sigma-Aldrich) containing 10% FBS and antibiotics/antimicrobials. siRNA transfections were performed the following day in OPTI-MEM medium, using Oligofectamine (Invitrogen, Paisley, UK) and siRNA duplexes that targeted a scrambled sequence or the type II BMP receptors BMPR2, ActRIIA and ActRIIB (Invitrogen). Three days post-transfection, cells were treated with vehicle or 10 ng/well recombinant human BMP-6, IL-6 or TNF- α (R&D Systems). Protein was extracted in radio-immuno-precipitation assay buffer (Sigma-Aldrich, Gillingham, Kent, UK). Pulmonary arterial endothelial cells and smooth muscle cells were obtained from PromoCell (Heidelberg, Germany) and cultured in the recommended endothelial cell and smooth muscle cell growth media with supplement mixes (PromoCell). RNA was extracted using the Trizol method (Invitrogen).

Immunoblotting

Immunoblotting was performed using a mouse monoclonal antibody for human BMPR2 at 1:400 dilution overnight at 4°C (BD Biosciences, Oxford, UK) followed by anti-mouse IgG (GE Healthcare, Bucks, UK) at 1:2000 for 1 hour at RT, as previously described (Upton et al. 2009).

RT-PCR

RNA was extracted in Trizol (Invitrogen) and reverse transcribed using ThermoScript RT-PCR kits (Invitrogen). 20ng cDNA product was used with 10 μ l of PCR MasterMix (Promega, Southampton, Hampshire, UK). Primer details are given in chapter 2.

BMP-6 measurements

BMP-6 levels were determined by ELISA (Insight Biotechnology, Wembley, UK) with a modification to the manufacturer's protocol incubating both samples and detection antibody overnight at 4°C. See Chapter 2 for more details on the optimization of this assay.

Data presentation and statistical analysis

Immunoblotting and RT-PCR results are presented as mean with bars for standard deviation, and were compared by ANOVA, with Tukey post-hoc analysis performed if $p < 0.05$. BMP-6 and hepcidin absolute values are shown in dot-plots, compared by t-test.

6.3 Results

Establishing an *in vitro* model of human hepcidin production

Human hepatocellular carcinoma HepG2 cells were used to study the regulation of hepcidin expression. HepG2 cells exhibit basal hepcidin expression, which can be further stimulated by IL-6 or BMP-6 treatment, as previously shown (Truksa et al. 2006; Yu et al. 2008), and inhibited by TNF- α treatment (Figure 6.2) as previously shown in monocytes (Zhang & Rovin 2010). Expression was normalised to actin. These data confirmed HepG2 cells as a viable model for studying hepcidin regulation at the mRNA level.

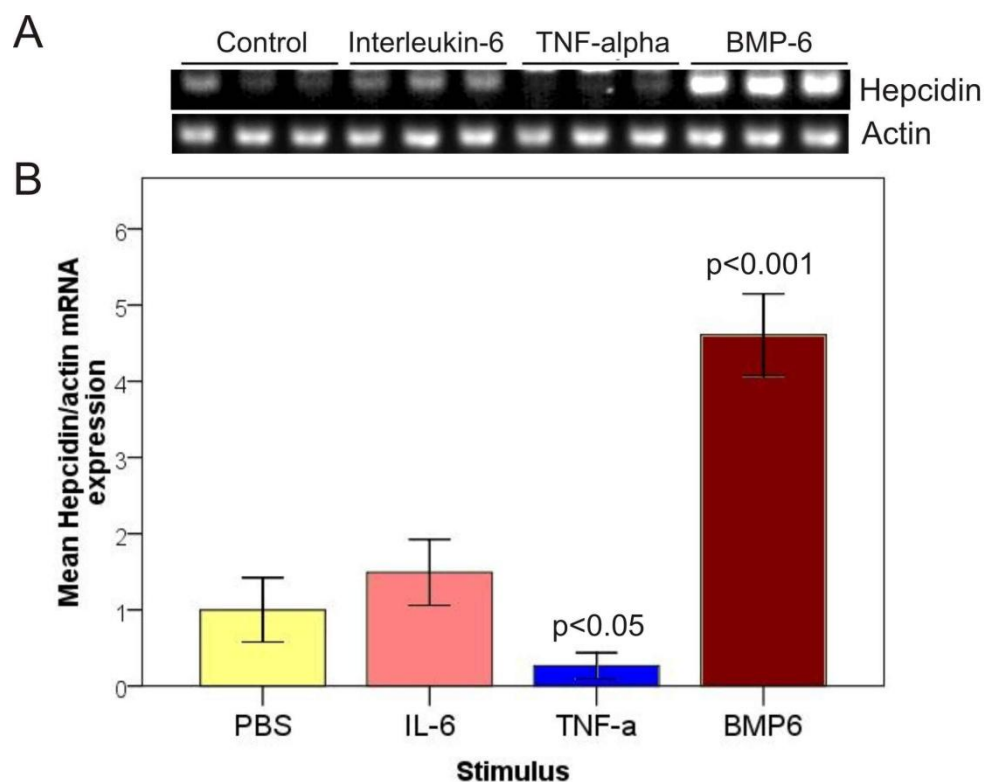


Figure 6.2– Establishing the experimental conditions for investigation of HepG2 hepcidin mRNA expression. A. HepG2 cells cultured in 10% foetal calf serum were treated with PBS or 20 ng/ml of IL-6, TNF- α or BMP-6 for 16h and then RNA was extracted by the Trizol method. RT-PCR results using specific hepcidin and actin (loading control) primers are shown above. B. Densitometry data from A. normalised to actin. Statistics shown are from Tukey post-hoc one way ANOVA analysis. N=6 replicates for each treatment.

To investigate the importance of the type II BMP receptors in regulating hepcidin, siRNA was utilised to knockdown expression of BMPR2 compared with type IIA and IIB activin ActRIIA and ActRIIB receptors. Significant reduction of mRNA expression 3 days following transfection was demonstrated (Figure 6.3A) for all 3 receptors (50-80% knockdown). Scrambled siRNA was used as a control. Successful reduction of BMPR2 protein expression (50-80% knockdown) at 3 to 4 days following transfection was confirmed by immunoblotting (Figure 6.3B&C).

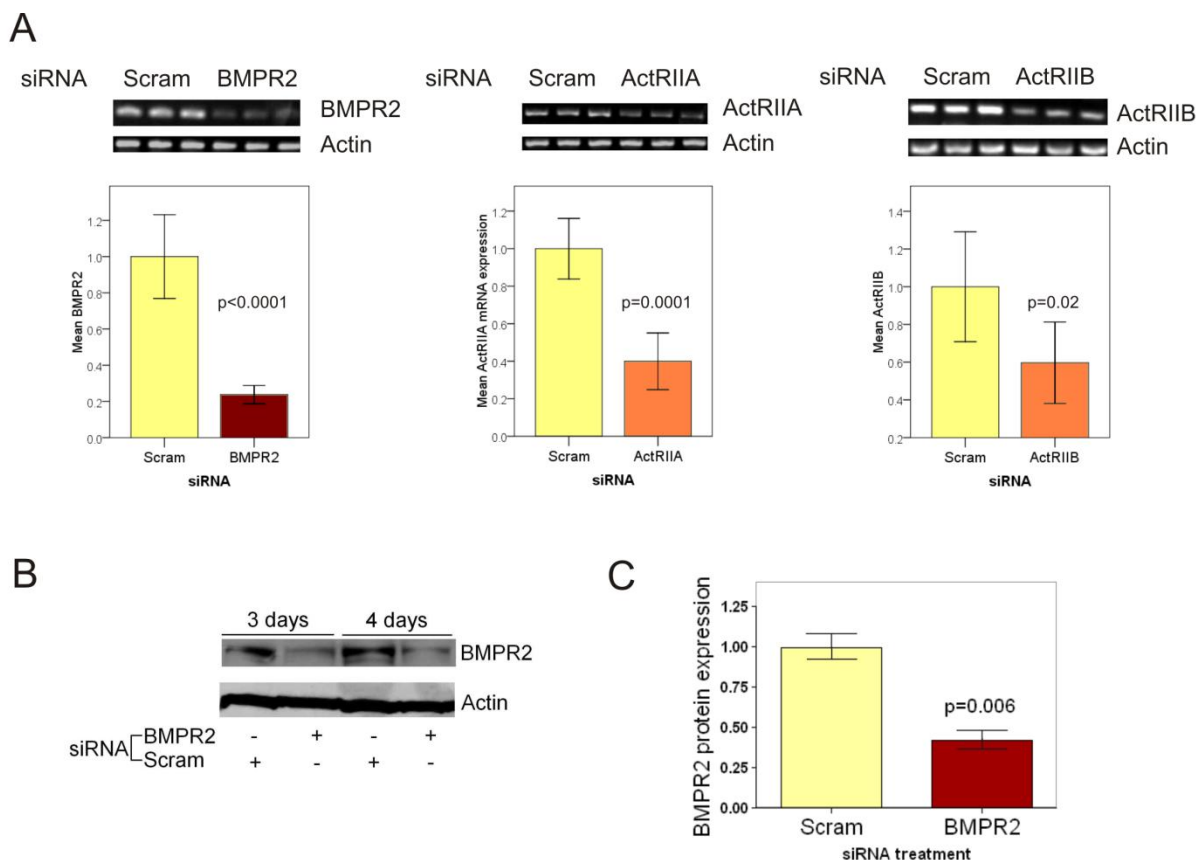


Figure 6.3 – Establishing siRNA-mediated knockout of type II BMP receptors. Pre-designed validated siRNA duplexes were transfected with Oligofectamine into HepG2 cells for 5-6 hours and cells were harvested 3-4 days after transfection. A. Type II BMP receptor mRNA expression levels in HepG2 cells 3 days after siRNA transfection. B. Analysis of BMPR2 protein expression over the treatment period. C. Densitometry measurements of B showing BMPR2 protein levels 3-4 days following transfection. Statistics shown are from t-tests. N=3 or more replicates in each comparison.

Hepcidin stimulation by BMP-6 is increased by BMPR2 knockdown

Basal expression of hepcidin was unaffected by knockdown of any of the 3 type II BMP receptors (Figure 6.4). However, the stimulation of hepcidin expression by BMP-6 was significantly augmented following BMPR2 knockdown ($p<0.001$), but not the knockdown of either activin receptor (all $p>0.8$). This effect was observed at both the mRNA (semi-quantitative, Figure 6.4B) and protein secretion (quantitative, Figure 6.4B) level.

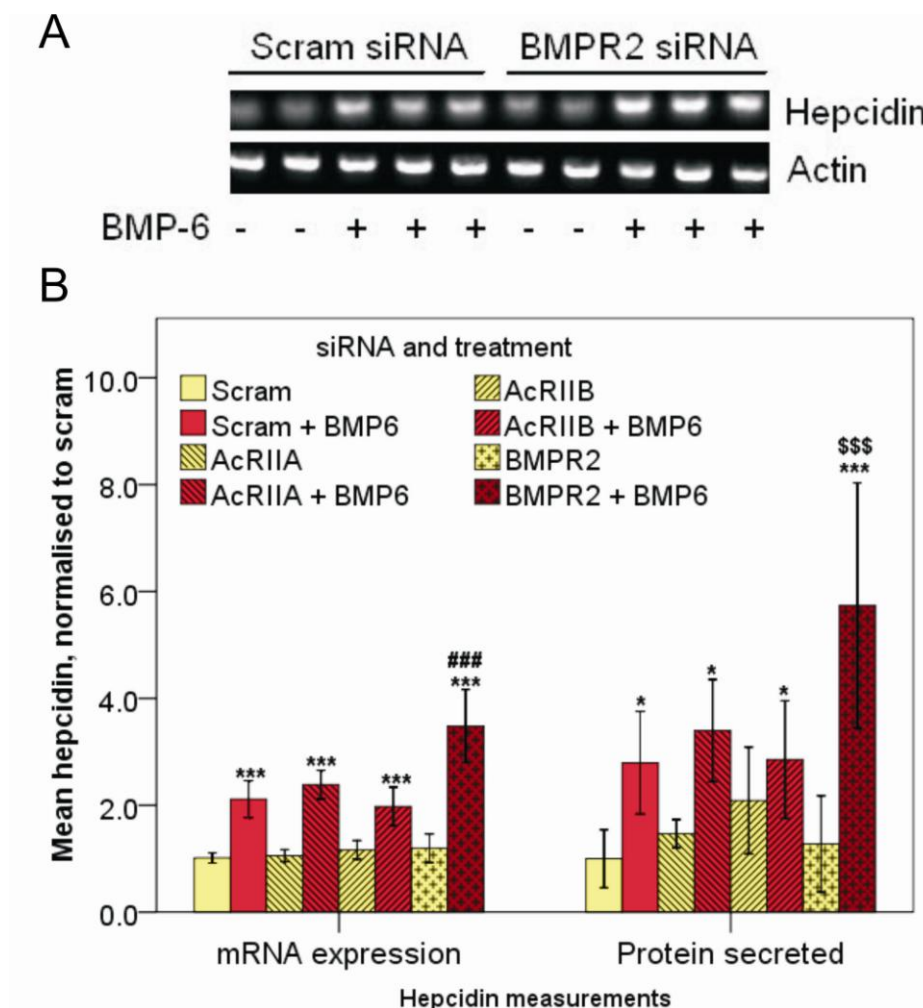


Figure 6.4 – Hepcidin expression in HepG2 cells following BMP type II receptor knockdown and BMP-6 treatment. A. RT-PCR data indicating hepcidin and actin expression following scramble/BMPR2 siRNA treatment followed by +/- BMP-6 treatment (10 ng/ml). B. Hepcidin mRNA expression and protein release from HepG2 cells treated with +/-BMP-6 following siRNA treatment. Results shown are from $n\geq 3$ experiments. ***, $p<0.001$ versus controls. ###, $p<0.001$ versus all other siRNA plus BMP-6 treatment groups. * $p<0.05$ versus scram baseline. \$\$\$, $p<0.001$ versus scram+BMP-6 and ActRIIB+BMP-6 and $p<0.05$ versus ActRIIA+BMP-6. Statistics from Tukey post-hoc analysis. Bars=Standard deviation.

Heritable PAH patients have very low circulating iron levels

To test the hypothesis that iron deficiency related to overexpression of hepcidin might be linked to BMPR2 deficiency in PAH, we analysed plasma samples taken from patients with heritable PAH, who have a more severe downregulation of BMPR2 expression than non-heritable IPAH patients (Atkinson et al. 2002). Of the 5 available patient samples, 3 had sTfR levels above the normal range, with the other 2 subjects at the upper end of the normal range (Table 6.3). The same 3 patients had very low ferritin levels (<30 µg/L) and all 5 patients had very low circulating iron levels, with 4 below the measurable range of the assay. Despite the presence of iron deficiency in these patients, all 5 maintained measurable hepcidin levels, although the 3 patients with the most significant iron deficiency did have hepcidin levels at the bottom end of the normal range. While very preliminary in their nature, these data suggest that heritable PAH patients also exhibit iron deficiency and inappropriate hepcidin expression, in the face of reduced circulating iron levels.

Sample	Hepcidin	Ferritin	Iron	sTfR
<i>Normal</i>	<i>2-55 ng/ml</i>	<i>30-300 µg/l</i>	<i>7-29 µmol/L</i>	<i>8.7-28.1nmol/L</i>
HPAH1	45	33	<0.9	21.4
HPAH2	3.6	7	<0.9	57.5
HPAH3	11.1	188	2.4	21.9
HPAH4	2.7	19	<0.9	36
HPAH5	4.5	8.6	<0.9	39

Table 6.3 – Iron status of heritable PAH patients. Plasma samples were collected from 5 patients with heritable PAH and analysed for iron status. Red highlighted cells indicate those outside the normal ranges.

BMP-6 does not correlate with hepcidin in IPAH plasma

Of the many BMPs, BMP-6 in particular is considered to be important in the regulation of hepcidin. To investigate this relationship in IPAH, circulating BMP-6 levels were measured in plasma samples from 71 healthy controls and 97 IPAH patients and related to hepcidin (Figure 6.5). Only around a third of both control and IPAH subjects had BMP-6 levels in the measurable range of the assay employed, with no apparent difference in the distribution of values between the two groups (Figure 6.5A). There was no difference in the hepcidin levels measured in the IPAH patients with or without detectable BMP-6 levels (Figure 6.5B). The range in BMP-6 levels measured was notable, with two apparent subgroups of both patients and controls with detectable or undetectable levels of BMP-6. No relationship between BMP-6 and hepcidin expression was found; IPAH cases with detectable BMP-6 did not correspond to those with raised hepcidin levels or vice versa.

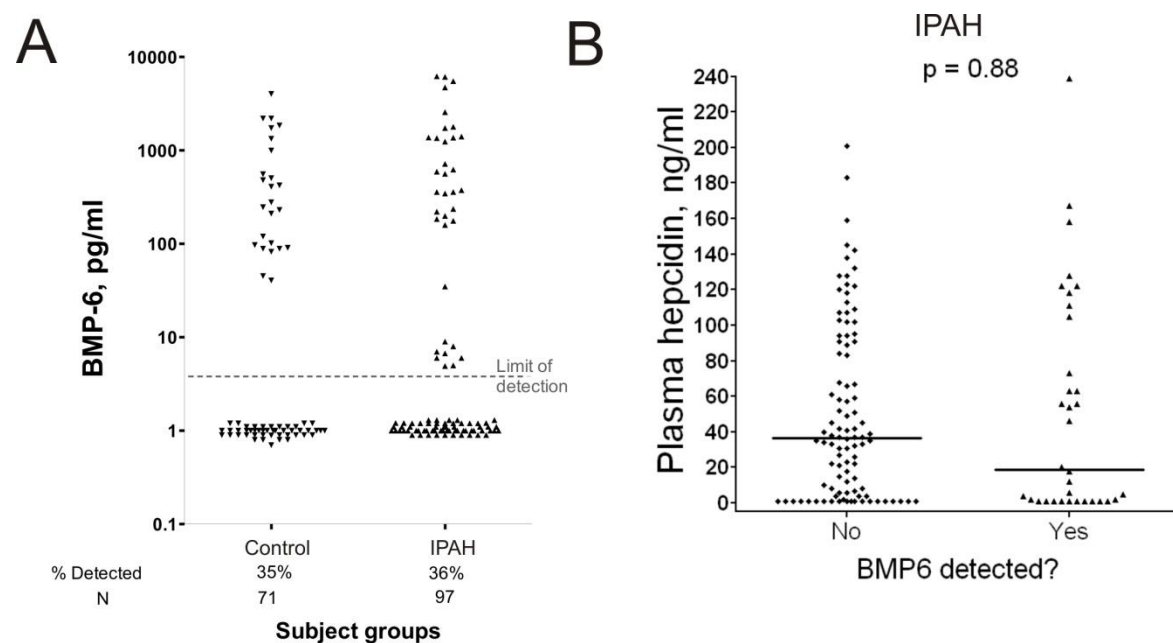


Figure 6.5 – BMP6 detection in plasma samples from controls and IPAH patients and its relationship to hepcidin levels in IPAH. A. BMP-6 levels were determined in plasma from 71 controls and 97 IPAH patients by ELISA. B. Plasma hepcidin levels in patients with IPAH stratified by detection of BMP6 in plasma. Statistic shown is from t-test.

BMPR2 protein cannot be detected in human and rat liver tissue

While significant mRNA expression of BMPR2 was seen in human liver tissues as well as HepG2 cells (Figure 6.6A), protein expression of BMPR2 in healthy human liver tissues could not be detected and has not been previously documented (Xia et al. 2008b). Immunoblotting of BMPR2, in the same liver tissue samples used to demonstrate mRNA expression, demonstrated very little BMPR2 protein when compared with HepG2 cell extracts (Figure 6.6B). In rat tissues, significant expression was observed in the lung and heart, with no detectable protein in liver samples. These data suggest that BMPR2 is differentially expressed between tissues, with relatively low expression in the liver compared with extra-hepatic sources such as the heart or lung.

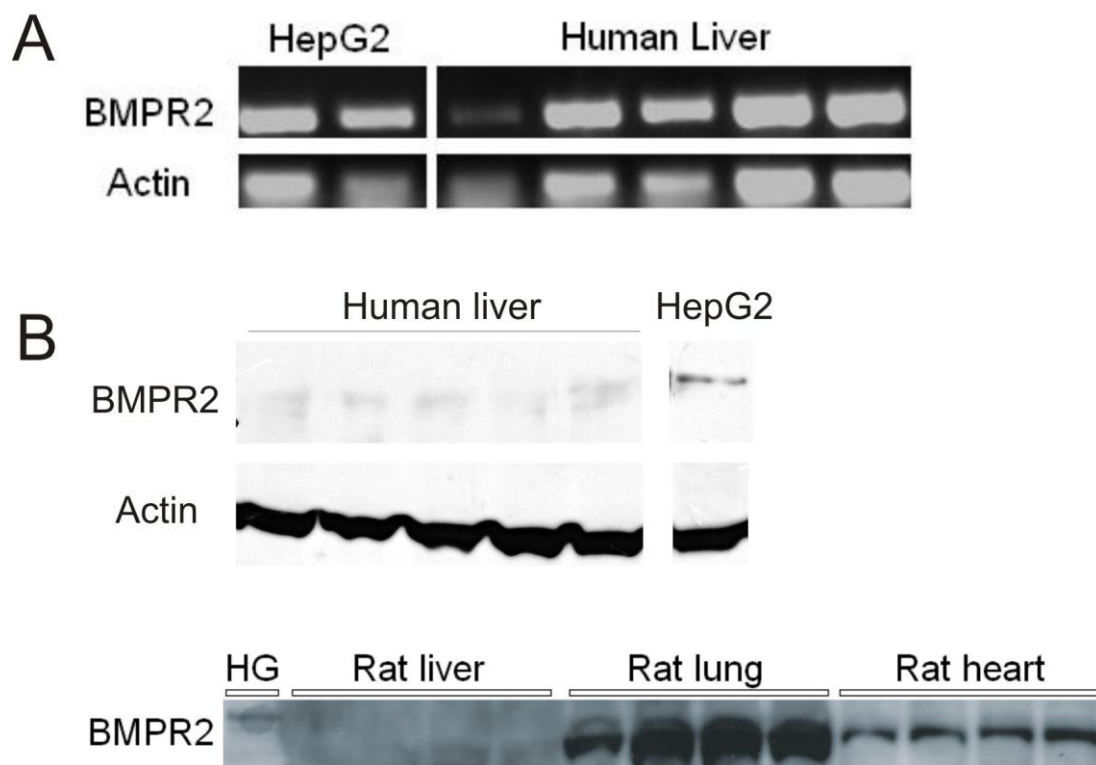


Figure 6.6 – BMPR2 mRNA and protein expression in human and rat tissues. A. BMPR2 and actin mRNA expression in HepG2 cells and human liver tissue homogenates. B. BMPR2 protein expression detected by immunoblotting in, human liver tissue homogenates, HepG2 cells, rat liver, lung and heart tissue homogenates. HG: HepG2 cells. Actin shows equal loading between HepG2 and human liver samples. Rat tissue samples were loaded equally from protein concentrations determined by the Bradford assay.

Pulmonary hepcidin and ferroportin expression increases during chronic hypoxia

To explore the potential importance of localised iron regulation in extra-hepatic tissues in PAH, mRNA expression of hepcidin and its target ferroportin was analysed in tissue samples from rats exposed to chronic hypoxia. Following 2 days hypoxia, pulmonary hepcidin mRNA was the same as baseline levels. At 1 and 2 weeks significant increases in hepcidin mRNA were seen ($p < 0.001$ and $p < 0.01$, respectively, Figure 6.7A&B) but no difference was seen at 3 weeks in hypoxia. Actin levels appeared to be higher in the 1 and 2 week hypoxic rat tissues but the increases in hepcidin were normalised to this and still highly significant. Conversely, after 3 weeks exposure to hypoxia, hepatic hepcidin mRNA expression appeared to be reduced by around 40% (Figure 6.7C&D). Liver tissue from other time points was not available.

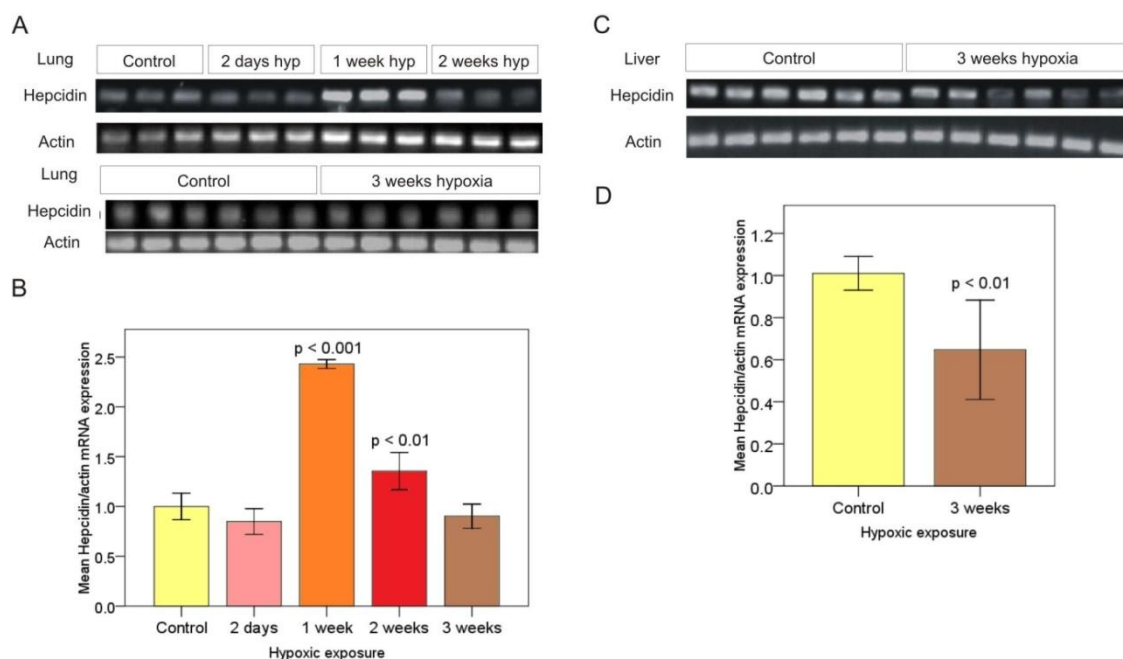


Figure 6.7 – Hepcidin mRNA expression in the lungs and livers of rats exposed to hypoxia.

A. Hepcidin mRNA expression in lung tissue samples from rats exposed to 10% hypoxia for 0 days (n=9), 2 days, 1 week, 2 weeks (all n=3) and 3 weeks (n=6). B. Hepcidin densitometry data from A. normalised to actin expression. Statistics from Tukey post-hoc analysis following one way ANOVA. C. Hepcidin mRNA expression in liver tissue samples from rats exposed to 10% hypoxia for 3 weeks. D. Hepcidin densitometry data from C. normalised to actin. Statistic is from a t-test.

Pulmonary ferroportin expression was significantly increased in rats following 2 and 3 weeks exposure to chronic hypoxia (Figure 6.8). These data suggest that during exposure to chronic hypoxia, hepcidin is transiently increased in the lungs of rats, which is followed by a later transient increase in ferroportin mRNA.

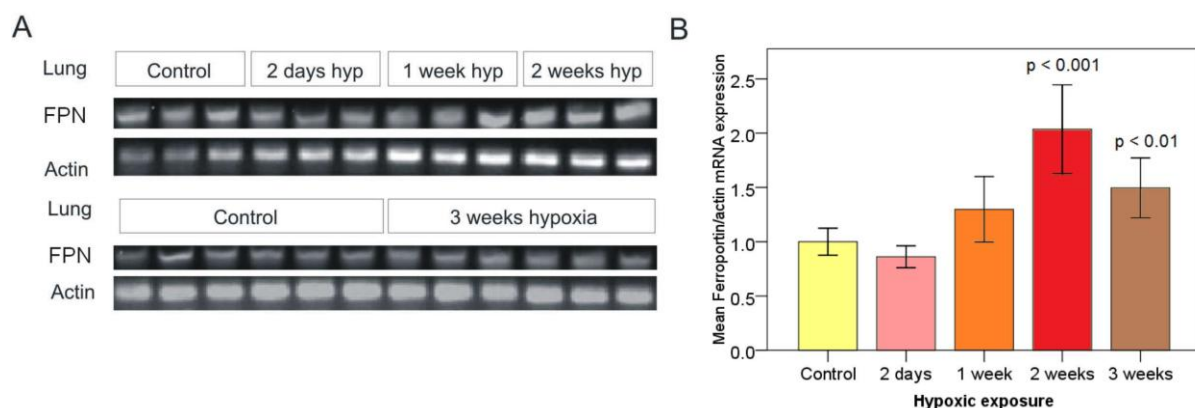


Figure 6.8 – Ferroportin mRNA expression in the lungs of rats exposed to hypoxia. A. Ferroportin mRNA expression in lung tissue samples from rats exposed to 10% hypoxia for 0 days (n=9), 2 days, 1 week, 2 weeks (all n=3) and 3 weeks (n=6). B. Ferroportin densitometry data from A. normalised to actin expression. Statistics from Tukey post-hoc analysis following one way ANOVA.

Hepcidin and ferroportin expression in IPAH lung tissue

To investigate whether the changes in hepcidin and ferroportin expression observed in hypoxic rat tissues might be replicated in IPAH lung, cDNA samples from 12 IPAH and 12 lobectomy lung tissue samples were analysed. Although variation in expression could be observed between samples, no significant difference in hepcidin or ferroportin expression was seen between the IPAH and lobectomy groups (Figure 6.9). This suggests that in end-stage IPAH, hepcidin and ferroportin levels are not abnormal at the whole lung tissue level, but the contribution of individual cell types is unclear. Hepcidin and BMP-6 expression was detected in pulmonary vascular cells, but contrary to

previous studies (Babitt et al. 2007) no BMP-6 was detected in the extract from HepG2 cells, though these data are preliminary in their nature (Figure 6.10).

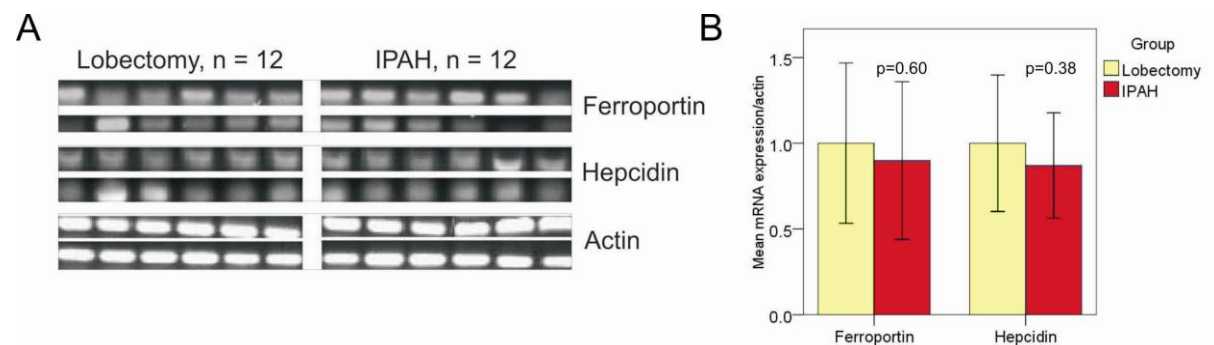


Figure 6.9 – Ferroportin and hepcidin mRNA expression in lobectomy and end-stage IPAH lung tissue samples. A. RT-PCR products visualised on gels using GelRed DNA stain. Actin is shown as a loading control. B. Densitometry data from A. normalised to actin. Statistics are from t-tests.

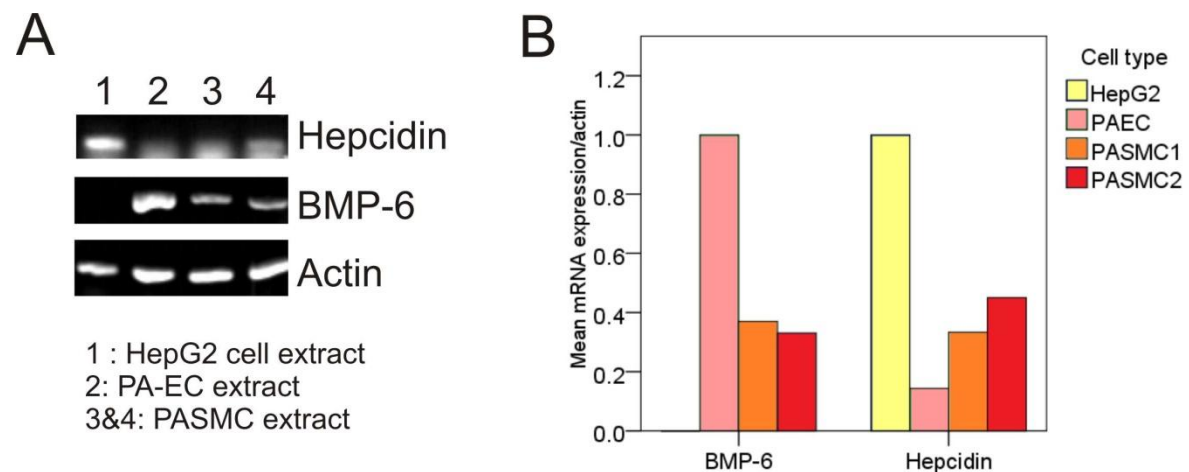


Figure 6.10 – Hepcidin and BMP-6 mRNA expression in pulmonary vascular cells. A. Human pulmonary arterial (PA) endothelial cells (ECs) and smooth muscle cells (SMCs) do express hepcidin and BMP-6 mRNA. HepG2 cells were used as positive controls for hepcidin expression. Actin is shown as a loading control. B. Densitometry data from A. normalised to actin. These data are preliminary (n=1 per measure).

Basal hepcidin expression in the livers of BMPR2 heterozygous mice

To investigate whether the increased hepcidin production in response to BMP-6 observed in BMPR2-knockdown HepG2 cells could be replicated in primary cells, hepatocytes were isolated from wild type and BMPR2 heterozygous mice. Despite maintaining viability in culture in preliminary studies, no response in hepcidin mRNA expression to BMP-6 stimulation was observed (Figure 6.11).

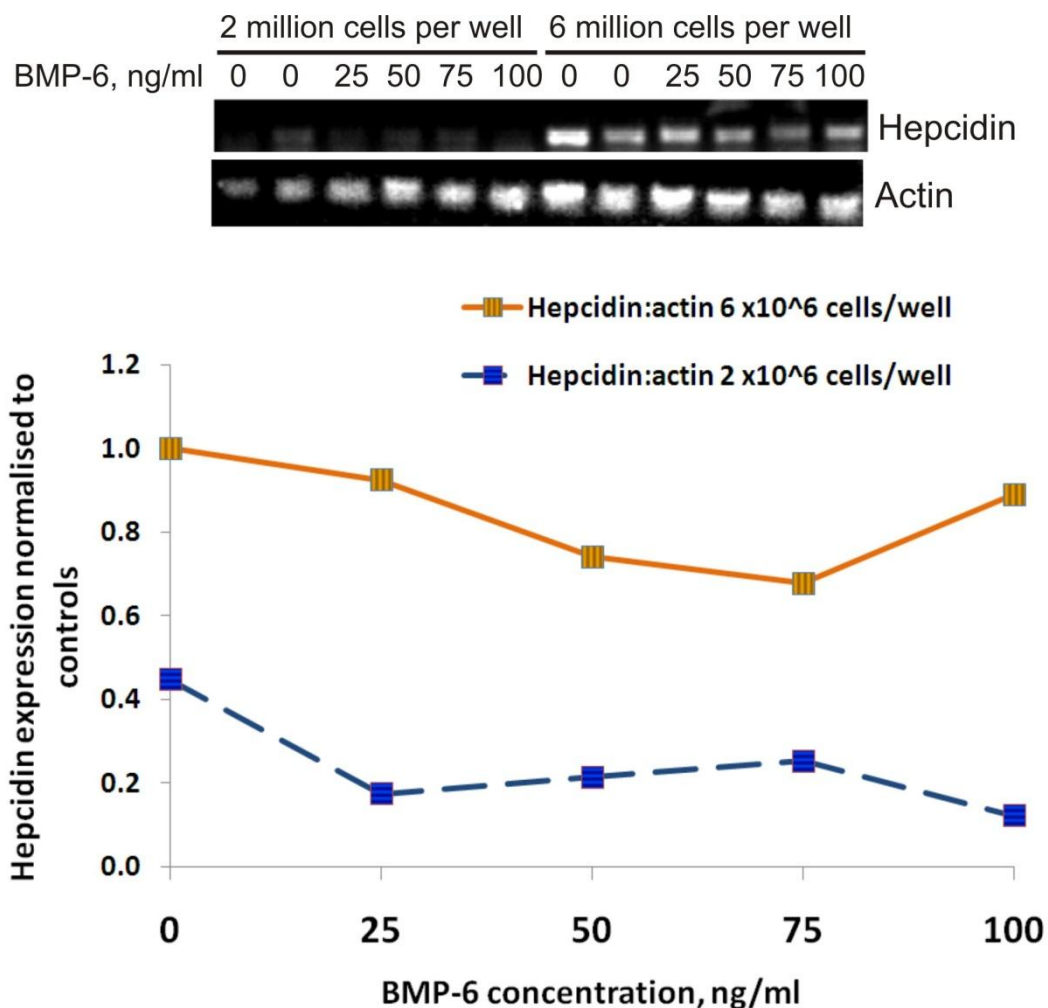


Figure 6.11 – Hepcidin expression following BMP-6 treatment in primary hepatocytes from mice. Hepcidin and actin mRNA expression were measured semi-quantitatively by RT-PCR. Densitometric measurements of hepcidin normalised to actin are shown across a range of BMP-6 treatment concentrations. These data are preliminary, n=2 for baseline n=1 for each treatment point.

Basal hepcidin mRNA levels were not significantly different in liver tissues from BMPR2 heterozygous mice versus wild type (WT) littermates (Figure 6.12).

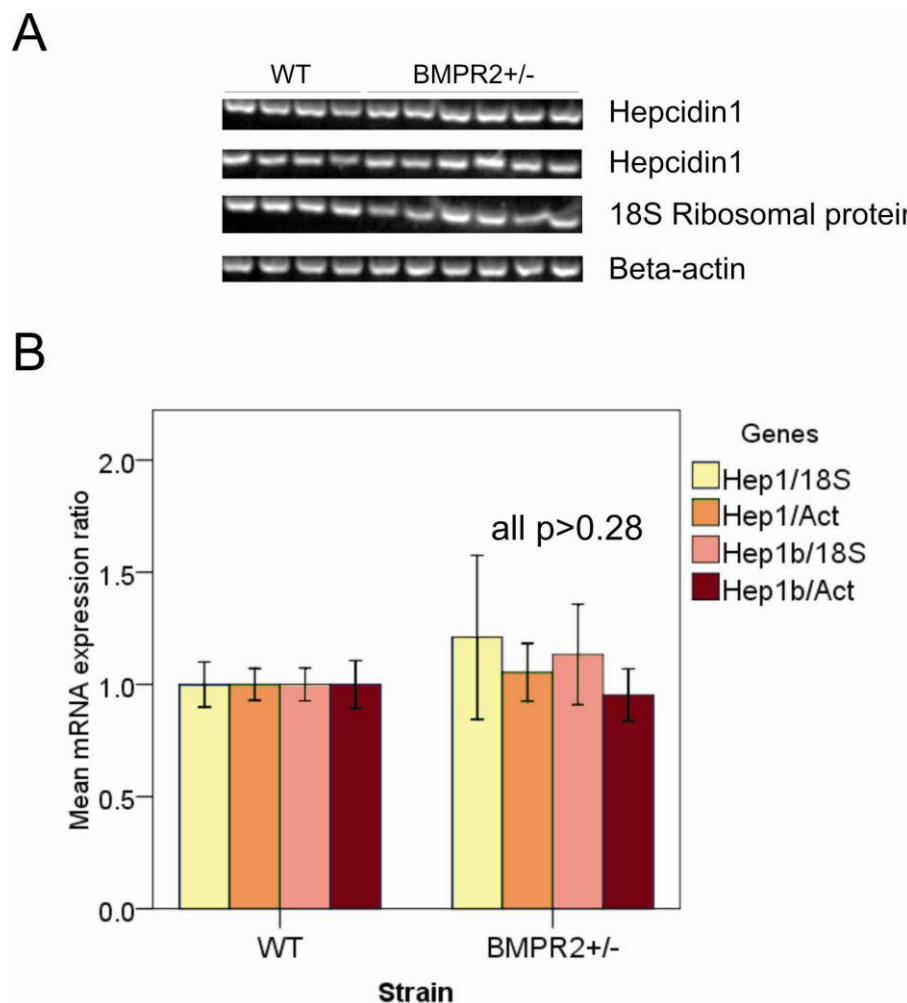


Figure 6.12 – Basal unstimulated hepcidin levels in liver tissues from BMPR2 heterozygous mice.

A. RT-PCR products of hepcidin1 with two different primer sets, 18S ribosomal protein and beta-actin as loading controls in liver tissues from wild-type (WT) and BMPR2 heterozygous mice. B. Densitometry data from A. with both hepcidin primer sets normalised to both loading controls. Statistics are from t-tests. N=4 wild type versus 6 BMPR2 heterozygous mice.

6.4 Discussion

In this chapter, I demonstrated that knockdown of BMPR2 but not the other type II BMP receptors (ActRIIA and ActRIIB) leads to increased hepcidin mRNA expression and protein production in response to BMP-6 stimulation in human hepatoma cells. I also found patients with heritable PAH displayed very low serum iron levels and high sTfR. Circulating levels of BMP-6 did not correlate with hepcidin expression in the IPAH cohort, suggesting this factor was not responsible for the altered hepcidin levels in IPAH. I also demonstrated BMPR2 mRNA but very little protein expression in human liver tissue; protein expression was also undetectable in rat liver whereas heart and lung displayed significant expression. I found chronic hypoxia triggered a transient increase in pulmonary hepcidin expression, which was followed by a transient increase in ferroportin mRNA production. These results were, however, not mirrored in lung tissue samples from end-stage IPAH patients versus lobectomy. Expression of hepcidin and BMP-6 in pulmonary vascular cells suggest local iron regulation could be of interest in the lung and in IPAH. Basal hepcidin mRNA expression was unchanged in liver tissues from BMPR2 heterozygous mice versus wild type controls.

BMP signalling and hepcidin regulation

A number of factors may influence hepcidin levels in PAH. Of particular interest is BMP signalling. BMP signalling regulates hepatic expression of hepcidin (Andriopoulos, Jr. et al. 2009; Kautz et al. 2008; Meynard et al. 2009). BMP-6 has emerged as the principal BMP regulating hepatic hepcidin expression *in vivo* and its levels are regulated by iron in the liver and intestine (Andriopoulos, Jr. et al. 2009; Arndt et al. 2010; Kautz et al. 2008; Meynard et al. 2009). Iron stimulates BMP-6 expression (Arndt et al. 2010), leading to increased hepcidin expression; conversely, BMP-6 knockout mice develop severe iron overload due to markedly reduced hepcidin expression (Andriopoulos, Jr. et al. 2009). BMPR2 expression is reportedly reduced in the pulmonary vasculature of all IPAH patients (Atkinson et al. 2002) and loss-of-function mutations in *BMPR2* have been linked to >70% of familial PAH and 10-20% of IPAH cases (Machado et al. 2009). Whether or not BMPR2 protein levels are reduced in tissues other than the lung in IPAH and HPAH has not been established, but mutations that pre-dispose the BMPR2 receptor to misfolding or that cause inactivation are likely to equally impair function in other tissues which express BMPR2. I hypothesised that reduced BMP signalling would predict raised hepcidin levels in PAH (Figure 6.1).

Indeed, using hepatocellular carcinoma cells, I have shown that BMPR2 but not ActRIIA or ActRIIB knockdown leads to a concomitant increase in BMP-6-stimulated hepcidin production. This effect

was seen at both the mRNA level and when protein secreted into the culture medium was quantified. I also demonstrated BMPR2 mRNA expression in 5/5 of the human liver samples we examined, which has not been consistently demonstrated in previous studies (Kawabata et al. 1995; Nohno et al. 1995; Rosenzweig et al. 1995; Xia et al. 2008b). These data suggest downregulation of BMPR2 may augment plasma hepcidin levels in PAH, predicting that heritable PAH may be associated with more severe iron deficiency and increased hepcidin expression.

BMPR2 mutations are not routinely determined in patients attending the National Pulmonary Hypertension Service at Hammersmith Hospital, so the BMPR2 status of the main IPAH cohort studied in this thesis is unknown. Plasma samples were available from 5 patients with heritable PAH and their iron status was assessed. As predicted, serum iron levels were significantly reduced in all 5 patients, and all 5 had relatively high sTfR levels; 3/5 were significantly raised above the upper end of the normal range and these 3 cases also had extremely low ferritin levels. Despite this, all 5 patients had detectable levels of hepcidin, although the 3 with high sTfR and low ferritin did have hepcidin levels towards the bottom end of the normal range. These data are preliminary in their nature but do indicate that, as seen in IPAH, heritable PAH may be associated with low circulating iron levels, and iron deficiency in a proportion of patients. Determination of the iron status of a larger cohort of HPAH patients with known BMPR2 mutations would establish more conclusively whether this subgroup display a more severe phenotype.

As discussed above, BMP-6 is thought to be the most important BMP regulating hepcidin (Andriopoulos, Jr. et al. 2009; Arndt et al. 2010; Kautz et al. 2008; Meynard et al. 2009). I found no significant difference in BMP-6 levels in IPAH patients in comparison with healthy volunteers, although this comparison was somewhat limited by the assay sensitivity/low expression level of BMP-6 in many subjects, as around 2/3 patients had undetectable levels. Those IPAH patients with detectable BMP-6 levels did not have significantly higher hepcidin levels. This suggested that alterations in circulating BMP-6 levels themselves were not responsible for the variations in hepcidin seen in IPAH. It would be of interest to investigate the possible causes of the large variation in BMP-6 levels which appear to segregate subjects into two subgroups; around two-thirds of samples contained undetectable levels of BMP-6 whereas some approached 1000 times the minimum detectable concentration.

Other circulating hepcidin regulators

Hepcidin expression is also stimulated by iron-transferrin (by switching human haemochromatosis protein binding from transferrin receptor 1 to transferrin receptor 2) and inflammation (primarily through IL-6 activation of the signal transducer and activator of transcription 3) and inhibited by erythropoietic activity (Nemeth 2008; Nemeth & Ganz 2009). The mechanisms by which erythropoietic activity inhibits hepcidin are not well established, but two proteins produced by erythroid precursors, the TGF- β superfamily member GDF-15 and BMP-binding protein TWSG1 have both been proposed as possible mediators (Tanno et al. 2007; Tanno et al. 2009). Hepcidin is also decreased by hypoxia and liver-specific HIF stabilisation leads to decreased hepcidin mRNA expression (Peyssonnaud et al. 2007). This could be an indirect effect; hypoxia is well known to stimulate EPO expression and infusion of EPO into healthy volunteers has been shown to decrease circulating hepcidin levels (Ashby et al. 2010). While hepcidin levels are raised in IPAH patients in relation to their iron status, this is not associated with inflammation (IL-6 levels), GDF-15 levels or loss of the inverse relationship between hepcidin and EPO (see Chapter 4). Other circulating factors that regulate hepcidin may be of importance in IPAH. It would be interesting to see whether differences could be observed in *in vitro* hepcidin expression following application of IPAH serum to the media compared to control serum. Plasma could be fractionated and the fraction responsible for any alteration in hepcidin expression analysed by mass spectrometry. This approach could potentially identify a novel circulating regulator of hepcidin.

Pulmonary iron regulation in PAH

Local tissue iron regulation could be as or more important than systemic changes in iron homeostasis. I explored the possibility of tissue-specific disturbances in iron handling in PAH using chronically hypoxic rats. Following 1 weeks exposure to 10% oxygen, a transient but significant increase in hepcidin expression was observed. This was not seen at 2 days, at which point the acute phase protein haptoglobin was raised (Chapter 3), which might suggest it was not purely an inflammatory response. In contrast, liver hepcidin expression was reduced by (3 weeks) hypoxic exposure, consistent with previous reports (Merle et al. 2007). Messenger RNA levels of the iron-exporter ferroportin were raised significantly at 2 and 3 weeks exposure, which might represent a compensatory response to restore normal protein levels of ferroportin after degradation by hepcidin. These data suggest that alterations in pulmonary hepcidin and ferroportin function accompany the development of pulmonary hypertension in the chronically hypoxic rat, though

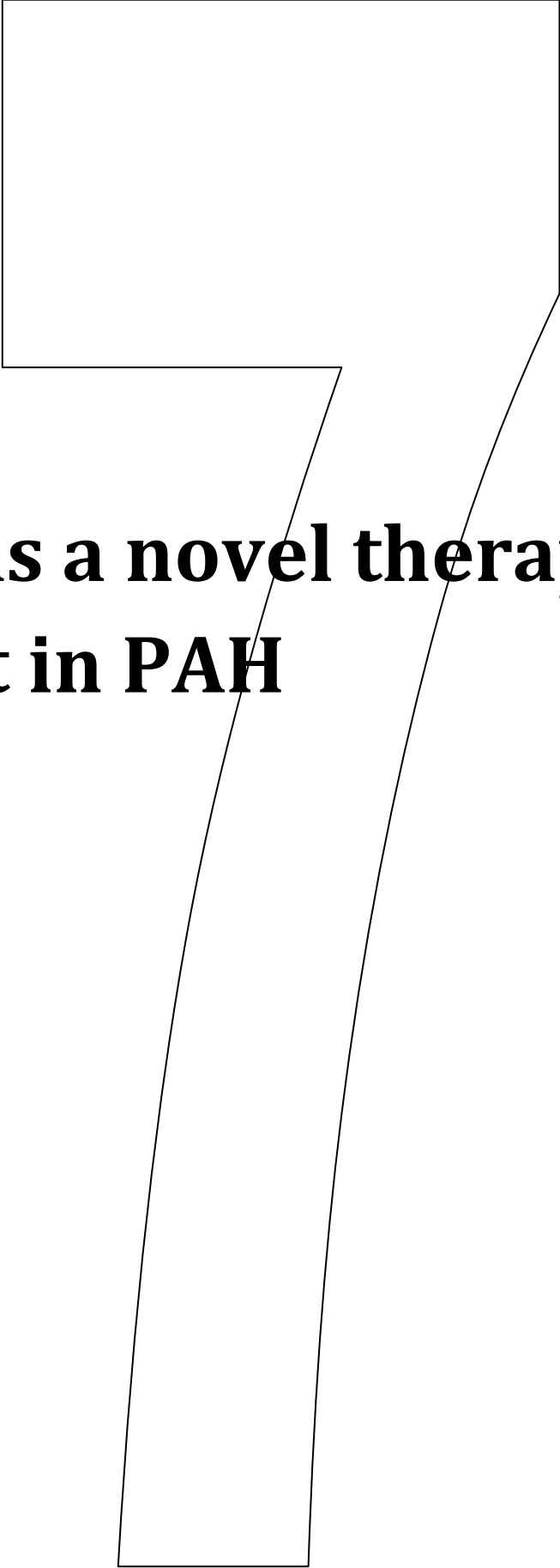
whether these changes represent a disease modifier or are just coincident remains to be established. It would be interesting to assess the development of experimental PAH in animals displaying these alterations basally, for example with lung-specific overexpression of hepcidin or ferroportin, to observe whether remodelling or pulmonary artery pressure changes were modified.

The alterations in ferroportin and hepcidin expression seen in experimental hypoxia-induced PH were not replicated in lung tissue samples from IPAH patients when compared to lobectomy samples. While this might dampen speculation of altered pulmonary iron handling in these patients, it is worth noting that these samples are taken from patients with advanced end-stage disease, which may not be representative of patients earlier on in the natural progression of PAH. Hepcidin and BMP-6 were detectable in human pulmonary vascular cells and it would be of interest to investigate whether hepcidin production in response to BMP-6 stimulation was similarly augmented by BMPR2 dysfunction in these cells as seen in HepG2 cells. In an attempt to see whether the effect was replicated *in vivo* I analysed the hepatic expression of hepcidin in BMPR2 heterozygous mice. No significant alteration was observed in liver tissue expression of hepcidin mRNA in these preliminary data. It may be that stimulation of hepcidin production by BMPs or inflammatory cytokines is necessary to identify a difference in hepcidin production in these animals. This might be achievable in primary cell culture.

In preliminary studies, I successfully isolated intact viable hepatocytes from wild type and BMPR2 heterozygous mice but did not see a response in hepcidin expression to up to 10 times the level of BMP-6 peptide used in the HepG2 experiments in either wildtype or BMPR2 heterozygous mice. Further experiments are required to optimise the protocol (developed from (Berry & Friend 1969; Truksa et al. 2006)). Possible improvements to the protocol could include the use of dexamethasone in the medium in the initial cell attachment phase to maintain cell enzyme expression, the introduction and optimisation of a period of a serum starvation period prior to BMP-6 addition, and optimisation of the length of the treatment period (Klingmuller et al. 2006). It would also be worth investigating whether the isolated primary cells responded to other stimuli of hepcidin such as other BMPs or IL-6, and indeed the inhibitory TNF- α . If the BMPR2 knockdown effect on BMP-6-hepcidin stimulation was observable in human primary hepatocytes, then this would also add weight to the argument that BMPR2 dysfunction could contribute towards hepcidin dysregulation in IPAH.

In summary, in this chapter I have established a possible mechanism of hepcidin dysregulation in IPAH; reduced BMPR2 expression can lead to increased production and release of hepcidin. Low serum iron levels were seen in familial PAH patients and alterations in pulmonary iron handling were

observed following exposure of rats to chronic hypoxia. Further work is required to establish whether these mechanisms are relevant to clinical PAH.



Iron as a novel therapeutic target in PAH

Chapter 7 – Iron as a novel therapeutic target in PAH

7.1 Introduction

Iron status is important in regulating pulmonary vascular tone and hypoxic pulmonary vasoconstriction (Balanos et al. 2002; Smith et al. 2008; Smith et al. 2009). In chapter 4 I showed iron deficiency measured by sTfR level is prevalent, predicts survival in IPAH and is related to worse WHO functional class and lower exercise capacity. I identified increased hepcidin in IPAH, which suggests that oral iron therapies would have low efficacy, as seen by Ruiter *et al.* (Ruiter et al. 2010). Intravenous iron replacement therapy has shown impressive results in left heart failure, improving 6 minute walk distances, quality of life and functional class in clinical trials (Anker et al. 2009; Bolger et al. 2006; Okonko et al. 2008; Toblli et al. 2007; Usmanov et al. 2008). This leads to the question: *Does intravenous iron therapy improve pulmonary haemodynamics, exercise capacity and/or symptoms in patients with IPAH and iron deficiency?*

Iron and the pulmonary vasculature

Several studies have demonstrated the importance of iron availability in the control of pulmonary artery pressure and pulmonary vascular responses to hypoxia (Balanos et al. 2002; Smith et al. 2008; Smith et al. 2009). Iron chelators mimic hypoxic pulmonary vasoconstriction in man (Balanos et al. 2002). Hypersensitivity to hypoxia produced by pre-exposure to 8 hours of hypoxia can be effectively inhibited by pre-infusion of iron, whereas iron chelation exacerbates the acute hypoxia response (Smith et al. 2008). Iron infusions also partially reversed the pulmonary hypertensive response to hypoxia at high altitude. In high altitude residents with chronic mountain sickness, iron venesection lead to increases in pulmonary artery systolic pressure, which were not significantly reversed by 400 mg iron infusions when compared to saline (Smith et al. 2009). These data indicated that modulation of iron bioavailability within the normal physiological ranges can affect pulmonary vascular tone, and has implications for the importance of iron status in pulmonary hypertensive disease in general.

It has also been proposed that iron deficiency could contribute towards pulmonary vascular remodelling through induction of HIF-responsive growth factors, and increased recruitment of circulating CD34+/CD133+ cells to the pulmonary arteries (Farha et al. 2011). The authors demonstrated elevated levels of these growth factors and increased numbers of CD34+/CD133+ cells

in the pulmonary arteries of PAH patients, but they did not demonstrate that this was due to iron deficiency in these patients. Iron chelation with desferrioxamine has also been shown to increase the pro-angiogenic activity of systemic vascular endothelial cells (Ikeda et al. 2011). This study showed that it was activation of endothelial nitric oxide synthase by the serine/threonine protein kinase B (Akt) rather than HIF stabilisation that lead to the effects seen. This emphasises the importance of iron in regulating signalling pathways independent of HIF. Collectively, these data suggest that iron could potentially regulate longer term changes in pulmonary vascular resistance than the relatively acute vasodilatory effects seen in human physiology studies conducted by Robbins and colleagues (Balanos et al. 2002; Smith et al. 2008; Smith et al. 2009).

While it is clear that iron availability is important in energy metabolism, being vital to the activities of many enzymes of the respiratory chain, it is unlikely that these effects are responsible for the increased mortality seen in patients with IPAH and iron deficiency (see Chapter 4). Effects of iron on pulmonary vascular resistance could exacerbate the increased afterload experienced by the right ventricle, failure of which is the main cause of mortality in patients with PAH. PVR therefore represents a key target for investigation in clinical studies of the efficacy of iron replenishment therapy in IPAH.

Iron in chronic heart failure

Anaemia is a powerful and independent prognostic indicator in CHF (Al-Ahmad et al. 2001; Horwich et al. 2002; Mozaffarian et al. 2003) and five key studies have attempted to address the potential therapeutic value of intravenous iron replacement therapy in these patients (Anker et al. 2009; Bolger et al. 2006; Okonko et al. 2008; Toblli et al. 2007; Usmanov et al. 2008). These studies have shown that iron replacement therapy can improve haemoglobin levels, exercise capacity, functional class, quality of life and biochemical markers of myocardial stress and inflammation. Benefits were seen in both anaemic and non-anaemic patients. This implicates iron deficiency itself as an important independent therapeutic target in cardiovascular diseases and emphasises the clinical relevance of the other roles of iron discussed earlier.

Iron deficiency in IPAH

Recently, iron deficiency defined by reduced serum iron and transferrin saturations has been characterised in 30/70 patients with IPAH (Ruiter et al. 2010). Iron deficiency with or without

anaemia was associated with reduced 6MWD. Interestingly, oral iron therapy was only effective at increasing ferritin levels in 8/18 patients treated. This supports the idea that dietary iron uptake may be dysfunctional in these patients. Soon *et al.* have also reported a high proportion of iron deficiency in IPAH, but not in CTEPH patients (Soon et al. 2011). These data are consistent with the data I have presented in this thesis, and focus attention on the idea that increased hepcidin may contribute to the iron deficiency identified in IPAH. Combined with the importance of iron in regulating pulmonary vascular tone and the success of intravenous iron replacement in left heart failure discussed above, iron is an attractive novel therapeutic target in IPAH, which might lead to improvements in pulmonary haemodynamics, symptoms and exercise capacity. The lack of efficacy of oral iron therapy in IPAH suggests that intravenous administration should be the subject of future clinical investigations.

Conversely, recent data suggests that iron chelation therapy may have anti-hypertrophic effects on the RV. Iron-catalysed carbonylation of annexin A1 leading to its degradation (Park et al. 2010), can stimulate increased expression of the *Gata4* gene (Mantovani 1999). GATA4 overexpression is associated with cardiac hypertrophy (Liang et al. 2001). Iron chelation with desferrioxamine inhibits hypoxic-PH mediated RV hypertrophy, coincident with the repression of *Gata4* (Park et al. 2010). GATA4 has also been implicated in the transcription of the S100 calcium-binding protein S100A4 (Lawrie et al. 2005), which is overexpressed in occlusive lesions of patients with pulmonary vascular disease and can drive the development of occlusive pulmonary vascular lesions in ~5% of transgenic animals (Greenway et al. 2004). These mechanisms posit a possible negative role for excess free iron in PH, emphasising the importance of maintaining iron levels within the normal range. Having said this, the clinical benefit of reversing RV hypertrophy without an accompanying reduction in PVR is unclear and could in fact be detrimental by reducing the ability of the RV to meet demand (Wilkins et al. 2010).

7.2 Designing a trial of intravenous iron replacement therapy in IPAH

Designing a clinical trial is a multi-disciplinary task and as such the design of this trial was performed as a collaborative effort between members of the PH research team at Imperial College (including myself), the clinical PH service at Hammersmith Hospital, London and the Statistical Advisory Service, Imperial College, with expert advice from the Haematology Department at Hammersmith Hospital, London. Several challenges to designing a trial for iron replacement therapy in IPAH are apparent. Dosing is a difficult issue as the Ganzoni formula (Ganzoni 1970), used to calculate iron deficiency in the FAIR-HF trial, is not of use in a patient population where haemoglobin levels are within the normal range, as it is based on the iron dose required to meet a target haemoglobin level. As mentioned above, studies in CHF and now IPAH have demonstrated the importance of iron in non-anaemic patients, so a new dosing regimen is required. Average total doses in the CHF studies ranged from 1-3 g administered over several weeks or months (Table 1.1). Ferric carboxymaltose (Ferinject®), the preparation of iron used in the FAIR-HF study, allows a single dose of up to 1 g to be administered by infusion to individuals with body weight >35 kg. Half of this dose would replenish the iron stores of an individual of body weight >35 kg. In anaemic patients, the second 500 mg is estimated to be sufficient to enable increases of haemoglobin of up to ~3 g/dL (Ganzoni 1970) though whether similar increases would be seen in PAH patients is unknown.

Another important issue is which endpoint to choose. Trials of new therapies in PAH have traditionally used 6 minute walk distance versus placebo in treatment-naïve patients, however with limited patient numbers and the ethical requirement not to restrict patient access to approved efficacious PAH therapies, novel therapeutic strategies must be tested on top of established treatments and novel endpoints which require fewer patient numbers must be considered (McLaughlin et al. 2009b). As discussed above, PVR represents a key target for investigation in this study and the relatively low variability in PVR determined by cardiac catheterisation (therefore the requirement for fewer patients to study changes in PVR) makes it an attractive choice. Other potential endpoints include cardiopulmonary exercise testing (CPET), which may provide further insight into how iron availability affects functional capacity (Arena et al. 2010), and cardiac magnetic resonance imaging, which like PVR gives relatively small variation and hence high power to detect significant differences in small patient populations (Wilkins et al. 2005; Wilkins et al. 2010).

The proposed study

For the reasons discussed above, a trial of intravenous iron therapy in patients with IPAH and iron deficiency is required. It is possible that patients with IPAH and no overt iron deficiency might also benefit from this therapy, but this would be for later, larger studies to investigate. The primary endpoint of the study would be saline-controlled change in pulmonary vascular resistance over a time frame suitable to assess both effects on pulmonary vascular remodelling and vasoconstriction (e.g. 12 weeks) following infusion of the maximum dose of ferric carboxymaltose that can be delivered in one visit (1 g for subjects > 35 kg in weight). No suitable placebo for intravenous iron therapy is available, hence the selection of saline as a control. Key secondary endpoints would include CPET measures, as well as WHO functional class, 6 minute walk distance, patient self-reported global assessment and quality of life questionnaires (Figure 7.1).

Patients with IPAH and iron-deficiency, who have been stable on their current therapy for the preceding 1 month, will be treated with 1000 mg ferric carboxymaltose or saline in a crossover design. The rest of this chapter will explain how this study design and its details were decided upon.

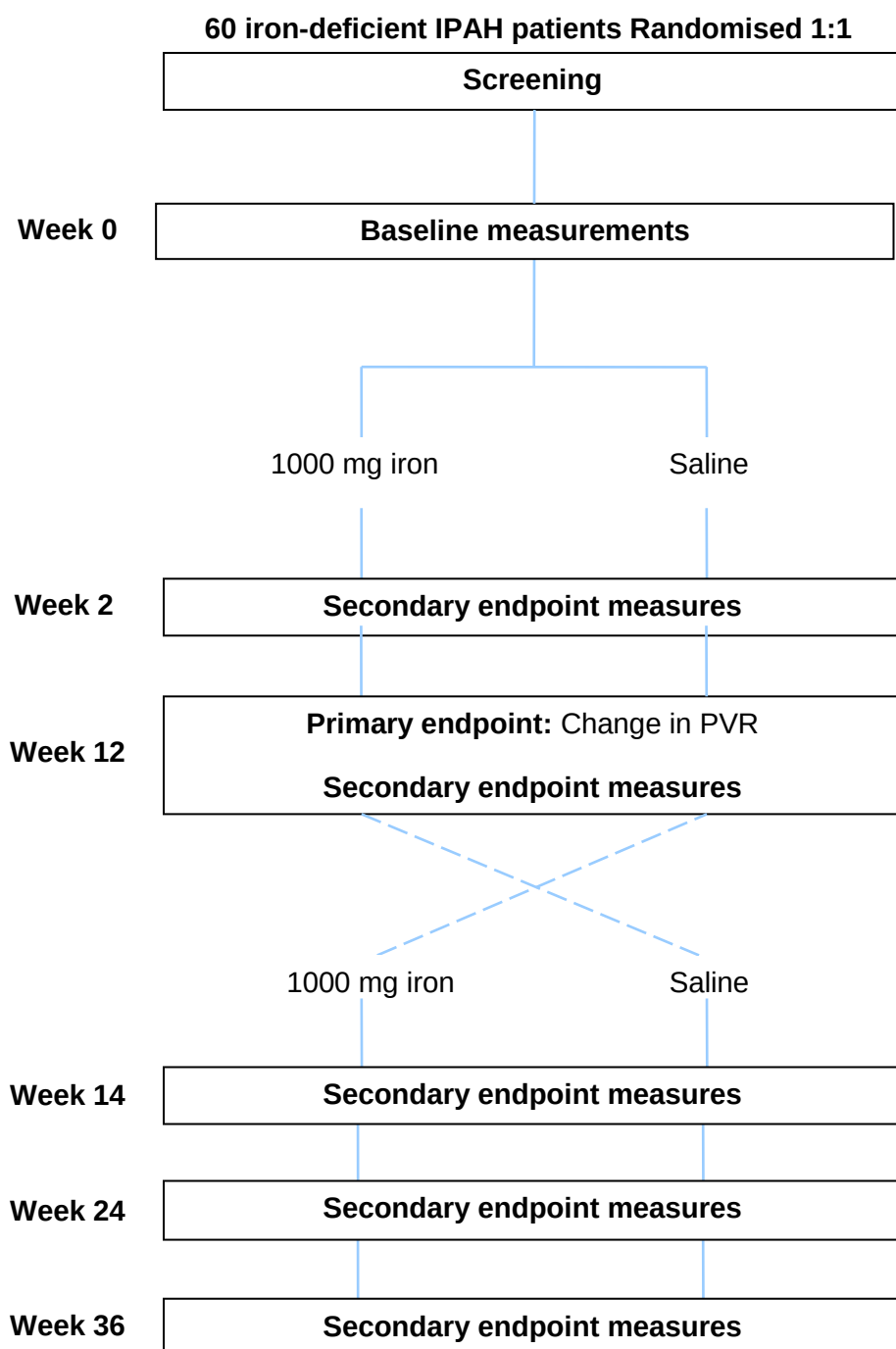


Figure 7.1 – Proposed study schematic. 60 patients with IPAH will be randomised 1:1 to saline or 1 g ferric carboxymaltose in a crossover design, so that when the trial is complete, all patients reaching 12 weeks or further will have received one dose of iron replacement therapy. The primary endpoint is change in PVR at 12 weeks. Changes in cardiopulmonary exercise testing parameters are key secondary endpoints which can be assessed over short-term (2 week) periods and longer term (12, 14, 24 and 36 weeks). The period after 24 weeks will be unblinded as it will be used primarily to collect information on the duration of effect of intravenous iron in IPAH patients, i.e. the maintenance of improved iron status.

Defining iron deficiency

As discussed in Chapter 4, soluble transferrin receptor (>28.1 nmol/l) is the best measure of iron deficiency in inflammatory diseases such as IPAH, but it is not routinely available. Iron deficiency will be based, therefore, on standard laboratory measures of iron. Ferritin levels in patients with sTfR >28.1 nmol/L were not markedly different. Transferrin saturations and serum iron distinguish sTfR >28.1 nmol/L well but EPO levels appear most distinct (Figure 7.2).

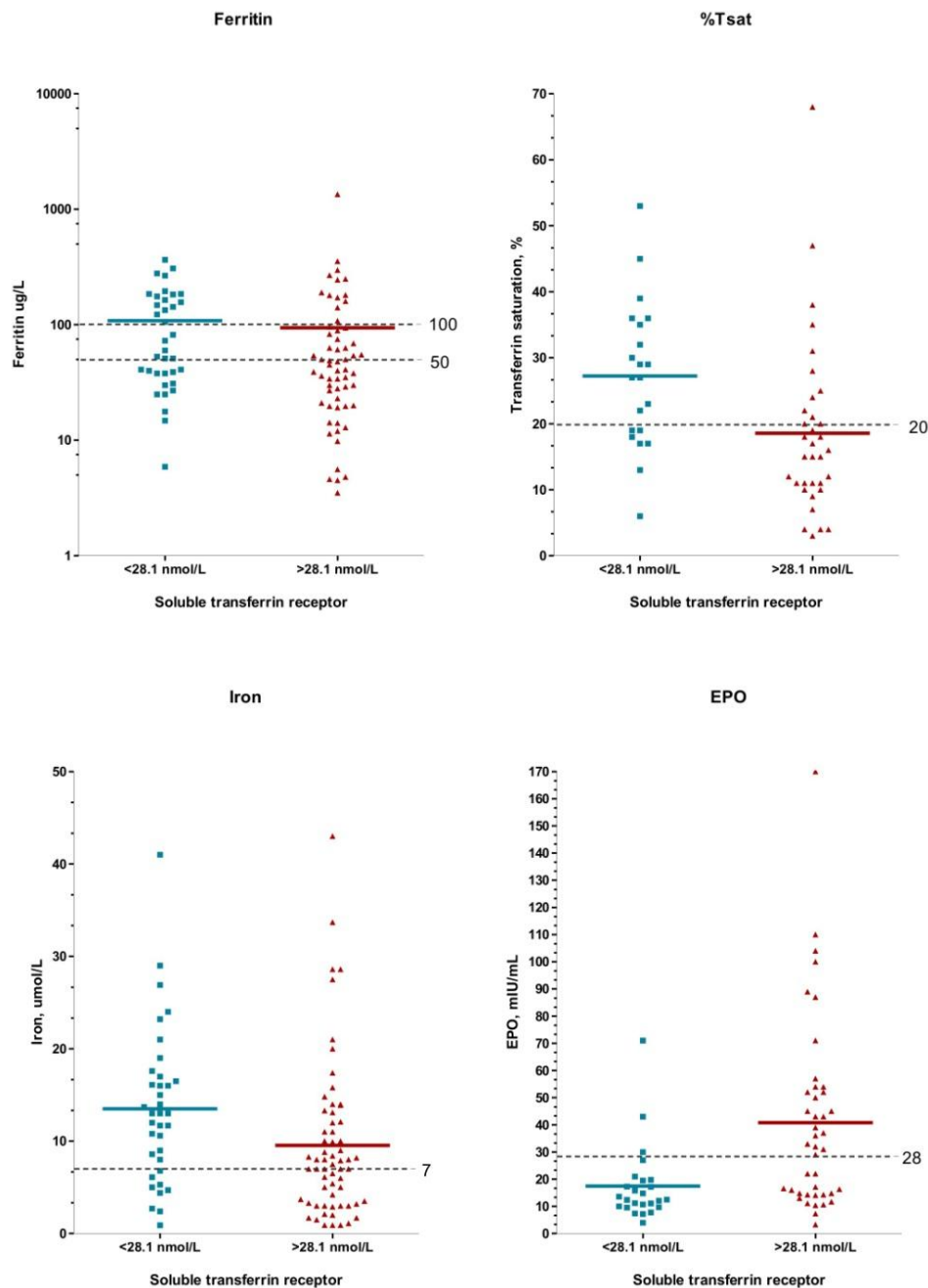


Figure 7.2 – Iron data by soluble transferrin receptor levels above or below the upper limit of the normal range, 28.1 nmol/L. All data from IPAH patients studied in Chapter 4.

ROC analysis confirmed ferritin as the weakest predictor of a sTfR reading above 28.1 nmol/L, with EPO and transferrin saturations being the strongest predictors (Figure 7.3).

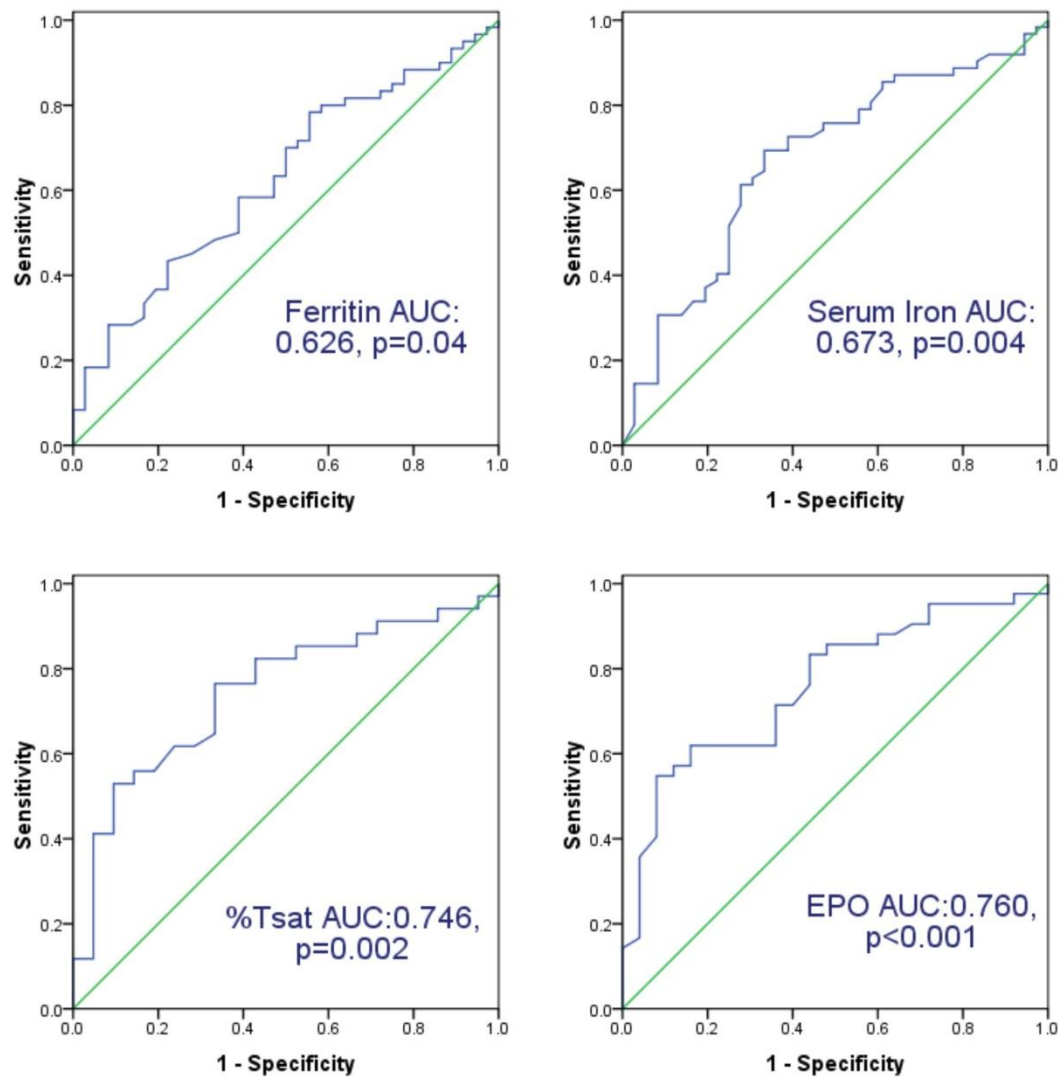


Figure 7.3 – ROC curves showing predictive power of iron measurements in determining sTfR levels above 28.1 nmol/L. All data from IPAHA patients studied in Chapter 4.

Cut-offs derived from these ROC curves and their sensitivities and specificities of predicting a raised sTfR level are shown in Table 7.1 below.

ROC-derived cut-off values	Sensitivity (positives ID'd)	Specificity (negatives not ID'd)
<i>Ferritin</i>		
37 µg/L	43	78
100 µg/L	78	43
<i>Serum Iron</i>		
10.3 µmol/L	69	67
<i>Transferrin sat.</i>		
16.4%	53	90
22.1%	77	67
<i>EPO</i>		
30.5 mIU/mL	55	92

Table 7.1 – Iron parameter cut-offs for predicting sTfR > 28.1 nmol/L as derived by receiver operating characteristic curve analysis. High sensitivity or high specificity alternatives are provided for ferritin and transferrin saturations whereas only one cut-off was derived for EPO and serum iron.

Combinations of parameters will perform better at selecting out patients with high sTfR levels. The predictive power of different entry criteria algorithms are detailed below in Table 7.2. Using highly specific cut-offs for all four parameters (any of Ferritin<37 µg/L, serum iron<10.3 µmol/L, transferrin saturations<16.4 % or EPO>30.5 mIU/mL) allows for highest correct identification rate. However, using sensitive cut-offs for just ferritin (100 µg/L) and transferrin saturations (22.1%) gives a high proportion of positives (85% vs. 67% for more stringent criteria) with a fairly reasonable accuracy (73% vs. 81% for more stringent criteria), therefore recruitment would be faster.

Measures	Cut-offs used	Criteria	Total positive	Correct ID	Positive correct (index specificity)
Ferr, EPO, Fe, Tsat	Specific	Any 1 positive	67%	81%	86%
Ferr, EPO, Fe, Tsat	Sensitive	All 4 positive	23%	51%	90%
Ferr, EPO, Fe, Tsat	Sensitive	Any 3 positive	44%	72%	95%
Ferr, EPO, Fe, Tsat	Sensitive	Any 2 positive	74%	74%	78%
Ferr, EPO, Tsat	Specific	Any 1 positive	63%	77%	85%
Ferr, Tsat	Specific	Either positive	51%	71%	82%
Ferr, Tsat	Sensitive	Both positive	47%	64%	77%
Ferr, Tsat	Sensitive	Either positive	85%	73%	70%

Table 7.2 – Predictive power of algorithms designed to identify patients with high sTfR. Specific cut-offs are more stringent i.e. select fewer patients with higher accuracy. Sensitive cut-offs are less stringent but will select a higher total proportion of patients. The highlighted index provides the highest specificity with good predictive power (correct ID = 72%).

Based on the data presented above, 72% of patients with sTfR >28.1 nmol/l can be identified with 95% specificity, if any 3 of the following criteria are met: ferritin < 100 µg/l; transferrin saturations < 22.1%; erythropoietin > 30.5 mIU/ml or iron < 10.3 µmol/l. More detailed entry and exclusion criteria are detailed in Table 7.3. These criteria are based upon ability to provide informed consent, confounders such as inflammatory stimuli, pregnancy, anaemia, or folic acid/vitamin B12 deficiency, significant bleeding or alterations in iron utilisation. IPAH patients in WHO class II-IV give scope for response to therapies and the ability to perform the necessary exercising testing is vital. Clinical stability on treatment is a pre-requisite for any trial, to reduce drop-out rates from patients not being able to complete the study protocol and provide a stable backdrop against which to judge the effect of an intervention.

Inclusion criteria	Exclusion criteria
Males or females 18 years or older	Unable to provide informed consent
PAH which is idiopathic, heritable or associated with anorexogens with resting mean pulmonary artery pressure >25mmHg, pulmonary capillary wedge pressure $\leq$ 15 mm Hg and normal or reduced cardiac output	Clinically-significant renal (Creatinine clearance < 30 ml/min per 1.73 m ² calculated from CKD-Epi http://www.qxmed.com/renal/Calculate-CKD-EPI-GFR.php) or liver disease (including serum transaminases > 3 times upper limit of normal)
Iron deficiency as defined by any 3 of the following criteria being met: ferritin < 100 µg/l, transferrin saturations < 22.1%, erythropoietin > 30.5 mIU/ml or iron < 10.3 µmol/l	Asthma, eczema or atopic allergies or known to have haemoglobinopathy e.g. sickle cell disease, thalassaemia.
WHO functional class II, III or IV, stable for 1 month prior	Haemoglobin concentration <10 g/dl
6 minute walking distance greater than 150m at entry	Acute or chronic infection or inflammation
Able to complete an incremental cardiopulmonary exercise test to a minimum of 40 Watts surpassing anaerobic threshold as evidenced by a peak lactate > 4 mmol/l	Evidence of left ventricular disease or significant lung disease on high-resolution CT scanning or lung function
Stable therapy (any combination of endothelin receptor antagonist, phosphodiesterase inhibitor or prostacyclin analogue) for at least 1 month	Admission to hospital or treatment change within 1 month
Able to provide written informed consent prior to any study-mandated procedures	Females who are lactating or pregnant
	Uncorrected deficiency of folic acid or vitamin B12
	Individuals known to have HIV, Hepatitis B or C or Creutzfeldt-Jakob disease.
	Known hypersensitivity to Ferinject® or any of its excipients.
	Evidence of disturbances in utilisation of iron.
	Significant blood loss (e.g. GI bleed) within the last 3 months or history of menorrhagia

Table 7.3 – Inclusion and exclusion criteria for a clinical trial of intravenous ferric carboxymaltose therapy in IPAH.

Two major constraints on the design of a study such as this are the acceptability to patients of repeated invasive cardiac catheterisation and the variability of non-invasive measures such as CPET and 6MWD. In the proposed study, a cardiac catheter will be performed at baseline and 12 weeks to assess change in pulmonary haemodynamics and a 6-minute walk test and incremental

cardiopulmonary exercise test to measure changes in cardiopulmonary performance. Patients will then receive the alternative treatment and 6-minute walk test and incremental cardiopulmonary exercise testing repeated after a further 12 weeks (Figure 7.1). A third right heart catheter is unlikely to be acceptable to patients. This crossover design maximises the data available on exercise testing at 12 weeks post-iron for analysis. Quality of life scores and patient global assessment will also be recorded. Thereafter patients will be followed routinely and iron and exercise data captured for up to 52 weeks after the iron treatment. When ferric carboxymaltose 1000 mg or saline will be administered patients will be blindfolded to prevent them identifying the infusion material. The infusions will be performed by a member of the healthcare team with no involvement in the design, data collection or analysis of this study.

Outcomes

The primary efficacy measure will be the change in resting pulmonary vascular resistance from baseline at 12 weeks, measured by cardiac catheterisation. It is possible that iron may be beneficial in PAH through mechanisms not directly related to pulmonary haemodynamics. Capturing other data, particularly exercise capacity and NT-proBNP data, on the study patients is therefore essential. These non-invasive tests can be repeated to detect evidence of early benefit (at 2 weeks) as well as follow serial changes.

A summary of the measurements that will be made is as follows:

- Incremental bicycle cardiopulmonary exercise testing - peak VO_2 (ml/min/kg) measures maximal oxygen extraction, VO_2 at metabolic threshold gives an effort-independent measure of oxygen extraction at a set point and VE/VCO_2 slope represents ventilator efficiency - Exercise testing is increasingly used to evaluate the level of exercise intolerance in patients with lung and heart diseases (Arena et al. 2010).
- Cardiopulmonary haemodynamics – right atrial pressure, right ventricular contractility (dP/dT and isovolumic maximal pressure), pulmonary arterial pressure, pulmonary wedge pressure, cardiac output and stroke volume. – These direct measurements of cardiac function will inform us of direct effects of iron therapy on the heart. Combined with the exercise testing we may be able to assess whether changes in oxygen delivery or other changes, for example in energy metabolism, are more important in determining patient functional capacity.

- Plasma marker measurements – serum iron, transferrin saturations, ferritin, soluble transferrin receptor, RDW and erythropoietin levels – These measures will confirm the efficacy of ferric carboxymaltose in replenishing body iron stores, thus meeting erythropoietic demand and also, over time, the rate of any iron loss that occurs in these patients. Inflammation status is known to affect hepcidin expression and will be determined by measuring CRP and IL-6 levels. Possible haemolysis will be assessed by measuring unconjugated bilirubin, LDH, haptoglobin and reticulocytes. Measurements will be made as described above or using standard clinical pathology accredited hospital assays.
- 6 minute walk distance, Borg dyspnoea scale, WHO functional class – These are standard clinical assessments of patient functional capacity in PAH (McLaughlin et al. 2009b)
- BNP, NT-proBNP – These plasma biomarkers will inform us whether iron therapy has any effect on right heart stress (Daniels & Maisel 2007)
- Quality of life (CAMPBOR questionnaire) and the self-reported Patient Global Assessment – Quality of life has been a focus of previous studies of iron therapy in left heart failure (Anker et al. 2009; Toblli et al. 2007) and the CAMPBOR questionnaire has been developed specifically to assess quality of life in patients with pulmonary hypertension (McKenna et al. 2006)
- Safety - the occurrence of adverse events. This study will provide safety data as to the tolerability of a single maximal dose of ferric carboxymaltose in IPAH with iron deficiency. 1000 mg has been approved as safe for treatment of iron deficiency anaemia.

Sample size and analysis

This is a proof-of-concept study, thus the sample size should be chosen with respect to safety (in terms of exposure to the drug and investigations) and feasibility (patient population) and with the aim of measuring effect size. A sample size of 60 should be sufficient to detect clinically meaningful changes in pulmonary haemodynamics. Trends in exercise end-points, BNP and quality of life should be observable in the direction consistent with benefit. The null hypothesis is that iron replacement has no effect on PVR in patients with iron deficiency and IPAH, with a two-sided alternative that iron replacement changes PVR in patients with iron deficiency and IPAH. Assuming a standard deviation of 250 dynes/s/cm⁵ and a drop-out rate of 10%, 60 patients randomised 1:1 would give this study an 80% power to detect a 194 dynes/s/cm⁵ reduction in PVR with iron treatment at a significance of $p=0.05$ using a two-sample t test. In previous trials in IPAH the mean treatment effect versus placebo using bosentan (Channick et al. 2001) was 415 dynes/s/cm⁵ (223 decrease vs. 191 increase) with iloprost (Olschewski et al. 2002) 335 dynes/s/cm⁵ after inhalation and sildenafil (Galie et al.

2005b) 310 dynes/s/cm⁵. Power calculations were performed using Russ Lenth's piface software (Lenth 2009; Lenth 2007).

The placebo-to-treatment crossover group provides an extra data set (60 each arm) for CPET 2 and 12 weeks post-iron dosing. Assuming a 12-weekly drop-out rate of 10% and standard deviation of 5 ml for peak VO₂ we would have 80% power to detect a mean change of 1.94 ml at a significance level of alpha=0.05 using a paired analysis. Other pre-specified secondary endpoints will be change in CPET at 2 weeks post-iron (2 and 14-week visits versus 0 and 12 week), change in cardiac output at 12 weeks, and change in 6 minute walk distance and circulating biochemical markers at 12 weeks (all versus baseline). Repeated measures regression models will be used to allow for intra- and inter-subject variation. Primary analysis will be intention-to-treat, with sensitivity analyses to explore the effect of dropout. Due to the exploratory nature of the study no multiplicity adjustment will be made to p-values for secondary analyses, though results will be interpreted with caution if the primary endpoint does not achieve significance.

Summary and conclusions

Iron status influences several important aspects of physiology relevant to pulmonary hypertension. The prevalence and prognostic power of iron deficiency in IPAH patients without overt anaemia suggests iron deficiency as a novel therapeutic target in this group. Dietary iron absorption may be repressed in IPAH and therefore a trial of intravenous iron therapy is required. Important considerations in the design of this trial must be made, with more novel endpoints such as pulmonary vascular resistance and cardiopulmonary exercise testing allowing assessment of iron therapy effects in smaller, treated PAH patient groups. A single large dose should be assessed as a simple therapeutic strategy, considering that there are no current guidelines for dosing in non-anaemic patients.

Conclusions and future work

Conclusions and future work

This thesis commenced with the finding that two proteins involved in iron regulation, haptoglobin (Hp) and haemopexin, were downregulated in lung tissue from patients with idiopathic pulmonary arterial hypertension. Reduced Hp expression was also found in plasma samples from IPAH patients and tissues from rats with chronic hypoxia-induced PH. A relationship with altered iron status in IPAH patients led to the discovery of a high prevalence of iron deficiency in IPAH, which was shown to be clinically relevant. Iron deficiency was associated with morbidity and mortality in the IPAH patients. Circulating biomarkers related to iron status, in particular red cell distribution width, predicted survival in patients with IPAH independent of established markers. Raised levels of the master iron regulator hepcidin (Nemeth & Ganz 2009), which might contribute towards the development of iron deficiency, were found in IPAH. Studies in a hepatoma cell line revealed that downregulation of BMPR2, as seen in the pulmonary vascular cells of IPAH patients (Atkinson et al. 2002), leads to selective overproduction of hepcidin following BMP-6 stimulation. The potential for alteration in iron regulatory factors such as hepcidin in local tissues was explored in PH models. Finally, a clinical trial designed to investigate the potential benefits of intravenous iron replacement therapy in iron-deficient IPAH patients was presented.

While the discovery of downregulated Hp in IPAH patients led to my study of iron status in these patients and its clinical importance, the significance of the original finding remains unclear. Confirmation of haemolysis as a potential cause of reduced Hp levels would require more in depth patient studies such as attaining reticulocyte counts or performing ^{51}Cr red cell survival studies. In terms of understanding other possible mechanisms of Hp downregulation, it might prove useful to determine the tissues that experience the highest loss of Hp in IPAH. While I was able to identify different tissues expressing Hp by immunostaining, I did not determine in which tissues levels were altered in comparison with healthy lung sections from lobectomy patients. Refinement of the staining protocol or expansion of studies to tissues from more subjects could help in this respect.

Hp levels did not relate to altered patient clinical status, but just to iron status and patient age at presentation (see Chapter 3). It is unclear as to whether reduced Hp levels are a cause or effect of higher serum iron in these patients. Measuring the Hp levels in IPAH patients before and after the infusion of ferric carboxymaltose could help establish whether iron levels directly affect Hp expression as seen in mice (Faye et al. 2007). In this study, the authors proposed the involvement of the unfolded protein response (UPR) in downregulating Hp expression following a high iron diet. If increasing iron concentrations does not affect Hp levels, this might suggest the higher levels of iron

in patients with low Hp are an effect of low Hp itself. Studies to establish the true importance of a lack of Hp in the pathology of PH could begin with the exposure of Hp-deficient mice (Lim et al. 1998) to chronic hypoxia.

Iron deficiency is becoming a topic of heightened interest in pulmonary vascular research and towards the end of my studies two studies investigating the iron status of patients with IPAH were published (Ruiter et al. 2010; Soon et al. 2011). These studies complement the data I present in this thesis, together demonstrating significant iron deficiency in 3 different IPAH populations and providing robust evidence of a link between IPAH and iron deficiency. Future studies should establish whether iron deficiency is prevalent and clinically relevant in other PAH subtypes. Soon *et al.* indicated that iron deficiency was not more prevalent in patients with CTEPH than in healthy populations (Soon et al. 2011). These patients also have raised pulmonary artery pressures, anticoagulation therapy and receive PH-targeted therapies such as endothelin receptor antagonists, and therefore represent an ideal control population for these studies.

Ruiter *et al.* investigated the value of oral iron therapy in patients with IPAH. Around half of the patients investigated did not respond to oral iron treatments (Ruiter et al. 2010), which is consistent with the hypothesis that raised hepcidin levels inhibit dietary uptake of iron in a proportion of patients with IPAH. However, further studies would be required to establish whether this is the case. Assessment of hepcidin levels prior to the commencement of oral iron therapy could confirm whether a poor response to this therapy could be attributed to high hepcidin levels. The importance of hepcidin in the anaemia of inflammation (Weiss & Goodnough 2005) suggests that raised hepcidin levels are sufficient to drive iron deficiency. Serial measurements of hepcidin and iron parameters in patients with IPAH could help determine their temporal relationship. If hepcidin upregulation precedes the development of iron deficiency in these patients, this would add to the evidence supporting a positive role for hepcidin in the development of this phenomenon in IPAH.

Intravenous iron therapy could circumvent the inhibition of dietary iron uptake by hepcidin, but it would not prevent the recurrence of iron deficiency from this cause nor prevent the downregulation of ferroportin and hence restriction of cellular iron mobilisation. Alternative strategies targeting directly the overexpression of hepcidin might be preferable in preventing the development of iron deficiency before clinical consequences occur. One such strategy, which has been investigated in a mouse model of hepcidin excess, is the use of anti-BMP-6 antibodies (Corradini et al. 2010). Treatment with anti-BMP-6 antibodies reversed the iron deficiency that was a consequence of raised hepcidin levels in these animals. A strategy such as this is being actively pursued for the regulation of iron homeostasis in humans (Lin & Babitt 2010) and could be more effective in IPAH in those

patients who had measurable circulating levels of BMP-6 (see Chapter 6). If these antibodies were shown to be safe for use in human subjects, then they could be used initially to determine whether they reduced hepcidin expression over the short term, with later studies then determining their effect on iron status over a longer period of time.

To develop other strategies for reducing hepcidin expression in IPAH, a better understanding of the factors stimulating hepcidin overproduction in IPAH is required. The experiments in hepatoma cells suggest a possible role for BMPR2 dysfunction, but it remains to be established whether this truly has any effect on systemic hepcidin levels in patients with IPAH. The prevalence of iron deficiency in IPAH patients with BMPR2 mutations was 60% (6/10) versus 27% (20/75) in those without a BMPR2 mutation in one study (Soon et al. 2011) and the 5 HPAH patients analysed in this thesis all displayed very low serum iron levels. This supports the hypothesis of more frequent iron deficiency in patients with HPAH, but the numbers are still too small to be conclusive. More basic studies to assess the effect of downregulated BMPR2 in primary hepatocytes from BMPR2-deficient mice or human hepatocytes treated with anti-BMPR2 siRNA could establish the possible importance of this mechanism in determining systemic hepcidin levels. Other possible mechanisms which could be explored in tissues from IPAH patients or animal models of PH include alterations in BMP signalling modulators or co-receptors (Corradini et al. 2009) or the UPR pathway (Oliveira et al. 2009). Altered expression of BMP co-receptors could potentially switch otherwise normal BMP signalling towards increased stimulation of hepcidin. As discussed in Chapter 3, the UPR pathway can affect the translation of mRNAs during hypoxia (Koumenis & Wouters 2006). It has also been shown that chronic activation of the UPR leads to upregulated hepcidin expression in HepG2 cells (Oliveira et al. 2009). Increased pulmonary hepcidin expression was coincident with decreased Hp expression in rats exposed to 1 week of hypoxia (see Chapters 3 and 6) and it would be interesting to see if this was related to the UPR. If it were, this might have implications for possible parallel changes in UPR function in IPAH. This is highly speculative but could provide a connection between the reduced Hp and increased hepcidin expression seen in patients with IPAH.

Measurement of circulating soluble transferrin receptor levels is arguably the best available method currently of defining iron status in patients with IPAH, due to the lack of effect of inflammation on its levels (Cook et al. 1990; Huebers et al. 1990). Analysis of sTfR levels in the IPAH cohort in this thesis revealed its prognostic value and suggested that lowering sTfR levels in patients with IPAH may represent a valid novel therapeutic strategy. The work of Robbins and colleagues (Balanos et al. 2002; Smith et al. 2008; Smith et al. 2009) has illustrated the importance of iron status in regulating pulmonary vascular tone and for these reasons a trial of intravenous iron therapy in patients with

iron deficiency and IPAH, with pulmonary vascular resistance as its primary endpoint, has been proposed. This study should reveal something of the clinical benefits and kinetics of iron repletion in these individuals and will commence in 2011. This will allow the relationship between changes in sTfR and PVR to be studied, as well as understanding more about other mechanisms that may be of importance such as oxygen delivery and muscle function.

Measurement of other circulating biomarkers in patients with IPAH revealed that indices related to iron status, namely RDW and GDF-15, predict survival in these patients, with RDW in particular outperforming other established prognostic measures in this study. Future studies should look to confirm the prognostic value of RDW alongside NT-proBNP and 6MWD in a prospective manner in separate groups of incident and prevalent patients. The value of these markers in stratifying risk in other aetiologies of PH should also be established individually. RDW has been shown to predict survival in PH of mixed aetiologies (Hampole et al. 2009), where diagnosis was not an independent prognostic variable. GDF-15 has been shown to be raised in patients with systemic sclerosis-associated PAH (Meadows et al. 2010) where it also predicted survival. Measurements of these biomarkers in serial samples from different patient groups will help us further understand the relative power of these markers in prognostication and in tracking disease progression or responses to therapy.

Further work will then be required to determine how they could be used to improve current day-to-day practice in the treatment of PH. In cardiovascular disease in general the additive value of novel biomarkers to established risk factors has been questioned (Wang 2011) with an emphasis on the necessary ability of biomarkers to re-classify patients into different risk brackets with implications for clinical action such as commencement of therapies. Rigorous assessment of biomarkers in PAH must be performed, with benefits of their use demonstrated in a prospective clinical trial setting. Lessons on designs of these studies can be learnt from oncology (Sargent et al. 2005). Patients should be randomised either to the use or absence of biomarker measurements followed by treatment strategies, or randomised to treatments following biomarker measurement to assess the improvement in response compared to current treatment algorithms.

The main conclusion of this thesis is that iron status is important in patients with IPAH, as evidenced primarily by the relationship of markers of iron status to survival. A trial of intravenous iron therapy in patients with IPAH and iron deficiency will investigate the clinical benefit of this approach and further studies of circulating markers related to iron status could help further improve risk stratification and treatment decisions in this disease.

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