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The Role of Apelin in the Pulmonary Circulation

UNIVERSITY
of
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MD Thesis

by Dr Lauren Brash

Scottish Pulmonary Vascular Unit

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Abstract

Background – Apelin agonism causes vasodilatation and increased cardiac contractility in humans and improves pulmonary arterial hypertension in animal models. We here aimed to determine the pulmonary haemodynamic effects of apelin in patients with pulmonary arterial hypertension (PAH) and to determine the effects of apelin on rat pulmonary artery fibroblasts in the lab.

Methods and Results – In a double-blind randomized crossover study, 19 patients with PAH received intravenous (Pyr[1])apelin-13 (10-100 nmol/min) and matched saline placebo during invasive right heart catheterization and measurement of pulmonary artery pressure, pulmonary artery wedge pressure, cardiac output. Acute (Pyr[1])apelin-13 infusion caused a reduction in pulmonary vascular resistance (p=0.001), increased cardiac output (p<0.0001) and an increase in stroke volume (p<0.0001).
Apelin also prevented the hypoxic hyperproliferation and migration of rat pulmonary artery fibroblasts and reduced the activity of p38 MAP kinase.

Conclusions – Acute intravenous (Pyr[1])apelin infusion reduces pulmonary vascular resistance and increases cardiac output and stroke volume in patients with PAH. It also prevented fibroblast proliferation and migration and reduced the activity of the p38 MAP kinase pathway. Apelin agonism is an novel potential therapeutic target for the treatment of PAH and appears to improve both the haemodynamic consequences of pulmonary hypertension as well as potentially improving the remodelling seen in the pulmonary vasculature of patients with pulmonary hypertension.

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The anti-proliferative effects of apelin on pulmonary adventitial fibroblasts. Eur Respir J 2012; 40: Suppl. 56, 3272. Oral presentation.

Apelin improves cardiac output in patients with pulmonary arterial hypertension. Eur Respir J 2015; 46: Suppl. 59, 2107. Poster Discussion.

The effects of apelin on serum NT-proBNP levels in pulmonary hypertension patients versus controls. Thorax 2015; 70: Suppl. 3: A213.

Acute haemodynamic effects of apelin in patients with pulmonary arterial hypertension. Brash I, Barnes GD, Brewis MJ, Church CA, Gibbs, SJ, Howard LSGE, Johnson MK, McGlinchey N, Simpson J, Stirrat C, Thomson S, Watson G, Welsh DJ, Wilkins MR, Newby DE, Peacock AJ. Paper pending submission.

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Finally and most importantly I would like to thank all the patients who took part in this research. Without them this would definitely not have been possible. I hope that the results of the trial will provide new and important knowledge to the world about pulmonary hypertension and help in the progression towards one day hopefully finding a cure.

Author's Declaration

I declare that, except where explicit reference is made to the contribution of

others, that this thesis is the result of my own work and has not been submitted

for any other degree at the University of Glasgow or any other institution.

Signature……………………………………………………………………..

Printed name………………………………………………………………

Definitions/Abbreviations

6MWD	6 minute walk distance
ABG	arterial blood gas
ANOVA	analysis of variance
AT1	Angiotensin receptor
BMP	bone morphogenetic protein
BMP-2	bone morphogenetic protein 2
BMPR2	bone morphogenetic protein type 2 receptor gene
BMPR-II	bone morphogenetic protein type 2 receptor
bpm	beats per minute
CBB	calcium channel blocker
ChIP-chip	chromatin immunoprecipitation with DNA microarray
CO	cardiac output
CPET	cardiopulmonary exercise test
cpm	counts per minute
CTD	connective tissue disease
CTEPH	Chronic thromboembolic pulmonary hypertension
CTIMP	Clinical Trial of Investigational Medicinal Products

CXR	chest xray
DLCO	diffusing capacity of the lung for carbon monoxide
DNA	deoxyribonucleic acid
dpm	disintigrations per minute
ECG	electrocardiograph
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
eNOS	endothelial nitric oxide synthase
ERS	European Respiratory Society
ESC	European Society of Cardiology
FEV_1	forced expiratory volume in 1 second
FC	functional class
FVC	forced vital capacity
GJNH	Golden Jubilee National Hospital
GPCR	G protein coupled receptor
HR	heart rate
HRCT	high resolution computed tomography
HRP	horse radish peroxidase
iNO	inhaled nitric oxide

IPAH	idiopathic pulmonary arterial hypertension
IV	intravenous
mPAP	mean pulmonary artery pressure
mRNA	messenger ribonucleic acid
mSv	millisievert
NO	nitric oxide
NT proBNP	N-terminal pro b-type natriuretic peptide
NYHA	New York Heart Association
PAEC	pulmonary artery endothelial cell
PAH	pulmonary arterial hypertension
PAP	pulmonary artery pressure
PAWP	pulmonary artery wedge pressure
PBS	phosphate buffered saline
PDE5	phosphodiesterase type 5 inhibitors
PFT	pulmonary function test
PH	pulmonary hypertension
PIS	patient information sheet
PPARγ	peroxisome proliferator-activated receptor gamma
PPHN	Persistant pulmonary hypertension of the newborn

PVR	pulmonary vascular resistance
RCT	randomised controlled trial
RHC	right heart catheterisation
RIPA	radioimmunoprecipitation assay
rpm	rotations per minute
RV	right ventricle
RVSP	right ventricular systolic pressure
SEM	standard error of the mean
SDS	Sodium Dodecyl Sulphate
siRNA	small interfering ribonucleic acid
SPVU	Scottish Pulmonary Vascular Unit
SV	stroke volume
SVR	systemic vascular resistance
WHO	World Health Organisation
Wnt/βC	wingless/beta-catenin

1 Introduction

1.1 Pulmonary Hypertension

This thesis focuses on a condition called pulmonary hypertension (PH) which is a disease in which functional and structural changes of the pulmonary vasculature cause a progressive increase in pulmonary vascular resistance (PVR), leading to pressure overload of the right ventricle (RV) and premature death. This is thought to arise from an imbalance between vasodilator and vasoconstrictor mediators. In addition, structural changes of the vessel walls (remodelling) due to proliferation of the endothelium, smooth muscle cells and fibroblasts cause progressive obstruction of the pulmonary vascular bed[1]. There are limited therapeutic options based on agents that reduce pulmonary vascular tone. Despite recent therapeutic advances, there remains an unacceptably high 3-year mortality of around 50%[2] and there is an urgent need to develop new effective therapies.

1.1.1 Definition of pulmonary hypertension

Normal pulmonary artery pressure (PAP) is defined by consensus opinion as a resting mean pulmonary artery pressure (mPAP) of ≤ 20 mmHg. Pulmonary hypertension is defined by a resting mPAP ≥ 25 mmHg as measured at right heart catheterisation[3]. Pulmonary hypertension can be caused by disease of the pulmonary arteries themselves (pulmonary arterial hypertension) or as a consequence of elevation of pressure in the pulmonary venous system or pulmonary capillaries. Table 1-1[4] details the haemodynamic definition of different classifications of pulmonary hypertension.

Definition	Characteristics	Clinical Groups
Pulmonary Hypertension (PH)	mPAP ≥ 25 mmHg	All
Pre-capillary PH	mPAP ≥ 25 mmHg PAWP ≤ 15mmHg	1. Pulmonary Arterial Hypertension 3. PH due to lung diseases and/or hypoxia 4. Chronic thromboembolic pulmonary hypertension 5. PH with unclear multifactorial mechanisms
Post-capillary PH	mPAP ≥ 25 mmHg PAWP > 15 mmHg	2. PH due to left heart disease

Table 1-1 Haemodynamic definition of pulmonary hypertension.
Adapted from "2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension" by Galiè et al. European Heart Journal, 2016, 37, pp 67-119. PH indicates pulmonary hypertension; mPAP, mean pulmonary artery pressure; PAWP, pulmonary artery wedge pressure.

1.1.2 Clinical classification and epidemiology of pulmonary hypertension

The first World Symposia on Pulmonary Hypertension was organised by the World Health Organisation in 1973 after an epidemic of pulmonary arterial hypertension (PAH) cases secondary to anorexigens. A simple classification was established at this time along with a haemodynamic definition[5]. This classification has developed over the subsequent years in order to categorise PH into different subgroups sharing similar pathological findings, haemodynamic characteristics and similar management strategies[6]. The current clinical classification divides PH into 5 distinct subgroups as seen in Table 1-2[6]. The group that I have concentrated on for the purposes of this thesis is group 1.

Updated Classification of Pulmonary Hypertension
Group 1.Pulmonary arterial hypertension
1.1 Idiopathic PAH
1.2 Heritable PAH
1.2.1 BMPR2
1.2.2 ALK-1,ENG,SMAD9,CAV1,KCNK3
1.2.3 Unknown
1.3 Drug and toxin induced
1.4Associated with:
1.4.1 Connective tissue disease
1.4.2 HIV infection
1.4.3 Portal hypertension
1.4.4 Congenital heart diseases
1.4.5 Schistosomiasis
1' Pulmonary veno-occlusive disease and/or pulmonary capillary haemangiomatosis
1'' Persistent pulmonary hypertension of the newborn (PPHN)
Group 2. Pulmonary hypertension due to left heart disease
2.1 Left ventricular systolic dysfunction
2.2 Left ventricular diastolic dysfunction
2.3 Valvular disease
2.4 Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies
Group 3. Pulmonary hypertension due 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 lung disease
Group 4. Chronic thromboembolic pulmonary hypertension (CTEPH)
Group 5. Pulmonary hypertension with unclear multifactorial mechanisms
5.1 Haematologic disorders: chronic haemolytic anaemia, myeloproliferative disorders, splenectomy
5.2 Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis
5.3 Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
5.4 Others: tumoural obstruction, fibrosing mediatinitis, chronic renal failure, segmental PH

Table 1-2 Clinical Classification of Pulmonary Hypertension.
Adapted from "Updated clinical classification of pulmonary hypertension" by Simonneau et al. Journal of the American College of Cardiology, 2013, vol. 62 (25 Suppl) ppD34-41. PAH indicates pulmonary arterial hypertension; BMPR2, bone morphogenic protein type 2 receptor; PPHN, persistant pulmonary hypertension of the newborn; CTEPH, chronic thromboembolic pulmonary hypertension.

1.1.2.1 Idiopathic pulmonary arterial hypertension

Group 1 PH is diagnosed at right heart catheterisation based on a mPAP ≥ 25 mmHg with pulmonary artery wedge pressure (PAWP) < 15mmHg and a normal or reduced cardiac output (CO). To fit into this classification group, left heart disease, hypoxic lung disease and chronic pulmonary thromboemboli must be excluded as causes. In this group the pulmonary hypertension is usually severe but it is also a rare cause of pulmonary hypertension. Due to the information gathered by several national registries the it is estimated that the incidence of PAH is 5-10 cases per million and its prevalence is 15-60 per million[4]. The pathobiological changes described in chapter 1.1.3 lead to a progressive increase in pulmonary vascular resistance, which consequently leads to right ventricular overload, hypertrophy, dilatation and failure.

Pulmonary hypertension should be considered in any patient with unexplained breathlessness, syncope and/or signs of right ventricular failure.

The onset of symptoms can be insidious and average time to diagnosis can be up to 4 years[7]. A high index of suspicion is therefore required in order to establish diagnosis at an earlier stage. There are specific groups that are considered at higher risk of developing pulmonary hypertension and screening programmes may be advocated in these groups. This includes patients with connective tissue disease, HIV, chronic liver disease and congenital heart disease.

1.1.2.2 Pulmonary hypertension due to left heart disease

Pulmonary hypertension due to left heart disease is defined as a mean pulmonary artery pressure ≥ 25 mmHg but with a pulmonary artery wedge pressure > 15 mmHg (Table 1-1). PH due to left ventricular systolic and diastolic dysfunction, valvular heart disease and cardiomyopathies are included in group 2 PH. PH initially develops due to a proportional increase in pulmonary artery systolic pressure which is an adaptation in order to maintain adequate flow across the pulmonary vasculature.

Pulmonary hypertension is a common consequence of left heart disease and when present is associated with more severe symptoms and poorer exercise tolerance[8-10]. This is probably the most common form of PH[11] and in one study of

246 elderly patients assessed for PH, only 15% had idiopathic PAH. The most frequent diagnosis was group 2 PH – secondary to left heart disease[12].

It can be difficult to differentiate group 2 PH from PAH when these patients have preserved ejection fraction. The haemodynamic definition of pulmonary hypertension due to left heart disease is based on a mean pulmonary artery pressure of ≥ 25 mmHg as measured at right heart catheterisation, a pulmonary artery wedge pressure > 15 mmHg and a normal or reduced cardiac output[9]. The patient may need a fluid/exercise challenge to elicit the raised pulmonary artery wedge pressure. Usually the transpulmonary gradient is low and as a consequence the pulmonary vascular resistance is normal or near normal.

There is a subset of patients, however, who develop a more severe phenotype[11,13-17]. At right heart catheterisation they are found to have a high transpulmonary gradient (> 12mmHg) with a high pulmonary vascular resistance. These patients seem to 'reactively' develop pulmonary vascular disease with remodelling of the pulmonary arteries. In fact, histologically, the pulmonary vasculature is no different to patients with PAH[17]. PH has been found to be an independent predictor of mortality in left heart failure[10,18,19].

Pulmonary hypertension due to left heart disease usually initially occurs due to backward transmission of filling pressures leading to a stepwise increase in venous, capillary and arterial pulmonary system pressures. The left atrial-venous pressure rise over time drives an increase in pulmonary artery pressure and therefore an increase in the pulsatile loading of the right ventricle leading to right ventricular dysfunction and failure[20].

Studies have shown that optimisation of heart failure treatment/surgical correction of valvular heart disease can lead to a regression in PH[13]. This is not usually the case in the subset of patients with more severe disease[14], and there are currently no available therapies which specifically treat the pulmonary vasculature and/or right ventricle in left heart disease. With this poorer prognosis in mind, several groups have investigated whether targeting the pulmonary vasculature with PAH specific therapies could be of benefit in left heart disease[21-24]. Most have been ineffective for treating left heart disease, though it should be noted that PH was not part of the inclusion criteria for many

of these studies. Sildenafil, however, has shown some potential in recent studies with evidence of haemodynamic and exercise improvement as well as fewer hospitalisations[25-27]. The development of new therapies to treat this population is urgently required.

1.1.3 Pathobiology and pathophysiology of pulmonary hypertension

The normal pulmonary circulation consists of thin walled, compliant vessels and is typically a high flow, low pressure circuit. This allows minimal changes in pressure when there is increased flow through the pulmonary vasculature during exercise. In pulmonary hypertension there is increased pulmonary vascular resistance which occurs due to a combination of vasoconstriction, vascular wall remodelling and thrombosis[28].

Pulmonary vascular wall remodelling involves pathological changes to all three layers of the pulmonary artery wall: the adventitia, media and intima and involves fibroblasts, smooth muscle cells and endothelial cells. These changes include fibroblast and endothelial cell proliferation, smooth muscle cell hypertrophy and muscularisation of small pulmonary arterioles, fibrosis as well as *insitu* thrombosis which leads to thickening of the pulmonary artery vessel wall and luminal occlusion[29]. There is an imbalance in vasoactive mediators in PAH. Endothelin-1 levels are elevated and are known to be a potent vasoconstrictor. Nitric oxide synthase (and hence nitric oxide) expression is decreased in PAH, again encouraging vasoconstriction. Prostacyclin has vasodilatory and antiproliferative effects and levels are low in PAH again leading to vasoconstriction of the pulmonary artery[30]. The combination of these processes leads to an increase in pulmonary vascular resistance. The precise cause of these processes are unknown but it is felt certain that the cause of PAH is multifactorial with genetic, environmental and inflammatory processes working in a "multiple-hit" mechanism[31].

This process of progressive increase in pulmonary vascular resistance leads to obstruction to pulmonary blood flow and therefore to an increase in right ventricular afterload. There is an initial adaptive response and the right ventricle hypertrophies and dilates (Figure 1-1) as it tries to compensate and

increase preload and myocardial contractility[32]. In the face of progressive increases in pulmonary vascular resistance, the right ventricle decompensates with reduced cardiac output and right ventricular failure. Unchecked this process culminates in death.
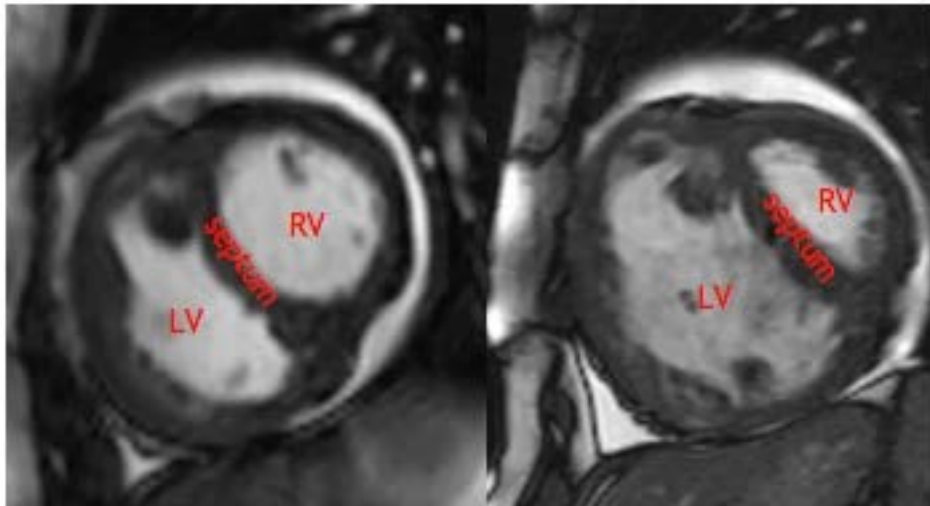
Figure 1-1 Short axis cardiac MRI.
Normal heart (left image) and heart of patient with pulmonary hypertension (right image). The right ventricle (RV) is bigger in pulmonary hypertension, compressing the left ventricle (LV) and causing bowing of the interventricular septum.

1.1.4 Investigation of pulmonary hypertension

The evaluation of a patient with suspected PH involves a series of investigations, which determines to confirm the diagnosis of PH, clarify the classification and aetiology and evaluate the severity. Figure 1-2 shows the process of evaluation set out by the ESC/ERS guidelines[4]. Right heart catheterisation is the gold standard test in the evaluation of PH and is the only way to accurately measure the pulmonary artery pressures. The procedure is safe and well tolerated with a mortality of 0.055% and morbidity of 1.1%[33].
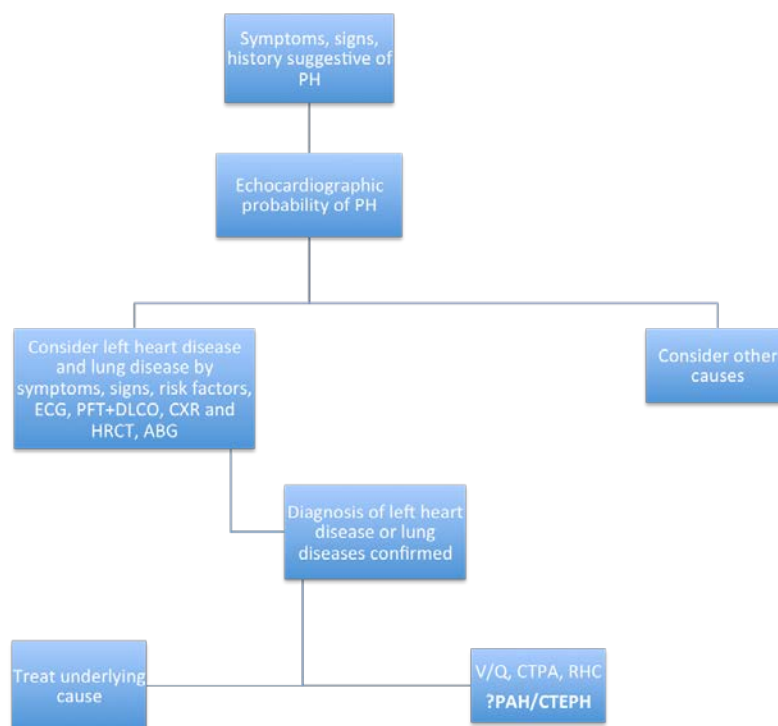
Figure 1-2 Diagnostic algorithm.
Adapted from "2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension" by Galiè et al. European Heart Journal, 2016, 37, pp 67-119. PH indicates pulmonary hypertension; ECG, electrocardiograph; PFT, pulmonary function test; DLCO, diffusing capacity of the lung for carbon monoxide; CXR, chest xray; HRCT, high resolution computed tomography; ABG, arterial blood gas.

1.1.5 Prognosis

PH is progressive and fatal if untreated. Historical data shows that untreated the median survival in patients with PAH is less than 2 years[34]. Now that a range of therapies are available, patients have improved mortality with a recent UK study showing a 5-year survival of 61.1%[35].

A risk assessment strategy (Table 1-3) has been proposed by the ESC/ERS guidelines to help provide prognostic information and therefore guide therapeutic strategy. The 'determinants of prognosis' can be evaluated for each patient at diagnosis and at each follow up visit and may help to guide treatment escalation if patients are deemed to be progressing poorly.

Determinants of prognosis (estimated 1-year mortality)	Low risk <5%	Intermediate risk (5-10%)	High risk >10%
Clinical signs of right heart failure	Absent	Absent	Present
Progression of symptoms	No	Slow	Rapid
Syncope	No	Occasional	Repeated
WHO functional status	I,II	III	IV
6MWD	>440m	165-440m	<165m
NT-proBNP plasma levels	<300 ng/l	300-1400 ng/l	>1400 ng/l
Pericardial effusion	none	none/minimal	present
Right Atrial Pressure	<8 mmHg	8-14 mmHg	>14 mmHg
Cardiac Index	≥ 2.5 l/min/m^3	2-2.4 l/min/m^3	<2 l/min/m^3

Table 1-3 Risk assessment in pulmonary arterial hypertension
Adapted from "2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension" by Galiè et al. European Heart Journal, 2016, 37, pp 67-119. WHO indicates World Health Organisation; 6MWD, 6 minute walk distance; NT proBNP, N-terminal pro b-type natriuretic peptide.

1.1.6 Treatment of idiopathic pulmonary arterial hypertension

There has been some development in the treatment of pulmonary hypertension since 1998 when there was only epoprostenol available. The evidence-based treatment algorithm recommended at the last World Symposium on pulmonary hypertension is summarised in Figure 1-3[36].
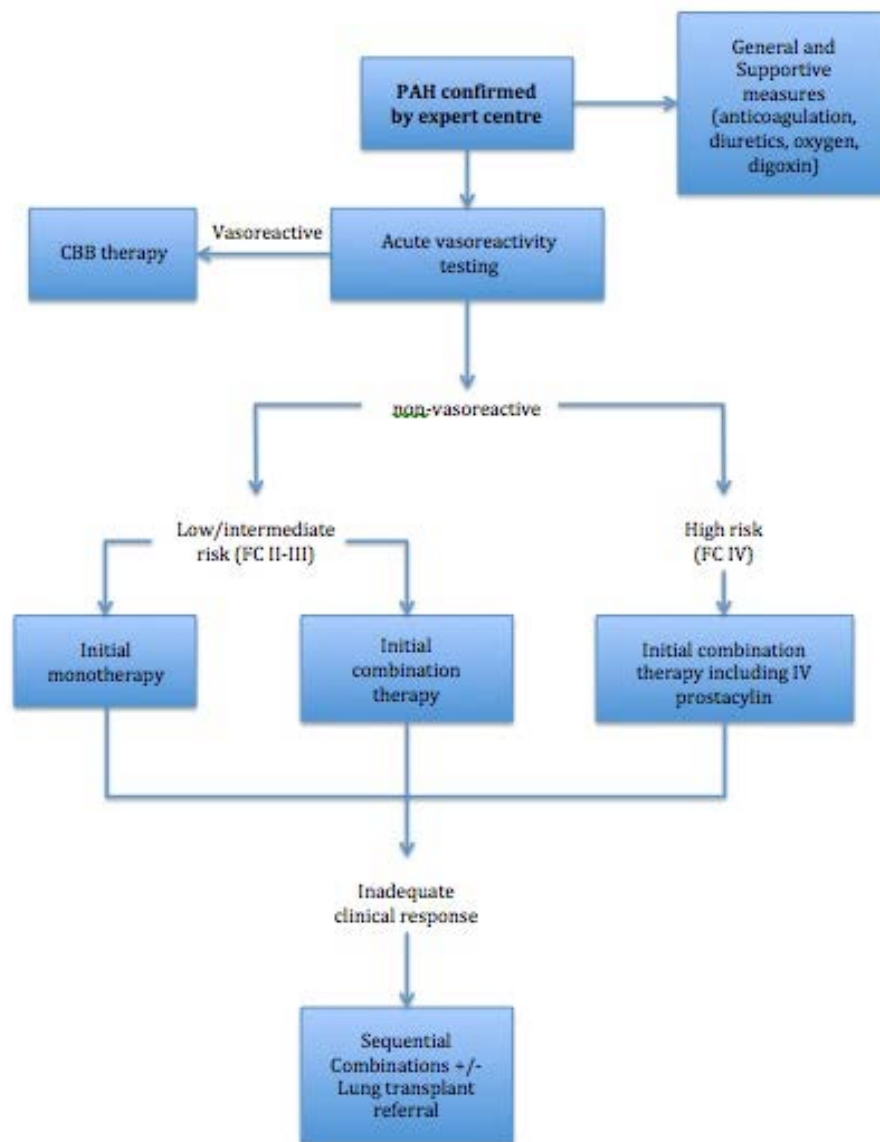
Figure 1-3 Treatment algorithm for pulmonary arterial hypertension patients
Adapted from "2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary
hypertension" by Galiè et al. European Heart Journal, 2016, 37, pp 67-119. PAH indicates
pulmonary arterial hypertension; CBB, calcium channel blocker; FC, functional class; IV,
intravenous.

This algorithm, as well as recommending pharmaceutical treatments also
recommends a number of general and supportive measures.

Physical exercise and rehabilitation alone have been shown to improve exercise
tolerance, quality of life and WHO functional class (severity scoring system)[37].
Supportive treatment in the form of oral anticoagulation, diuretics, oxygen and
digoxin are also recommended.

At diagnosis, patients have pulmonary artery pressure measurements taken at baseline and during inhalation of nitric oxide, a potent pulmonary artery vasodilator. Patients who have a significant improvement in pulmonary artery pressures in response are so-called vasoreactive. They have a significant benefit in prognosis compared with those who do not respond and do well on treatment with high dose calcium channel blockers.

Drugs which specifically treat pulmonary arterial hypertension act on three distinct pathways in the pulmonary circulation. The endothelin (endothelin receptor antagonists), the nitric oxide pathway (soluble guanylate cyclase stimulators, phosphodiesterase 5 inhibitors) and the prostacyclin pathway (prostanoids). Principally these drugs work by causing vasodilation through relaxation of the smooth muscle cells in the pulmonary artery. This therefore allows improvement of blood flow through the pulmonary vasculature and reduces right ventricular overload but there is little evidence that there is any reversal of the pulmonary vascular remodelling seen in pulmonary arterial hypertension.

Of the number of drugs recommended in the algorithm only two have been shown in clinical studies to provide clear morbidity and mortality benefit; epoprostenol[38] and macitentan[39]. The other pulmonary arterial hypertension specific drugs demonstrated improvements in exercise capacity, functional class and haemodynamics but did not look at morbidity and mortality.

Initial choice in drug therapy is based on a number of factors including disease severity, patient preference and comorbidity. In general patients are started on a single agent although in those with more severe disease at diagnosis, combination therapy is possible. Patient response to treatment is assessed at intervals by exercise capacity (six minute walk test), WHO functional class etc. When the response is inadequate then other pulmonary arterial hypertension specific agents can be added.

Other possible interventions include lung transplantation and balloon atrial septostomy. Lung transplantation is reserved for when patients have an inadequate response to maximal combination treatment. Balloon atrial septostomy is a procedure in which a hole is created in the septum between the

right and left atria causing a right to left shunt and therefore reduces right ventricular overload and can improve left ventricle preload and cardiac output. As a consequence the shunt can cause hypoxaemia and as such is not recommended in patients with O_2 saturations of <85%. It can lead to significant symptom improvement particularly in those with refractory right ventricular failure and syncope and so can be useful for palliation or as a bridge to definitive treatment such as transplantation.

1.2 Apelin

1.2.1 The history of apelin in cardiovascular disease

Almost all the known peptide hormones/neuropeptides exert their biological activity by acting on G protein coupled receptors (GPCRs). The APJ receptor was first identified in 1993 via the human genome project; an international scientific research project with the goal of DNA sequencing. Genome research unveiled a total of about 300 GPCRs of which 160 had ligands. The rest had no known ligand and are described as orphan receptors[40]. Apelin was first discovered in 1998 and is the endogenous ligand for the previously orphaned GPCR, APJ[41]. Tatemoto et al isolated the ligand from bovine tissue and named it apelin (APj Endogenous LIgaNd)[41]. APJ itself most closely identified with angiotensin (AT1) receptor sharing 54% homolgy[42] but angiotensin II was unable to activate the receptor. The apelin gene encodes a 77-amino acid peptide (Apelin-36). Shorter C-terminal fragments of apelin-36 have also been detected, including the pyroglutamated form of apelin-13, (Pyr^1)apelin-13, which exhibits greater affinity for the APJ receptor and may represent the principal endogenous ligand[43].

Apelin and APJ receptor mRNA is expressed extensively in body tissue. The highest expression appears to be vascular tissue[44]. APJ receptors have been found to be present on vascular endothelial cells, vascular smooth muscle cells, and cardiomyocytes[45]. This has lead to studies of its significance in the cardiovascular system. Human studies have also shown apelin to be present in pulmonary arteries[46].

In preclinical models, apelin signalling exerts major effects on both vascular tone and cardiac contractility. In *ex vivo* myography studies, apelin causes

vascular tone to be reduced by up to 50% in both systemic arteries[47,48] and veins[49]. This response is attenuated by inhibition of nitric oxide (NO) but not by inhibition of prostacyclin[48] suggesting a mechanism of action via the NO pathway. In isolated rat hearts, apelin is the most potent endogenous inotrope described so far[40].

In rodents, apelin also increases cardiac contractility *in vivo* and causes a rapid fall in both arterial blood pressure and systemic venous tone, with corresponding reductions in left ventricular afterload and preload[50-53]. This effect was prevented in Rodents treated with L-NAME (nitric oxide synthase inhibitor)[51] again supporting a mechanism of action via the NO pathway.

Apelin-deficient mice develop premature heart failure unless plasma apelin concentrations are restored[54]. Reductions in myocardial apelin and APJ expression have also been demonstrated in experimental rodent models of heart failure[40]. Despite the apparent down regulation of apelin-APJ activity, the haemodynamic effects of exogenous apelin are maintained or even augmented in rodents with heart failure[55].

Failing human hearts also exhibit altered patterns of Apelin and APJ gene expression, and plasma apelin concentrations appear to be markedly reduced in patients with severe chronic heart failure[56]. Following mechanical offloading of the heart with left ventricular assist devices the APJ expression increases[57] This has excited interest in the apelin-APJ pathway as a target for therapeutic intervention in this condition.

Professor Newby, with whom I collaborated in this study, and his team at The University of Edinburgh Centre of Cardiovascular Science recently provided the first evidence that apelin has vasoactive actions in humans. They have shown that apelin causes vasodilatation *in vivo* in the human forearm circulation of healthy volunteers through predominantly nitric oxide–dependent mechanisms, and that this effect is preserved in patients with heart failure[58,59]. Furthermore, apelin is a direct coronary vasodilator and increases myocardial contractility in humans. It causes a reduction in both peak and end-diastolic left ventricular pressures[59].

In addition they have recently assessed the effect of prolonged systemic infusion of apelin in man[60]. During 6-hour infusions the inotropic actions of apelin were maintained. Furthermore, they monitored pulmonary artery blood flow during the first hour of apelin infusion using echocardiography and found this to be increased with apelin infusion versus saline placebo.

In all of human studies apelin was well tolerated with no serious adverse events or ECG changes.

1.2.2 Pulmonary arterial hypertension and the role of apelin

Mutations in the bone morphogenetic protein receptor (BMPR)2 gene which encodes the cell receptor BMPR-II are found in 25% of sporadic and up to 80% of familial forms of idiopathic PAH (IPAH)[61]. Dysfunction of BMP signalling is linked to the pathogenesis of PAH. Patients with PAH demonstrate reduced expression of BMPR-II in their lungs whether they have BMPR2 gene mutation or not[62]. Reduced BMPR2 signalling promotes apoptosis of pulmonary artery endothelial cells (PAEC) and reduced endothelial cell survival may underlie the loss of microvessels, both in human disease as well as in animal models of PAH[63].

BMPR2-mediated signalling recruits the Wingless/beta-catenin (Wnt/βC) pathway to mediate expression of genes that promote PAEC survival, proliferation and angiogenesis[64]. BMP-2, a natural ligand for BMPR-II, mediates PAEC survival through the formation of a nuclear complex between PPARγ and βC. Inhibition of PPARγ and hence inhibition of the formation of this complex impairs PAEC survival. By using ChIP-chip in combination with whole genome wide gene-expression microarrays, the apelin gene was identified as one of a number of transcriptional targets of the PPARγ–βC complex[65]. Therefore apelin is a product of BMPR2 signalling and its production can be affected by dysfunction of this signalling pathway.

The highest level of apelin mRNA tissue expression is in the lung and this is thought to be the predominant source of circulating Apelin[44]. Immuno-staining techniques demonstrate distinct apelin staining within human vascular endothelium[45]. The expression of apelin is reduced in the PAEC of patients with IPAH compared with donor control lungs[65]. Reducing apelin in PAEC by siRNA

impairs PAEC survival and promotes pulmonary artery smooth muscle cell proliferation[65]. Furthermore, apelin can directly suppress vascular smooth muscle cell proliferation in response to growth factors, and is pro-apoptotic[65]. Plasma apelin-36 concentrations are also reduced in patients with IPAH[46]. The combination of raised plasma NT proBNP concentrations and reduced plasma apelin concentrations has been proposed as a prognostic biomarker for PAH.

Consistent with these observations, TIE2CrePPARγ$^{flox/flox}$ mice, a murine model in which PPARγ is selectively deleted in endothelial cells, show reduced apelin mRNA and protein expression in lungs and PAEC, and exhibit increased pulmonary arterial muscularisation, increased right ventricular systolic pressure and right ventricular hypertrophy consistent with PAH. A two-week course of treatment with apelin significantly reversed these signs of PAH in this murine model[65].

An apelin-null murine model was developed and exposed to chronic hypoxia which is well known to establish PH in wild-type mice. The apelin-null mice were found to develop a more severe phenotype of PH when compared to the wild-type mice kept in the same conditions[66]. They had comparatively higher right ventricular systolic pressures (RVSP) and increased muscularisation of the arterial walls. There was no difference in systolic blood pressure between the 2 groups[67]. Serum nitrate levels were also measured as a surrogate for NO and the apelin-null mice demonstrated significantly lower nitrate levels[66] which was secondary to down-regulation of endothelial nitric oxide synthase (eNOS)[66], again signifying that the NO pathway plays an important role in apelin signalling.

Another model of PH is the monacrotaline rat. A prevention study in which rodents were injected with monacrotaline, and then randomised to receive (Pyr^1)apelin-13 versus placebo, showed that apelin prevented the downregulation of RV apelin and APJ expression as well as preventing the haemodynamic changes seen in pulmonary hypertension and the right ventricular hypertrophy[68].

Patients with idiopathic pulmonary arterial hypertension have been shown to have reduced plasma apelin levels when compared with controls[46,65,66]. However in chronic hypoxic rats it has been shown that apelin plasma levels did not

correlate with the apelin content in lung or with right ventricular systolic pressure[69].

The precise physiological role of apelin, and hence the consequences of reduced plasma concentrations in PAH and left heart disease are unknown. Given the biological properties of apelin and the data from heart failure studies, it is reasonable to suppose that reduced apelin activity contributes to the pathology of these diseases. Consequently I hypothesis that APJ receptor agonism with apelin would benefit patients with PAH by reducing pulmonary vascular resistance and increasing cardiac output. In doing so apelin may have a role in the treatment of left heart disease with associated PH.

1.3 The aims and hypothesis

In healthy volunteers and patients with heart failure, it has been demonstrated that systemic intravenous apelin infusions increased cardiac index and lowered mean arterial pressure and peripheral vascular resistance. However, to date, there are no data on the effect of apelin on central filling pressures and specifically the vasomotor effects on the pulmonary circulation. My aim was to examine the effects of intravenous apelin administration on the pulmonary haemodynamics of patients with pulmonary arterial hypertension (PAH) which is idiopathic, heritable or associated with connective tissue disease (CTD) or drugs/toxins (group 1)[70]. The study was done in collaboration with Professor Newby from the university of Edinburgh Centre of Cardiovascular Science who's role it was to investigate the effects of Apelin in patients with pulmonary hypertension associated with left heart disease (group 2) and normal healthy controls.

It was also my aim to study the effects of apelin in the pulmonary circulation, in particular to see what effects it would have on pulmonary artery fibroblasts and see if apelin can reduce the proliferation of these cells seen with hypoxic stress.

My hypothesis was that apelin would:

i.   Cause pulmonary arterial vasodilation, reduce pulmonary vascular resistance and increase cardiac output.

ii.   Have greater haemodynamic effects in people with pulmonary hypertension, especially PAH, than in people with normal pulmonary circulation.

iii.   Have antiproliferative effects on pulmonary artery fibroblasts.

2 Materials and Methods

2.1 Clinical study

2.1.1 Study design

This was an investigational double-blind randomised crossover trial to investigate the acute pulmonary haemodynamic effects of apelin in patients with group 1 pulmonary arterial hypertension (PAH) and pulmonary hypertension (PH) associated with left heart disease. This was set up as a multisite trial and recruitment was carried out at the Scottish Pulmonary Vascular Unit (SPVU) at The Golden Jubilee National Hospital (GJNH) in Glasgow, The National Pulmonary Hypertension Service at Hammersmith Hospital in London and The University of Edinburgh Centre of Cardiovascular Science at The Royal Infirmary of Edinburgh. Prior to commencement of the trial I wrote the study protocol, submitted to the West of Scotland Research and Ethics committee for approval and applied for a funding grant from the British Heart Foundation.

2.1.2 Study populations

We recruited three populations of patient in order to determine the potential differential effects of intravenous apelin on pulmonary arterial haemodynamic parameters:

a) patients with PAH

b) patients with chronic left heart failure and associated PH

c) normal healthy controls

2.1.2.1 Pulmonary arterial hypertension

Patients with PAH were recruited from the Golden Jubilee National Hospital in Glasgow and Hammersmith Hospital in London, both of which are specialised national centres for the management of patients with pulmonary arterial hypertension. As this was an invasive study being carried out in patients with a rare disease, we felt that optimising recruitment potential by involving 2 sites was necessary to realistically meet our calculated sample size.

Participants recruited for the study were either new patients attending for diagnostic work up (which includes right heart catheterisation) or patients with known PAH who have been receiving stable doses of approved mono- or combination PAH therapy for 2 months prior to the study.

Inclusion criteria:

a) PAH that is idiopathic, associated with drugs/toxins, associated with connective tissue disease or heritable (group 1 PAH)

b) mean pulmonary artery pressure ≥ 25 mmHg, pulmonary capillary wedge pressure ≤ 15 mmHg and a normal or reduced cardiac output

c) stable WHO functional capacity of grade II to IV for 3 months prior to study

Exclusion criteria:

a) significant left ventricular dysfunction

b) chronic lung disease (FEV_1/FVC < 60%; abnormal lungs on computed tomography)

c) chronic thromboembolic pulmonary hypertension

d) bleeding diathesis

e) women of child bearing potential

f) systolic blood pressure >190 mmHg or <100 mmHg

g) malignant arrhythmia

h) renal or hepatic failure

i) haemodynamically significant valvular heart disease

j) severe or significant co-morbidity

2.1.2.2 Pulmonary hypertension associated with left heart disease

Patients with PH associated with left heart disease were recruited from the GJNH in Glasgow and the Royal Infirmary of Edinburgh. The GJNH is home to the Scottish National Advanced Heart Failure Service and we were working in collaboration with Professor David Newby of the Royal Infirmary of Edinburgh who has carried out previous systemic apelin studies in patients with heart failure. The results of these studies are not available in this thesis.

Participants recruited for the study were either hospital admissions undergoing assessment of their heart failure or recruited from the outpatient clinic.

Inclusion criteria:

a) Stable NYHA grade II to IV heart failure due to left ventricular systolic dysfunction

b) maintained on maximally tolerated doses of ACE inhibitor and beta-blocker for at least 3 months prior to the study

c) baseline echocardiography ejection fraction <35%, left ventricular end diastolic diameter >5.5cm, shortening fraction <20% and tricuspid regurgitant velocity ≥ 3.0 m/s

Exclusion criteria

a) bleeding diathesis

b) women of child bearing potential

c) systolic blood pressure >190 mmHg or <100 mmHg

d) malignant arrhythmia

e) renal or hepatic failure

f) haemodynamically significant valvular heart disease

g) severe or significant co-morbidity

2.1.2.3 Healthy control subjects

Healthy control subjects were recruited at the Royal Infirmary of Edinburgh. They were identified from patients attending for elective cardiac catheterisation with atypical chest pain and who were found not to have obstructive coronary artery disease (<50% luminal stenosis)

Inclusion criteria:

a) baseline echocardiography pulmonary artery systolic pressure <25 mmHg (tricuspid regurgitant velocity <2.5 m/s) confirmed at time of invasive right heart catheterisation

Exclusion criteria:

a) bleeding diathesis

b) women of child bearing potential

c) systolic blood pressure >190 mmHg or <100 mmHg

d) malignant arrhythmia

e) renal or hepatic failure

f) haemodynamically significant valvular heart disease

g) severe or significant co-morbidity

2.1.3 Consent

If the subject met all the inclusion criteria they were offered participation into the trial. They were given a patient information sheet (Appendix A) and allowed at least 24 hours to consider the information prior to consent being obtained.

2.1.4 Drug

The effects of APJ agonism were determined using synthetic-grade (Pyr[1])apelin-13 (Clinalfa AG, Laufelfingen, Switzerland) [58,59]. The apelin was stored at -20°C until ready to use. It was administered after dissolution in 0.9% physiological saline under aseptic conditions on the day of the study. Doses were chosen based on previous systemic human studies carried out by Professor Newby's team based at Royal Infirmary of Edinburgh[58-60].

Drug prescription, preparation and accountability documents were prepared (Appendix B).

2.1.5 Study conditions

All subjects were fasted for at least 4 hours prior to the procedure and avoided alcohol and caffeine for 24 hours prior to the study. The participants also delayed taking their routine medication on the morning of the right heart catheterisation until completion of the study protocol. Each study was carried out in an appropriate investigation room at the designated centre. The rooms were quiet and temperature-controlled with subjects in the supine position.

2.1.6 Study protocol

All haemodynamic measurements were made with the mid-axillary point used as the 0 mmHg reference point. Throughout the study there was continual monitoring of heart rate, electrocardiogram, pulse oximetry and every 5 to 15 minutes systemic arterial blood pressure was measured by automatic cuff inflation.

An 8F introducer sheath (Figure 2-1, Medtronic, USA) was placed in the right internal jugular vein using ultrasound imaging or brachial vein using standard aseptic techniques.
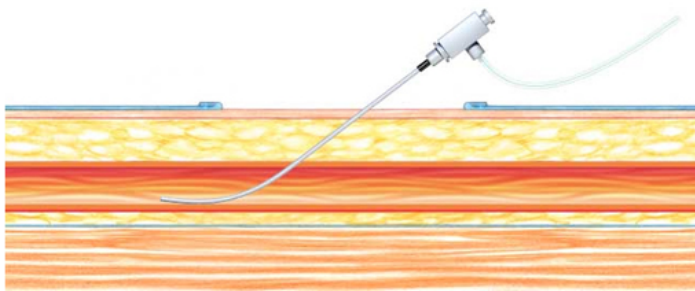
Figure 2-1 Medtronic introducer sheath

A 7F, 2 lumen thermodilution, pressure-measuring tipped Swan-Ganz catheter (Figure 2-2, Edwards Lifesciences, USA) was passed with the aid of fluoroscopy and pressure tracings (which are characteristic at each site) through the right atrium of the heart into the right ventricle and subsequently into the pulmonary artery.
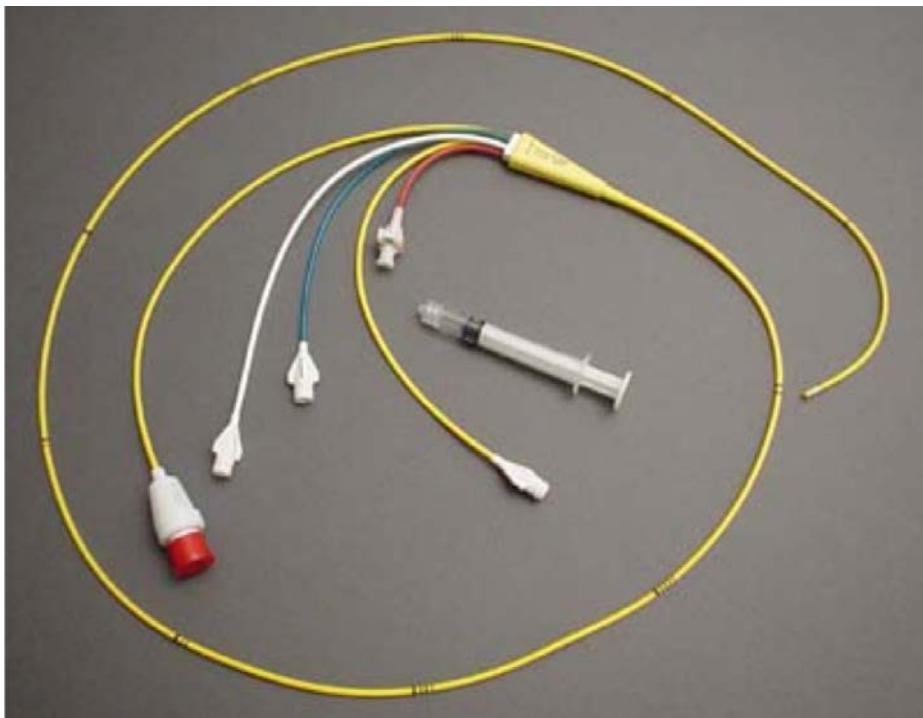
Figure 2-2 Swan-Ganz catheter

All centres had a universal data collection document to try and ensure universal standards between the sites (Appendix C).

2.1.6.1 Pulmonary haemodynamic measurements

Pulmonary haemodynamic measurements were made by invasive right heart catheterisation, which is the gold standard technique. All centres involved were experienced at carrying out cardiac catheterisation and there is low morbidity and mortality associated with this procedure in experienced hands (1.1% and 0.055% respectively[33]). The baseline measurements taken initially included right atrial pressure, right ventricular pressure, pulmonary artery pressure (systolic, diastolic and mean) and pulmonary arterial wedge pressure. Figure 2-3 shows a schematic representation of the study protocol.
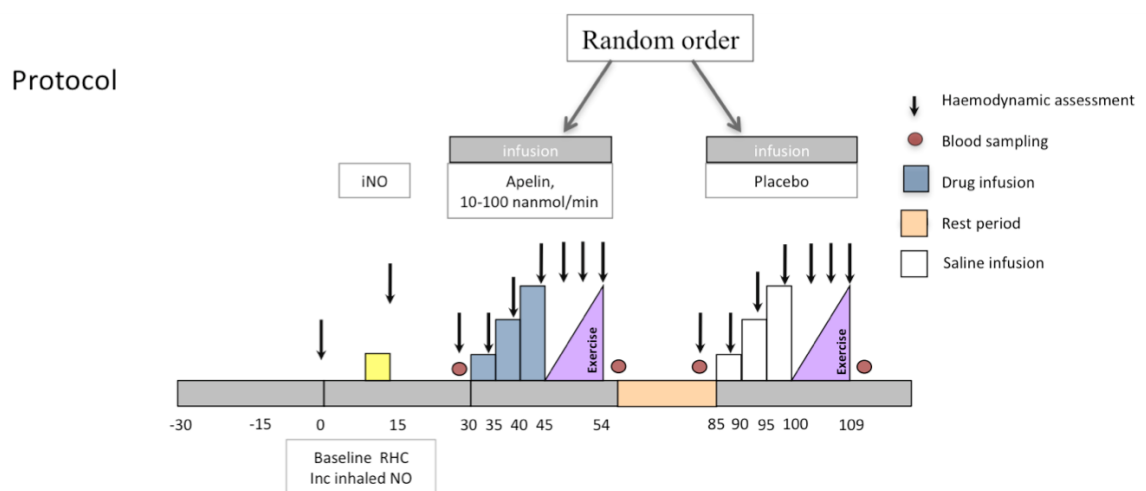
Figure 2-3 Schematic of study protocol
iNO indicates inhaled nitric oxide; RHC, right heart catheterisation; NO, nitric oxide.

Cardiac output was measured by either thermodilution (Glasgow/Edinburgh) or the Fick method (London). Both methods of measuring cardiac output are well accepted and validated and there should be no consequence for the validity of the results of the study by using 2 different techniques because the measurements were all within subject comparison.

Thermodilution cardiac output was determined by injecting a known quantity of cold (temperature measured) physiological saline through a proximal port (port 1 in Figure 2-2) in the Swan-Ganz into the right atrium. A more distal port (port 3 in Figure 2-2) has a thermistor attached and as the cooler fluid travels from the right atrium to the proximal end of the catheter, a brief drop in temperature is recorded. This data is used to plot a thermodilution curve and consequentially cardiac output can be calculated.
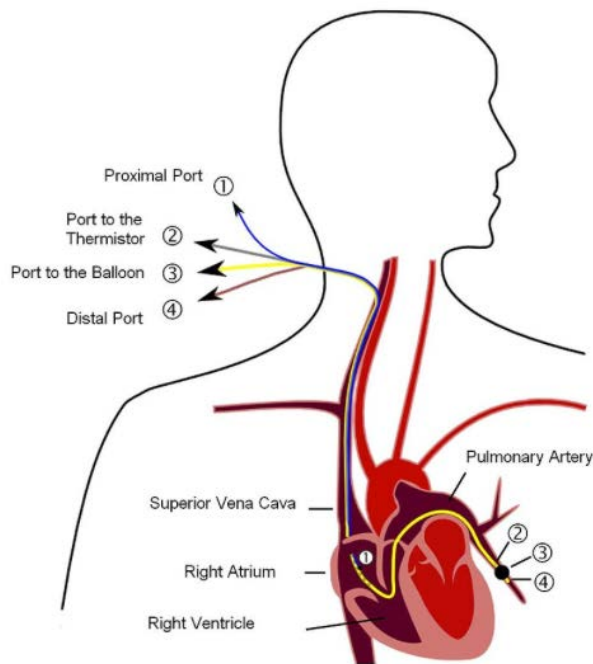
Figure 2-4 Swan-Ganz catheter ports

The Fick method determined cardiac output by arterial blood sampling and sampling of mixed venous blood from the pulmonary artery via the Swan-Ganz catheter. Oxygen consumption was measured using the Fitmate (Cosmed, Italy). The cardiac output was then calculated using the modified Fick equation (Equation 2-1):

$$CO = \frac{Oxygen\ Consumption}{(O2\ content\ of\ arterial\ blood\ -\ O2\ content\ of\ mixed\ venous\ blood)}$$

Equation 2-1
CO indicates cardiac output; O2, oxygen.

Pulmonary Vascular resistance was calculated by dividing the difference between the mean pulmonary artery and pulmonary arterial wedge pressures by the cardiac output (Equation 2-2):

$$PVR\ = \frac{mPAP - PAWP}{CO}$$

Equation 2-2
PVR indicates pulmonary vascular resistance; mPAP, mean pulmonary artery pressure; PAWP, pulmonary artery wedge pressure; CO, cardiac output.

Systemic vascular resistance (SVR) was calculated by dividing the difference between mean arterial pressure and mean right atrial pressure by cardiac output (Equation 2-3). SVR was chosen for analysis as we were interested in the vasodilatory effects of apelin whether they be pulmonary or systemic. Mean arterial pressure was considered for analysis but is dependent on SVR and cardiac output (CO) so any change in this variable could not be put down to any vasomotor change in the vasculature:

$$SVR = \frac{MAP - mRAP}{CO}$$

Equation 2-3
SVR indicates systemic vascular resistance; MAP, mean arterial pressure; mRAP, mean right atrial pressure; CO, cardiac output.

At rest each participant received, in a double-blinded randomised manner, three 5-minute intravenous infusions of apelin at 10, 30 and 100 nmol/min and three 5-minute intravenous infusions of saline placebo. Haemodynamic data was collected at the end of each 5-minute infusion. Following each 15-minute infusion period, if the participant was functionally capable they underwent an exercise protocol during which the 100 nmol/min infusion and saline placebo during crossover was continued.

To ensure uniformity across the different sites I produced an apelin manual (appendix D) which provides a step-by-step breakdown of how to run the study.

2.1.6.2 Exercise pulmonary haemodynamic measurements

Patients with PAH are usually asymptomatic at rest and develop symptoms of breathlessness when active. We therefore felt it would be interesting to study the acute haemodynamic effects of apelin during exercise as well as at rest.

In order to do this, in the days prior to the right heart catheterisation, subjects underwent a progressive incremental cardiopulmonary exercise test (CPET) on

an ergometer. Starting at 0 watts (W) for 3 minutes and adding 10W every minute until the symptom limited maximum was reached. The purpose of the incremental exercise test was to determine the maximal exercise capacity. This was used to calculate the exercise work rates for individuals during the exercise component of the study. Not all participants had the functional capacity to do this and therefore they underwent the resting component of the study only.

The exercise study was carried out in the supine position with a cycle ergometer secured to the catheterisation table (Figure 2-5).

Figure 2-5 Lode Corvial Supine Ergometer with cardiac stress table

Haemodynamic data was first collected with the participants feet strapped into the pedals but before the participant begins to cycle. This is because the raising of the participants legs alone would increase venous return to the heart and may alter pulmonary haemodynamics in itself. The participant was then instructed to start turning the pedals at a rate of 60 rotations per minute (rpm) while the workload was increased to 20% of the previously determined maximal erect CPET workload (Figure 2-6). After 2 minutes of cycling further haemodynamic data was collected (while the participant continues to cycle). The workload was then increased to 40% of the previously determined maximal erect CPET workload and after an additional 2 minutes of cycling further haemodynamic data was collected.

Figure 2-6 Subject carrying out the exercise protocol

A 30 minute washout/rest period was then carried out before the protocol was repeated for the second infusion (Figure 2-3).

2.1.7 Radiation materials

As the study involved right heart catheterisation which is carried out with fluoroscopy screening (Figure 2-7), details of the ionising radiation exposure had to be provided to the research and ethics committee with risks explained to the patient in the patient information study. I had to ask a local lead Medical Physics Expert to prepare a report detailing the possible ionising radiation exposure (Appendix E).
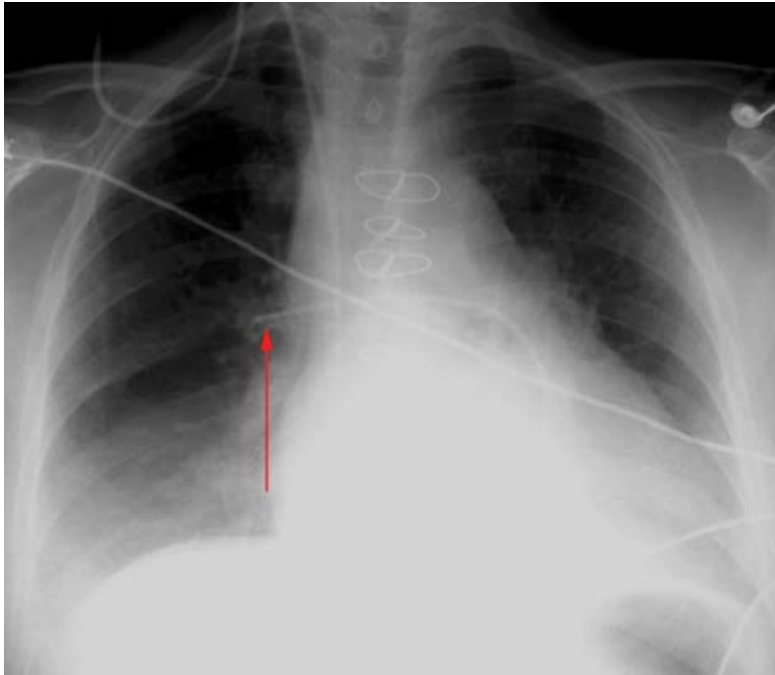
Figure 2-7 Fluoroscopy of right heart catheterisation procedure
Red arrow indicates tip of swann ganz in right main pulmonary artery.

The two possible procedures that subjects participating in the trial may undergo would be:

1. Right heart catheterisation with fluoroscopy screening.

All subjects recruited will undergo this procedure with an estimated procedure dose of 2.1 mSv. This equates to an additional lifetime fatal cancer risk of 1 in 9600 which is the equivalent of less than 1 years background radiation exposure.

2. Coronary angiogram with fluoroscopy screening.

The healthy controls may be recruited when undergoing a coronary angiogram for diagnostic reasons. The coronary angiogram was NOT part of the study protocol but was included so that total effective dose was known. This exposure was likely to affect up to one third of the patients recruited. This procedure provided an estimated procedure dose of 5.7 mSv or 7.8 mSv for coronary angiogram + right heart catheterisation. A 7.8 mSv effective dose equates to an additional lifetime fatal cancer risk of 1 in 2560.

2.1.8 Randomisation

All study participants were allocated an individual study number when recruited. Each study number had been randomly allocated an infusion order prior to commencement of the study. They were allocated to either receive apelin as infusion A then saline placebo as infusion B or vice versa. There was one contact who allocated the study number and randomisation order for recruits at all sites and a delegate from each site prepared the apelin and saline infusions, labelling them infusion A or B as per the randomisation order so that the delegates carrying out the study remain blinded.

2.1.9 Adverse event reporting

An adverse event is any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment. An adverse event can therefore be any unfavourable and unintended sign (including an abnormal finding, for example), symptom, or disease temporally associated with the use of a medicinal product, whether or not considered related to the medicinal product.

A serious adverse event is any untoward occurrence that:

   a) results in death

   b) is life-threatening

   c) requires hospitalisation or prolongation of existing hospitalisation

   d) results in persistent or significant disability or incapacity

   e) consists of a congenital anomaly or birth defect

   f) is otherwise considered medically significant by the investigator

Possible adverse events:

Apelin:

a) A change in mean arterial blood pressure of more than 30 mmHg on two consecutive measurements

b) A change in heart rate of more than 30 beats per minute (bpm) during resting data collection on two consecutive measurements

c) Electrocardiograph (ECG) abnormalities:

 a. second or third degree heart block

 b. sustained bradycardia (<40bpm) or tachycardia (120bpm)

 c. new atrial fibrillation

 d. atrial or ventricular bigeminy

 e. ST segment shift

 f. new T wave inversion or bundle branch block

d) Discomfort of the study participant

e) Request of the study participant

Right heart catheterisation:

a) Temporary pain/discomfort/bleeding/bruising at catheter insertion site

b) bleeding

c) infection

d) heart rhythm changes

e) vascular damage

f) damage to heart valves

g) pneumothorax

h) rarely death

Fluoroscopy (x-ray) during the right heart catheterisation:

Minimal amount of exposure to radiation. The typical radiation dose during right heart catheterisation is 2.1 mSv. The effective dose is equivalent to 11 months background radiation and represents an additional risk of lifetime fatal cancer of 1 in 9600. In terms of comparative lifetime risk of death, it is similar to the lifetime risk of death from work in a service industry, and is in the Intermediate risk category for bio-medical research.

Exercise testing:

Very low risk of harm. Occasionally causes sore muscles and/or breathlessness due to the effort involved.

Blood drawing:

a) temporary pain/discomfort from needle stick

b) tenderness

c) redness

d) swelling

e) bruising

f) rarely infection

g) rarely bleeding

h) rarely light-headedness

i) rarely fainting

j) rarely dizziness

Any adverse event which occurred should have been documented in the participants health records and on an adverse event reporting form (Appendix F) which should be sent to the chief investigator and all adverse events were to be reviewed and reported in the annual report to the West of Scotland Research and Ethics committee/sponsor.

All serious adverse events were to be recorded in the participants health records. Serious adverse events were to be reported to the chief investigator within 24 hours of occurrence. The serious adverse event would then be analysed as to whether it was related to study procedures and if expected or unexpected. All related unexpected events would have been documented on an NRES 'report of serious adverse event' form and sent to the West of Scotland Research and Ethics committee within 15 days of the event.

2.1.10   Ethical review

This study was classed as a Clinical Trial of Investigational Medicinal Products (CTIMP) and required approval from the West of Scotland Research and Ethics Committee before recruitment could go ahead. Initial application prompted a reply asking for changes to be made to Patient Information sheets and consent forms. Once these changes were made and the documents were resubmitted a favourable outcome was achieved.

A couple of minor amendments were made and approved but there were 2 more substantial amendments made at the start of study recruitment.

The first was regarding the dosing of apelin. Initially we had chosen apelin doses of 30 nmol/min, 100 nmol/min and 300 nmol/min which was the doses used in the original studies carried out by Professor Newby in Edinburgh. This was used during the first subject recruited and the unblinded data showed that there was a difference in PVR and Cardiac output (CO) between infusion A and infusion B at

30 nmol/min and 100 nmol/min but that the effect was lost at the higher dose of 300 nmol/min (Figure 2-8). On reviewing the published data from Japp et al[59], they had seen a similar plateau effect at 300 nmol/min (Figure 2-9). Based on this information we believed that a maximal infusion concentration of apelin at 100 nmol/min would be enough to expose the physiological actions of apelin on the pulmonary vasculature. This amendment was accepted by the West of Scotland Research and Ethics committee.
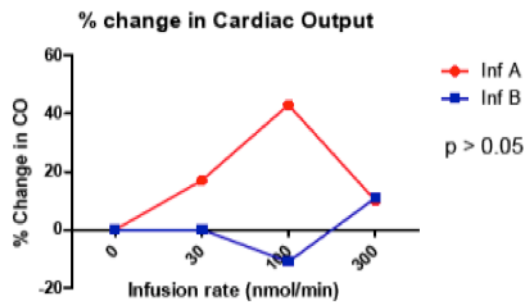
Figure 2-8 Initial study showing percentage change from baseline in cardiac output during infusion A and Infusion B.
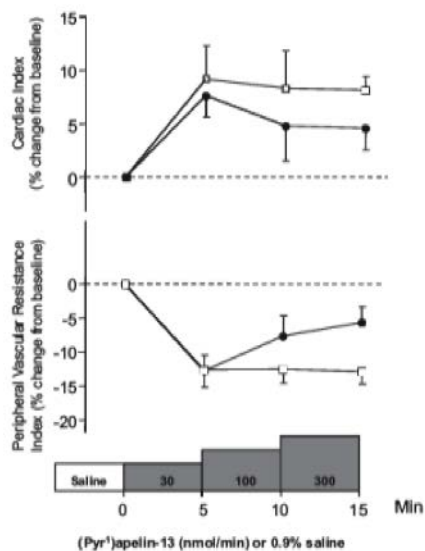
Figure 2-9 Percentage change from baseline in cardiac index (top) and peripheral vascular resistance (bottom) with apelin infusion in patients with chronic heart failure (open squares) and matched control subjects (closed circles).

A further substantial amendment was required regarding consent for patients stopping warfarin. In the initial protocol submission I had not considered that because of logistical reasons, patients may have to stop warfarin which they may be on for PAH or atrial fibrillation prior to signing the written consent form. In

some cases patients were being recruited for the study from some distance away meaning multiple attendances to the recruitment site were impossible and inconvenient for the patient. In some occasions the study protocol was discussed over the phone and the patient information sheet was sent to them by mail. If they then wished to participate in the study they could possibly attend the site specifically to take part in the trial and sign the consent within 24 hours of the study taking place meaning they may have to stop their warfarin in advance prior to signing written consent. The question we asked the ethics committee was whether we could take verbal consent for this over the phone when circumstances made it easier for the patient. They came back to me with a few questions detailed below before responding with a favourable outcome.

The specific questions I had to answer regarding this amendment were:

1. *'The study team should check to establish the reasons patients are on warfarin and not assume that they are on this medication because of Pulmonary Arterial Hypertension (PAH)':*

We would never make assumptions about why a patient is on warfarin and when they have been identified as a potential recruit for the study in clinic, they are screened to make sure they meet the inclusion and exclusion criteria. Significant co-morbidity is part of the exclusion criteria. Their past medical history is reviewed at this point and any other reason for being on warfarin will be identified. We also take the patient through the patient information sheet at this time, which in the new version includes a paragraph about warfarin, the reasons for stopping it and the risks this may entail.

2. *'If patients are established to taking warfarin for reasons other than PAH, then it is suggested that they should not be included in the study. If the patient is taking warfarin for PAH then the risks of coming off warfarin should be fully explained in the Patient Information Sheet (PIS), namely the risk of stroke or peripheral embolism':*

As stated in the Project Protocol version 4, any patients who are on warfarin for essential reasons, such as mechanical prosthetic valves, pulmonary embolism or

chronic thromboembolic pulmonary hypertension will not be included in the study.

PAH group

The European Respiratory Society (ERS) Guidelines for the management of pulmonary hypertension advise supportive therapy with warfarin in the absence of contraindication[71]. Their reasons for this are based on post-mortem studies in patients with IPAH, showing a high prevalence of vascular thrombotic lesions in the lungs. The theory is that warfarin prevents these lesions from developing and thereby helps to stabilise pulmonary vascular resistance. The guidelines also state that there is a "possible presence of non-specific risk factors for venous thromboembolism, including heart failure and immobility" and so there is a rationale for warfarin as thromboprophylaxis. The target for INR varies around the world, in Europe we aim for the standard target of 2-3, whereas in North America they aim for 1.5 – 2.5 in most centres. The ERS guidelines quote two retrospective, single centre studies as reason for their recommendation; Rich et al were assessing the effect of high dose calcium channel blockers on patients with IPAH. They carried out a lung scan on the patients and if the scan revealed nonuniformity of pulmonary blood flow, they were treated with anticoagulant therapy. Patients had additional supportive treatments added depending on their other signs and symptoms. Survival was found to be better at 1 year in the warfarin treated group than the group not treated with warfarin[72]. The study was not designed to assess the influence of anticoagulation and the authors suggest that the results be interpreted with caution as the group treated with warfarin may represent a subgroup with greater likelihood of survival. The second paper was the postmortem study by Fuster et al. Again this study was retrospective and not designed to look at the effects of anticoagulation, but there did appear to be a survival advantage in the group treated with warfarin though the numbers were small and didn't reach statistical significance[34]. A qualitative systemic review of 7 small retrospective observational studies evaluating the effectiveness of warfarin in PAH found that 5 studies supported the effectiveness of anticoagulation therapy in PAH with regards to survival, whereas 2 did not[73]. They conclude that a randomised controlled trial is needed to definitively address this important clinical issue. To date, an randomised control trial (RCT) has yet to be carried out. There is no data regarding the risk

of stroke or peripheral embolism in patients with PAH. The reason behind starting warfarin is that there is a possible long term survival advantage to those on warfarin. This is explained to patients when they are diagnosed and will be made clear to them again in the PIS.

In summary the evidence base for anticoagulation in PAH is historical and not based on validated RCT. Therefore it is difficult to quantify any risk the patient may have off this treatment. The rationale is based on the theory that due to slower blood flow in the pulmonary circulation in PAH as a result of a reduced cardiac output, there is an increased risk of in situ thrombosis rather than embolic events. We will fully inform the subject that there is a probable low risk of any embolic event while off warfarin. It is commonplace for us to stop warfarin in this patient group for right heart catheterization and we have not had any patients suffer adverse effects as a consequence.

PH secondary to left ventricular failure

Some patients in the group with left heart disease causing pulmonary hypertension may also be on warfarin. This may be because of their pulmonary hypertension with similar indications for anticoagulation as explained above although anticoagulation is not even mentioned in guidelines for the management of this disease[13,16,74].

They may also have atrial fibrillation as a co-morbidity and be on warfarin for this. Dunn and Turpie carried out a systematic review on patients receiving long-term anticoagulant therapy who stopped it for invasive procedures[75]. 29 thromboembolic events occurred among 1868 patients (1.6%), including 7 strokes (0.4%)

We will calculate the risk of stroke in each possible study subject with atrial fibrillation using the $CHADS_2$ score. We will explain the risk to each individual patient – annual risk will vary from 1.9 to 18% depending on their score. The patients will only be off warfarin for a matter of days so their actual risk will be minimal.

3. *'A full explanation must also be given that patients who are taken off warfarin for a period of time will require to be re-loaded with warfarin, which can take a number of days for them to come up to full anti-coagulation':*

This has been put in the PIS.

4. *'For patients coming off warfarin, a letter should be sent to their GP, explaining why and for how long':*

Changes have been made to the GP information sheet to include this.

5. *'An explanation should also be given in the PIS of what will happen should the date for their right heart catheterisation is changed or cancelled, so that patients are not left off warfarin indefinitely'.*

The PIS has been changed to explain to patients that on the rare occasion that their right heart catheterisation is delayed or cancelled, they should restart their warfarin and that they will be contacted by a member of the study team who will advise them on how to do so.

2.1.11   Blood assays

Blood samples (10ml) were collected before and at the end of each drug infusion into ethylene diamine tetraacetic acid (EDTA) and gel and clot activator tubes. The samples were kept chilled until transported to the laboratory where they were centrifuged to obtain plasma and serum and stored at -80°C.

The samples from a selected group of patients were then sent to colleagues at Bristol Myers Squibb, Princeton, USA (Joelle Onorato and Carrie Xu) who then measured apelin concentrations and passed on the results. This is their method of analysis:

(Pyr[1])apelin-13 plasma concentration was determined using immunoprecipitation followed by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) analysis. Briefly, human plasma (500 μL, kept on ice) was first treated with 2 x 0.75 mL cold 0.1% formic acid (FA) in isopropanol. Following

centrifugation, the supernatants were dried under nitrogen then reconstituted in phosphate buffered saline (PBS)/Tween. For immunoprecipation, an anti-(pyr[1])apelin-13 monoclonal antibody (Bristol Myers Squibb, Princeton, USA) was bound to high capacity Protein G magnetic beads (Promega). The reconstituted human plasma was incubated overnight at room temperature with 25 µL of the antibody-bead slurry. After multiple washes (pyr[1])apelin-13 was eluted from the bead complex using 0.1% bovine serum albumin (BSA)/0.1% FA in 90:10 water:methanol. The eluates were analyzed by LC/MS/MS on a Sciex API6500 mass spectrometer coupled to a Shimadzu Nexera X2 LC-30AD pump and Sil-30AC MP autosampler. Gradient elution was performed on a Waters BEH300 2.1 x 50 mm column using a mobile phase of 0.1% FA and 0.1% FA in acetonitrile. The transitions monitored were m/z 384.2–424.8 for (pyr[1])apelin-13 and m/z 387.4→426.8 for the internal standard (^{13}C,[15]N-(pyr[1])apelin-13 from Innovagen). For normalization, internal standard was added to all human plasma and calibration curve samples prior to starting the sample extraction protocol. Calibration curves were generated by spiking (pyr[1])apelin-13 standard into blank human plasma (purchased from Bioreclamation and heated at 37°C overnight to remove any endogenous apelin) and extracting apelin following the identical sample extraction protocol described above. The concentration of (pyr[1])apelin-13 in clinical human plasma was calculated based on the calibration curves, using the peak area ratio of measured apelin to internal standard. The limit of quantification of the assay was approximately 4 pM.

2.1.12   Data and statistical analysis

The primary end-point of the study is the change in PVR from baseline. This is a standard and well validated primary or secondary end-point in clinical trials for PAH therapies. Secondary outcomes included change in CO and systemic vascular resistance (SVR). Based on previous studies[76-81] investigating the acute pulmonary haemodynamic effects of PAH treatments, we calculated that to see an effect size of 20% (standard deviation of 149 dyn·s·cm[-5]), we would need a sample size of around 21[81] at 80% power and two-sided $P<0.05$.

Parameters are reported as mean ± SEM and analysed with repeated-measures ANOVA with posthoc Bonferroni corrections (Graph-Pad Prism, GraphPad

Software Inc, San Diego, California). Statistical significance was taken two-sided p<0.05.

2.1.13   Funding

In order to fund the study I applied for funding from the British Heart Foundation, which included funding for myself to run the pulmonary arterial hypertension arm of the study, as well as for a salary to recruit a clinical fellow at the University of Edinburgh Centre for Cardiovascular Science site, which was planned to recruit patients with Pulmonary Hypertension secondary to left heart disease and the normal controls. It also allowed funding to purchase an ergometer (Figure 2-5, Lode Corvial Supine Ergometer with cardiac stress table) for the Edinburgh site and the National Service for Pulmonary Hypertension at Hammersmith Hospital in London. The Scottish Pulmonary Vascular Unit in Glasgow already had this ergometer. The funding also paid for consumables for the right heart catheter studies including staffing required for the studies, for (Pyr[1])-Apelin-13 (Clinalfa AG, Laufelfingen, Switzerland), and for the pharmacy costs in storing this which included purchase of a freezer.

2.2 Basic Science Study – *in vitro*

2.2.1 Materials

All general chemicals were of analar grade.

Tissue plastic-ware were obtained from Greiner Labortechnik Ltd (Gloucestershire,UK), Costar (Uk) and Corning Incorporated (NY,USA).

Solution/chemical	Composition/Source
10% and 20% Culture medium	500ml Dulbecco's Modified Eagles Medium (DMEM) (Sigma,UK) 50mls (10%) or 100mls (20%) fetal calf serum (FCS) 800iu penicillin-streptomycin (Sigma,UK) 8mM L-glutamine (Invitogen,UK)
Serum free medium	500ml Dulbecco's Modified Eagles Medium (DMEM) (Sigma,UK) 800iu penicillin-streptomycin (Sigma,UK) 8mM L-glutamine (Invitogen,UK)
Phosphate buffered saline (PBS) solution	2x Phosphate buffered saline tablets (Sigma, UK) 400mls distilled water composition: 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4.
Trypsin solution	100mls 0.5% Trypsin-EDTA 10x (Fisher Scientific, UK) 400mls PBS solution
cryopreservation medium	1ml dimethyl sulphoxide (Sigma, UK) 9mls 10% culture medium
5% Trichloroacetic acid	4.1g powdered trichloroacetic acid 500mls distilled water
0.3M sodium hydroxide solution	6g sodium hydroxide pellets (Sigma, UK) 500mls distilled water
Radioimmunprecipitaion assay (RIPA) buffer	Thermo Fisher Scienticfic
methyl-[3][H] Thymidine	Amersham (UK)
Trypan blue stain	Gibco (UK)
Ethanol	
Ecoxcint XR Scintillation fluid	National Diagnostic
SDS sample buffer	Bioland scientific
10% non-fat milk in PBS	200ml distilled water

	1x Phosphate buffered saline tablets (Sigma, UK) 20 gram milk powder (Marvel)
Wash buffer (0.2% PBS Tween wash)	10x Phosphate buffered saline tablets (Sigma, UK) 2 litres distilled water 4 mls Tween detergent (Thermo Fisher Scientific)
5% non-fat milk in wash buffer	200mls wash buffer (as above) 10 grams milk powder (marvel)
Enhanced Chemoluminescence (ECL) solution. Reagents 1 & 2	Thermo Fisher Scientific
Apelin (experimental)	genscript
APJ receptor antibody	MBL Int Corp.
Total p38 MAP kinase antibody	cell signaling
phosphorylated p38 MAP kinase antibody	cell signalling

Table 2-1 Details of commonly used experimental solutions

Galaxy R incubators were supplied by Wolf Laboratories (Figure 2-12, York, UK).

To minimise chances of fungal and bacterial contamination all procedures involving making up of solutions or changing media were performed in sterile conditions using a clean Microflow laminar flow hood (Figure 2-10, Model number M25121/1). Non-sterile equipment was sterilised using a Prestige Medical 'classic 210' autoclave. Solutions were warmed to 37°C in a waterbath before using with cell cultures.

Figure 2-10 Microflow laminar flow hood

2.2.2 Animal models

2.2.2.1 Rat model

Rat pulmonary arteries were supplied by my colleague Dr Colin Church. Sprague-Dawley rats were used and all animals were kept in pathogen-free conditions in facilities managed by the biological services staff at the University of Strathclyde, under strict accordance with the guidelines laid out in the United Kingdom Home Office Animals (Scientific procedures) Act 1986. Dr Church supplied pulmonary arteries from normal healthy rats, as well as two rat models of pulmonary hypertension; a chronic hypoxic model and a monocrotaline model. Both are classic models of pulmonary hypertension and well established in the literature[82].

The chronic hypoxic model was developed by placing rats in a hypobaric hypoxic chamber for a period of 14 days. The monocrotaline model was developed by injecting rats subcutaneously with 60mg/kg of monocrotaline. The monocrotaline animals developed signs of pulmonary hypertension over 2-3 weeks. Both animal models showed evidence of pulmonary hypertension both haemodynamically (right ventricular systolic pressure was measured under anaesthesia) and histologically.

2.2.3 Primary cell culture

The principal cells utilised for the experiments in this thesis were pulmonary artery fibroblasts (Figure 2-11). These were derived from rat or cow. The explant technique is well established in the Scottish Pulmonary Vascular Unit laboratory and has been previously published[83].
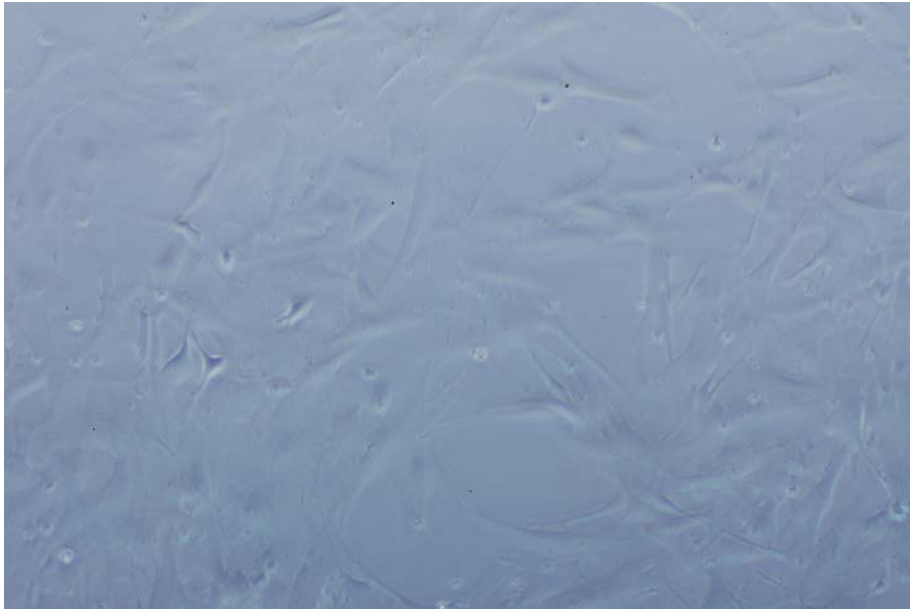
Figure 2-11 Typical appearance of rat pulmonary artery fibroblasts

The main pulmonary artery was dissected from the lung. This was cleaned of adipose tissue then cut longitudinally and opened into a flat sheet. Muscular tissue and endothelial cell layers were removed by gentle abrasion of the vessel using a sterile razor blade to leave the adventitia. The remaining tissue was dissected into 5mm^2 pieces. These were evenly distributed in a 25cm^3 tissue flask containing 1.5mls of 20% culture medium. The flasks were placed in a humidified atmosphere in the laboratory's incubator with 5% CO_2 and 21% O_2 at a controlled temperature of 37°C. Within a few days cells were observed growing out from the explants. When required a further 0.5mls of 20% culture medium was added to the flask. Once 50% of the flask was covered in a monolayer of cells they were detached from the flask by trypsinisation (as described in chapter 2.2.4.1) in order to transfer them to a fresh 75cm^3 culture flask to allow expansion of the number of fibroblasts. The explants were removed before transfer to the 75cm^3 culture flask.

Cells had the typical appearance and morphology of fibroblasts, were negative for smooth muscle α–actin and positive for vimentin at western blot.

2.2.4 Cell maintenance

Cells were routinely grown in 75cm^3 culture flasks with 10% culture medium. The cells were kept in the laboratory's incubator with 5% CO_2 and 21% O_2 at humidified 37°C. The culture medium was changed every 2-3 days and the cells were passaged just prior to being fully confluent in the flask.

2.2.4.1 Cell passage

Cells were used in experiments between passages 2 and 6. To passage the cells the culture medium was removed and they were washed with serum free medium. This was removed and the cells were then washed with 2mls of trypsin solution. This was left for 10 seconds then the excess was removed. The flask was then placed back in the incubator at 37°C for 2-5 minutes until the cells had detached from the flask surface (inspected by light microscopy). The flask was gently agitated to aid cell detachment. The trypsin was then deactivated by adding 10mls of 10% culture medium to create a cell suspension. 1-2mls of the cell suspension was added to a new 75cm^3 culture flask and topped up to 10mls with fresh 10% culture medium. The remaining cell suspension was further diluted with 10% culture medium and aliquoted into 6, 12 or 24 well plates for experimentation.

2.2.4.2 Cell storage

Confluent cells at an early passage were trypsinised with the same protocol as for cell passage and resuspended in 10mls of 10% culture medium. The suspension was then centrifuged at 3000rpm for 10 minutes at room temperature. The culture medium was carefully removed leaving a cell pellet at the bottom of the centrifuge tube. This cell pellet was resuspended in 1 ml of cryopreservation medium which was transferred to a 2ml cryotube and cooled for 1 hour each at +4°C, -20°C then at -80°C overnight before transfer into liquid nitrogen chamber (-200°C).

When required, frozen cells were removed from the liquid nitrogen chamber and thawed in a water bath at 37°C. The cell suspension was then transferred into a 75cm^3 culture flask containing 9mls 10% culture medium. Once the cells had adhered to the flask the culture medium was changed.

2.2.5 Acute hypoxia

The Galaxy R incubator (Figure 2-12) allowed control of oxygen levels between 0-21% due to nitrogen supplementation as well as providing a humidified, temperature-controlled atmosphere with CO_2 levels being maintained at 5%.

Figure 2-12 Galaxy R incubator

The lab contained 2 Galaxy R incubators, one which was maintained at normoxic experimental conditions, whilst the other was maintained at 5% O_2 to provide hypoxic experimental conditions for the cells. The laboratory has previously established that cells maintained in this environment for 48hours achieves a PO_2 of 35mmHg and a stable physiological pH.

2.2.6 Determination of cell viability and proliferation

2.2.6.1 Cell counting and viability

Cells were seeded into plates and appropriate experiments were performed. At completion of the experiment the cells were trypsinised with 400µl of trypsin solution. The detached cells were then resuspended in 1ml of PBS. 10µl of the mixture was then mixed with 10µl of 0.4% trypan blue stain. 10µl of the resultant mixture is transferred to a Neubauer Haemocytometer. non-viable cells are permeable and take up the blue stain. The haemocytometer is placed under a light microscope at x10 power and the grids etched onto the haemocytometer allow counting of the viable and non-viable cells.

The equation for total number of cells in the original solution is shown as Equation 2-4:

$$Number\ of\ cells\ in\ original\ solution = 2 \times \frac{Total\ number\ of\ cells\ counted\ in\ 4\ squares}{4} \times 10000$$

Equation 2-4

2.2.6.2 [3H] Thymidine proliferation assay

[3H] thymidine is a radioactive labelled thymidine. Under different experimental conditions [3H] thymidine is incubated with cells. During each cell division the cells will incorporate [3H] thymidine into its DNA. The more cell divisions (ie the higher the proliferation rate) the more radioactivity will be incorporated into the DNA. This can then be measured.

Cells were seeded into 24 well plates at a density of 5×10^3 - 1×10^4 cells/well with 500µl of 10% culture medium. The plates are returned to the incubator until they reach 60% confluency. The cells were then quiesced (growth arrested) by replacing the medium with serum-free medium and placed in the incubator for 24 hours. The cells were then ready for their experimental conditions. They were then stimulated with appropriate agonists and incubated in the normoxic incubator or in the hypoxic incubator where the O_2 was reduced to 5% for 24 hours.

Dilute [³H] thymidine was prepared by adding 10µl of stock solution (1 mCi/ml) to 2.5mls of serum-free medium. At 20 hours 25µl of the dilute [³H] thymidine solution is added to each well to allow incorporation of the radiolabeled thymidine into the cells DNA during the final 4 hours of the experiment.

To end the experiment the reaction was stopped by washing the cells twice with ice-cold PBS solution. The wells were then washed three times with trichloroacetic acid which precipitates nucleic acid polymers longer than ~20 nucleotides and therefore separates radiolabeled nucleotides incorporated into nucleic acid from unincorporated label. The lipid fractions were then solubilised by washing twice with 100% ethanol. The final precipitants were then dissolved in 0.3M sodium hydroxide solution and left for 30 minutes. 500µl of each solution was added to each well per wash.

The resultant solution was aspirated from each well and added to a 1ml eppendorf tube. 0.9mls of Ecoscint XR scintillation fluid. The vials were agitated then left to settle overnight. The resultant radioactivity was measured by a scintillation counter and expressed as either counts per minute (CPM) or disintigrations per minute (DPM).

2.2.7 Detection and analysis of proteins

2.2.7.1 Preparation of samples for protein analysis

Cells were grown on 6 well plates and grown to 90% confluency with 10% culture medium. They were then quiesced with serum-free medium for 24 hours before being stimulated by agonist and placed in either normoxic or hypoxic incubators for 24 to 48 hours.

The culture plates were removed from the incubators and placed on ice. The culture medium was removed and the cell monolayer was washed twice with 500µl of ice-cold PBS. The cells were then lysed and the proteins solubilised with 50µl of radioimmunoprecipitation assay (RIPA) buffer for 10 minutes. The cells were then mechanically disrupted by using a cell scraper and the contents of each well were transferred to 1ml eppendorf tubes sitting in ice then stored at -80°C.

2.2.7.2 Western blot procedure: Gel electrophoresis

The samples for protein analysis were prepared by adding SDS-sample buffer and reducing agent at an appropriate concentration. The proteins were then denatured by heating to 70°C for 10 minutes. 20 µL of sample was loaded into Sodium Dodecyl Sulphate Polyacrylamide (SDS-PAGE) 10% gels (Invitrogen) along with a molecular marker in a separate well (See-blue Plus 2, Invitrogen) which allowed determination of the protein sizes.

The gels were loaded into a Novex Xcell mini-cell electrophoresis unit (Figure 2-13) with running buffer (Invitrogen). 150V were applied for 1 hour or until the proteins reached the bottom of the gel.

Figure 2-13 Novex Xcell mini-cell electrophoresis unit with gel loaded

2.2.7.3 Western blot procedure: Transfer to nitrocellulose membrane

The gels were carefully removed from their cassettes and transferred to a nitrocellulose membrane using Novex Xcell blot module and transfer buffer (Invitrogen). 30V was applied for 1 hour.

2.2.7.4 Western blot procedure: immunoblotting

The nitrocellulose membrane is then blocked with 10% non-fat milk (Marvel) in phosphate buffered saline (PBS) by agitating at room temperature on a Gyro Rocker (Stuart Scientific) then incubating at 4°C overnight.

The membranes were then placed in a solution of 5% non-fat milk in wash buffer with primary antibody at a dilution of 1:500 to 1:1000. The membranes were agitated at room temperature for 2 hours in primary antibody before being washed with 2 litres of washer buffer.

The membrane was then incubated for a further 1 hour in 5% non-fat milk in wash buffer with 1:1500 dilution of secondary antibody conjugated to horseradish peroxidase (HRP).

To detect the protein bands the membranes were then incubated in Enhance Chemiluminescence (ECL) solution (Amersham) for 1 minute and placed in a developing cassette with a xray film for different periods of exposure. The film was developed using a Kodak X-OMAT processor.

2.2.7.5 Western blot procedure: densiometry

Using Image J software (a Java based freeware by Wayne Rasband from National Institute of Health, USA) the protein bands were relatively quantified from the western blot films. The quantification reflects the relative amounts as a ratio of each protein band relative to the lane's loading control.

2.2.8 Cell migration

The migratory capacity of pulmonary artery fibroblasts was investigated with the use of Cytoselect 24-well cell migration assay kit (Cell biolabs, Inc.).

A fibroblast cell suspension of 0.5-**1.0 x 106 cells/ml in serum free media.** 300 μL
of this solution was added to each insert which had a porous membrane as its
base. The membrane acts as a barrier to discriminate migratory cells from non-
migratory cells. The inserts are placed in a lower chamber containing media
and/or chemoattractant(s) and incubated in a cell incubator at 37°C for 24 hours
(Figure 2-14 A). Migratory cells will pass through the porous membrane (Figure
2-14 B). The inserts were then washed to remove all non-migratory cells (Figure
2-14 C). The insert is then stained using Cell Stain Solution for 10 minutes at
room temperature (Figure 2-14 D).

Figure 2-14 Cytoselect Migration assay

The cells on the bottom of the membrane could then be counted directly using
light microscopy.

2.2.9 Data and statistical analysis

For normally distributed values, responses were reported as mean +/- SEM.
Student t-test was used. For multiple comparisons of means across different
experimental groups analysis of variance (ANOVA) was performed with
Bonferonni *post hoc* analysis. (Graph-Pad Prism, GraphPad Software Inc, San
Diego, California). Statistical significance was taken two-sided $p < 0.05$.

3 Results: Clinical study

3.1 Pulmonary Arterial Hypertension Group

For the pulmonary arterial hypertension group I managed to recruit 20 subjects. As explained in the methods & materials chapter, because PAH is a rare disease, to make recruitment of our sample size of 21 realistic and feasible we collaborated with The National Pulmonary Hypertension Service at Hammersmith Hospital in London.

The Glasgow site recruited 15 subjects for the PAH group. Subject 301 (the first recruited) was excluded from analysis as this study was carried out with the original apelin doses of 30, 100 and 300 nmol/min before the amendment to change the doses to 10, 30 and 100 nmol/min. The London site recruited 6 subjects for the PAH group. Unfortunately for one of the studies in London, there was a miscommunication between members of the study team meaning only half of the study protocol was carried out before the study was abandoned. The subject only received infusion A but did not enter the crossover part of the study and so did not receive infusion B. This was reported to the National Research Ethics Service in the annual report as a breach of protocol and the study was excluded from analysis.

The total number of study recruits included in analysis for the PAH arm of the study was 19.

3.1.1 Baseline demographics

This study had a crossover design meaning that each subject served as his or her own control. Table 3-1 shows the baseline demographics of the subjects recruited.

Table 1. Demographic and haemodynamic characteristics at baseline.

	Male (n=7)	Female (n=12)	All (n=19)	<median age	>median age
Age, y	52.8	51.3	51.8		
Gender					
Male				3/10 (30%)	4/9 (44%)
Female				7/10 (70%)	5/9 (56%)
Diagnosis					
IPAH	7/7	10/12	17/19	9/10	8/9
CTD PH	0/7	2/12	2/19	1/10	1/9
Smoker					
never	6/7	5/12	11/19	7/10	4/9
current	0/7	4/12	4/19	3/10	1/9
ex	1/7	3/12	4/19	0/10	4/9
Hb, g/dL	15	13.6	14.1	14.3	14
BMI, kg/m²	29.1	29.2	29.1	27.7	34.8
Medication, n					
PDE5i	4/7 (57%)	7/12 (58%)	11/19 (58%)	9/10 (90%)	2/9 (22%)
ERA	3/7 (43%)	6/12 (50%)	9/19 (47%)	5/10 (50%)	4/9 (44%)
Prostacycline	0/7	3/12 (25%)	3/19 (16%)	2/10 (20%)	1/9 (11%)
treatment naïve	3/7 (43%)	2/12 (17%)	5/19 (26%)	1/10 (10%)	4/9 (44%)
Baseline haemodynamics					
Heart rate, bpm	65	76	72	76	67
MAP, mmHg	89.6	86.9	87.9	84.6	91.7
RAP, mmHg	7.6	5.1	6.1	5.8	6.3
RVP, mmHg	84	79	80	86	75
mPAP, mmHg	49.3	47.9	48.4	54.3	41.9
PAWP, mmHg	7.9	6.8	7.2	6.8	7.7
CO, L/min	4.7	4.9	4.8	4.6	5.1
PVR, WU	9.2	9.1	9.1	11.1	7.0
SV ,ml	74.7	66.9	69.7	62.5	77.7
SVR, dyns	1455.5	1404.3	1423.2	1467.4	1374.1

BMI indicates body mass index; Hb, haemoglobin; NYHA, New York Heart Association; PDE5i, phosphodiesterase inhibitor; ERA, endothelin receptor antagonist; MAP, mean arterial pressure; RAP, right atrial pressure; RVP, right ventricular pressure; mPAP, mean pulmonary artery pressure; CO, cardiac output; PVR, pulmonary vascular resistance; SV, stroke volume; SVR, systemic vascular resistance; Data are expressed as mean when appropriate.

Table 3-1 Demographic and haemodynamic characteristics at baseline

3.1.2 Results from resting studies

In comparison to saline placebo, (Pyr[1])apelin-13 infusion reduced PVR (p=0.001) with an effect that appeared maximal at 30 nmol/min (Figure 3-1). (Pyr[1])apelin-13 infusion increased CO (p<0.0001) and stroke volume (SV) (p<0.0001) but did not affect heart rate or mean pulmonary artery pressure (mPAP) Pulmonary artery wedge pressure was also not affected by (Pyr[1])apelin-13 infusion (data not shown). Consistent with Professor Newby's previous systemic studies[58,59], (Pyr[1])apelin-13 caused a reduction in systemic vascular resistance (SVR) (p<0.05).
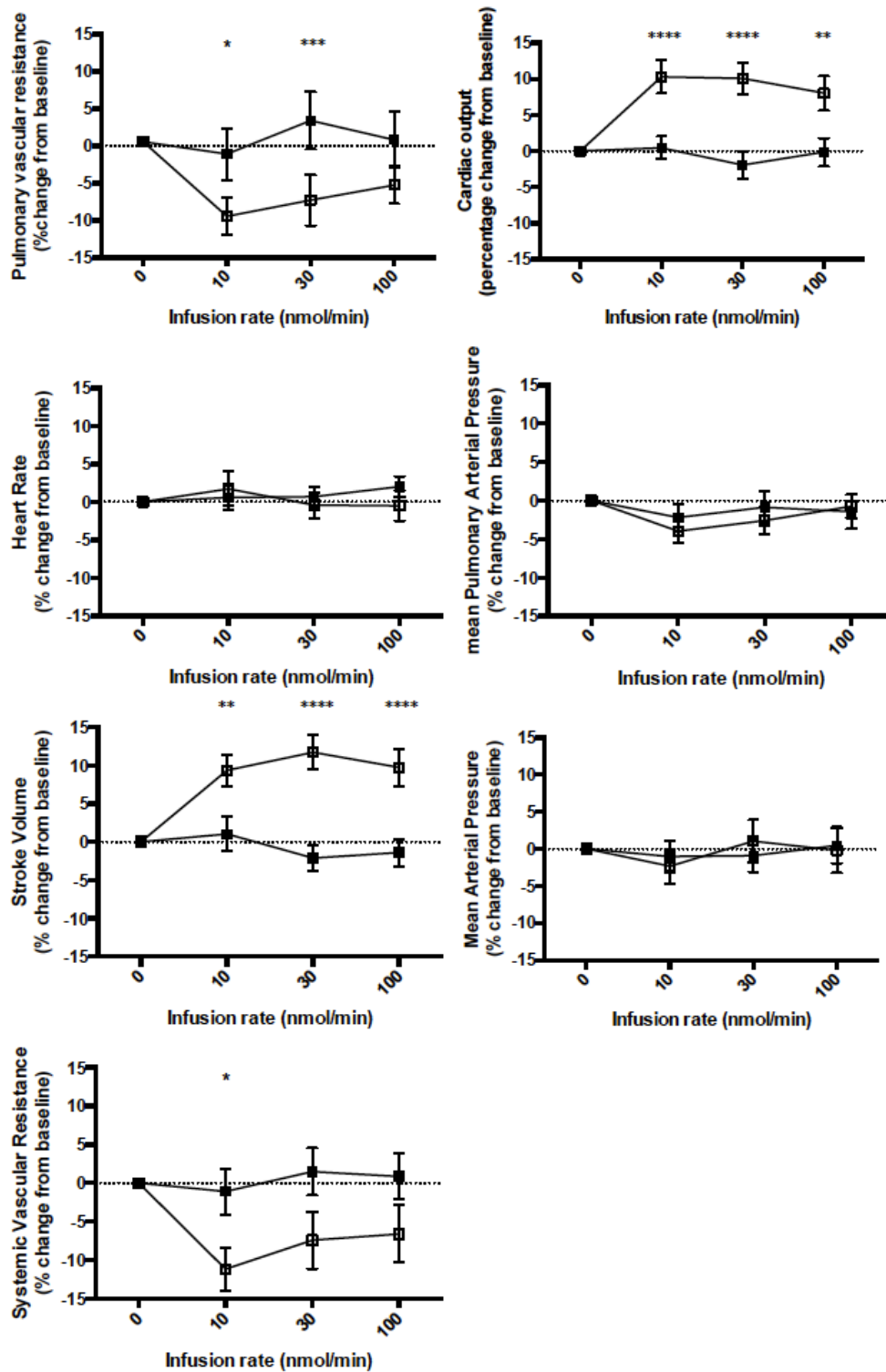
Figure 3-1 Percentage change in baseline haemodynamics during resting infusions
Percentage change from baseline in pulmonary vascular resistance, cardiac output, heart rate, mean pulmonary artery pressure, stroke volume, mean arterial pressure and systemic vascular resistance during infusion of (Pyr[1])apelin-13 (☐) or matched saline placebo (■) in patients with pulmonary arterial hypertension. *p<0.05, **p=0.01, ***p=0.001, ****p=0.0001 2-way ANOVA with posthoc bonferroni tests.

3.1.3 Results from exercise studies

No difference was seen in any of the haemodynamic variables during exercise except PVR (Figure 3-2) which fell during (Pyr[1])apelin-13 infusion (p<0.05). This was associated with an increase in heart rate (Figure 3-2, p<0.01).
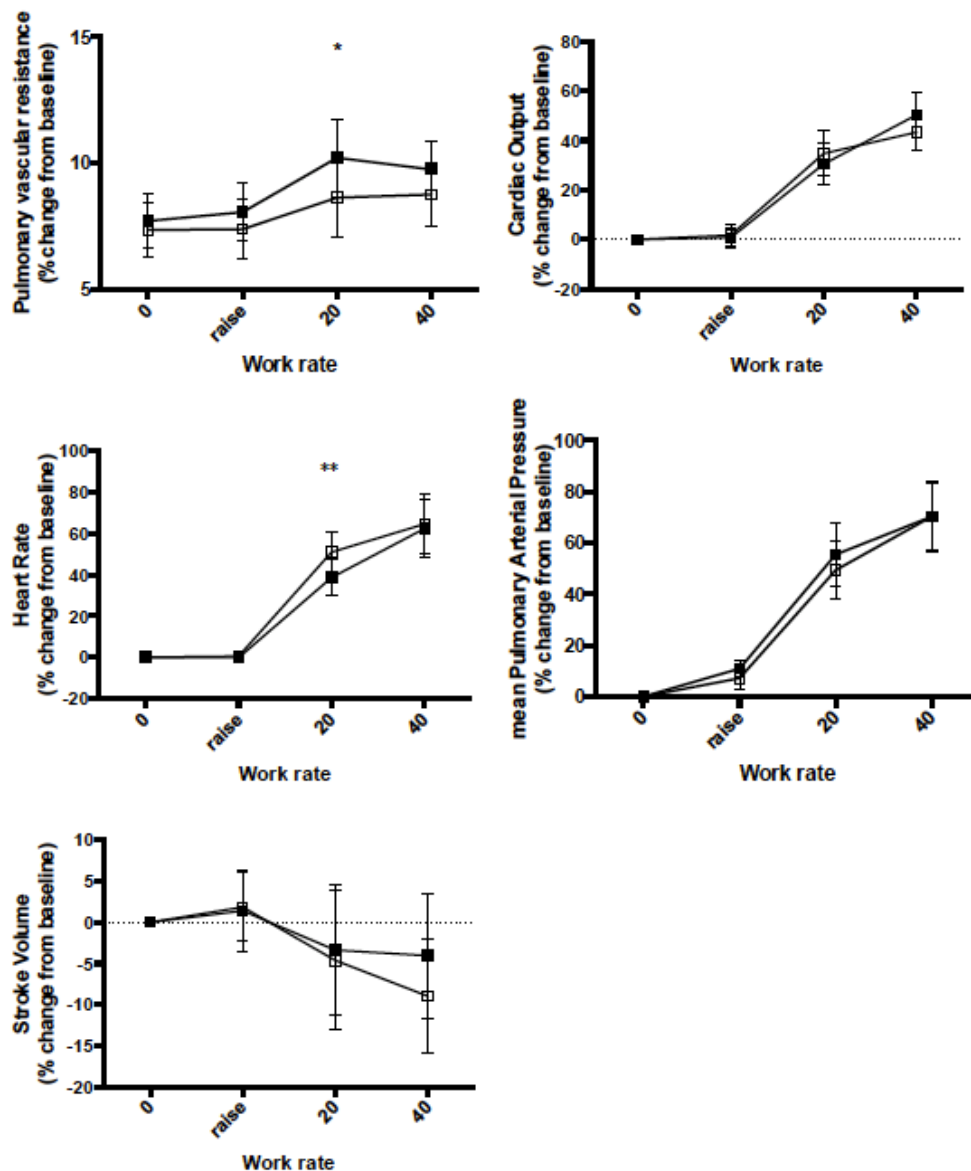
Figure 3-2 Percentage change in haemodynamics during exercise
Percentage change from baseline in pulmonary vascular resistance, cardiac output, heart rate, mean pulmonary artery pressure and stroke volume during infusion of (Pyr[1])apelin-13 (□) or matched saline placebo (■) in patients with pulmonary arterial hypertension while undergoing exercise protocol. *p<0.05, **p<0.01 2-way ANOVA with posthoc bonferroni tests.

3.1.4 Post-hoc analysis: Results analysed by age group

3.1.4.1 Haemodynamics in PAH patients ≤ median age
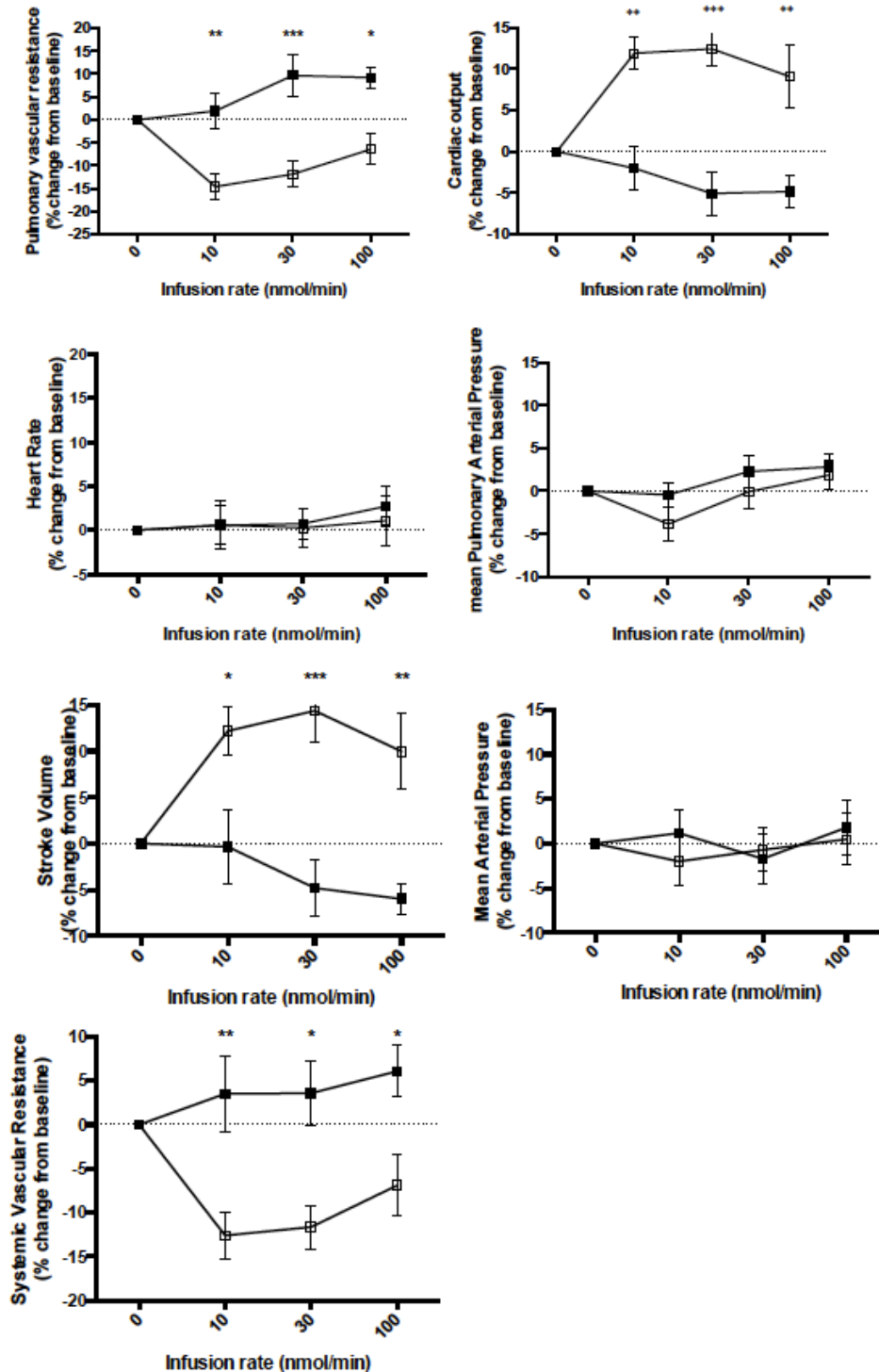
Figure 3-3 Haemodynamics in PAH patients ≤ median age
Percentage change in pulmonary vascular resistance, cardiac output, heart rate, mean pulmonary artery pressure, stroke volume, mean arterial pressure and systemic vascular resistance during infusion of (Pyr[1])apelin-13 (□) or matched saline placebo (■) in patients with pulmonary arterial hypertension ≤ median age of 49 years old. *p<0.01, **p<0.001, ***p<0.0001, 2-way ANOVA with posthoc bonferroni tests.

3.1.4.2 Haemodynamics in PAH patients > median age
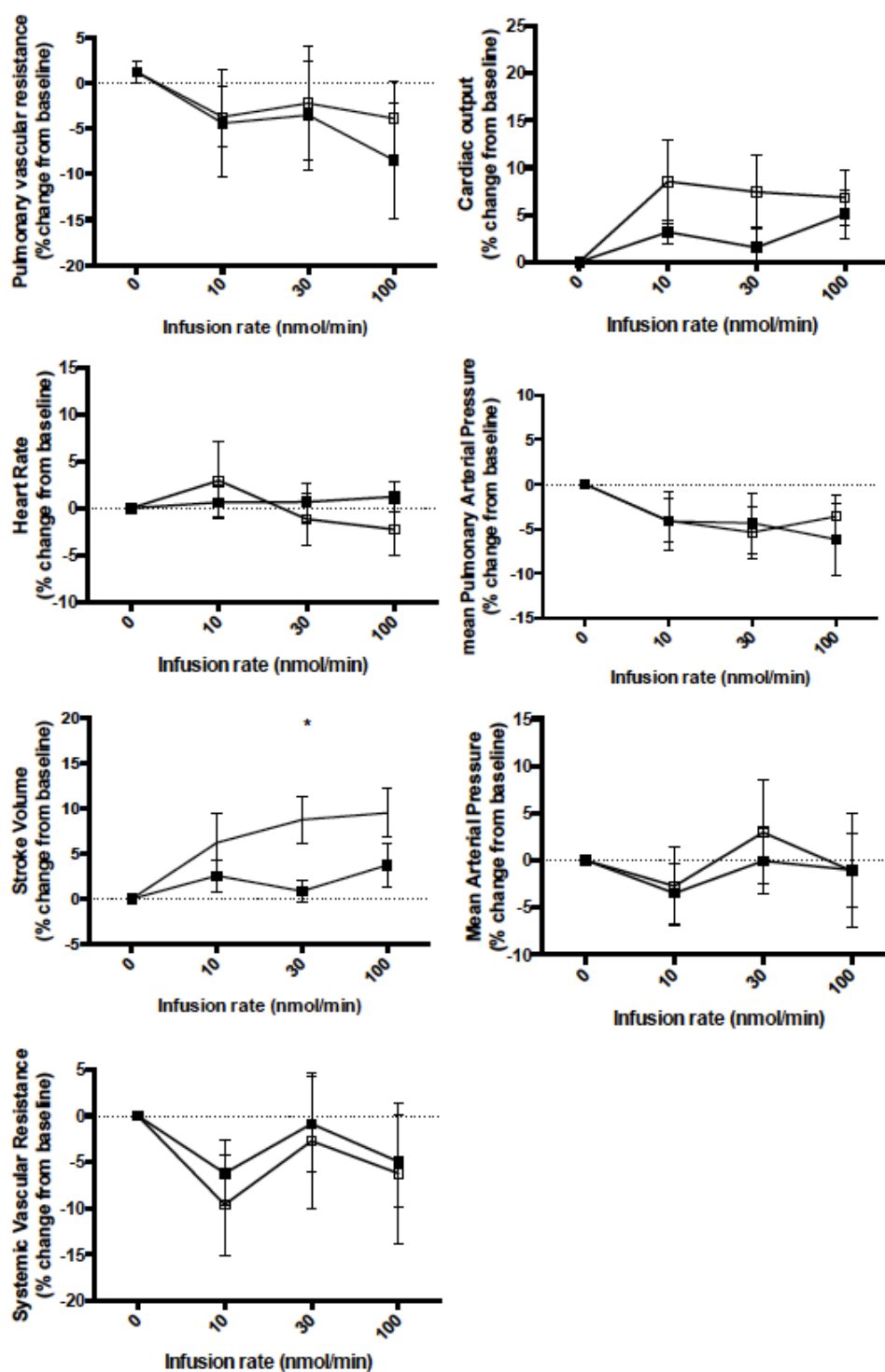
Figure 3-4 Haemodynamics in PAH patients > median age
Percentage change from baseline in pulmonary vascular resistance, cardiac output, heart rate, mean pulmonary artery pressure, stroke volume, mean arterial pressure and systemic vascular resistance during infusion of (Pyr[1])apelin-13 (□) or matched saline placebo (■) in patients with pulmonary arterial hypertension > median age of 49 years old.

3.1.4.3 Results: Analysed by age group

The median age of the subjects is 49 years old and the mean age 52. (The age range was 29-73). I did post-hoc analysis on the data to see if there was any suggestion that age influenced the results. Subjects who were 49 years old or younger had a significant reduction in pulmonary vascular resistance compared with the older age group with (Pyr[1])apelin-13 infusion (Figure 3-3, p<0.0001). This was again with an increase in CO (p<0.0001) and SV (p<0.0001) but with no affect on heart rate or mean pulmonary artery pressure (mPAP). (Pyr[1])apelin-13 caused a much more significant reduction in SVR in the younger subjects (p<0.001). Patients were asymptomatic but were lying supine on the cardiac catheterisation table.

On reviewing Table 3-1, the main differences between the younger group when compared with the older group, is that the younger patients were more likely to be female and more likely to be established on treatment prior to undergoing the study, in particular they were more likely to be on phosphodiesterase-5 (PDE5) inhibitors.

3.1.5 Post-Hoc Analysis: Results analysed by gender

3.1.5.1 Haemodynamics in females with PAH

Figure 3-5 Haemodynamics in females with PAH
Percentage change from baseline in pulmonary vascular resistance, cardiac output, heart rate, mean pulmonary artery pressure, stroke volume, mean arterial pressure and systemic vascular resistance during infusion of (Pyr[1])apelin-13 (□) or matched saline placebo (■) in female patients with pulmonary arterial hypertension. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 2-way ANOVA with posthoc bonferroni tests.

3.1.5.2 Haemodynamics in males with PAH
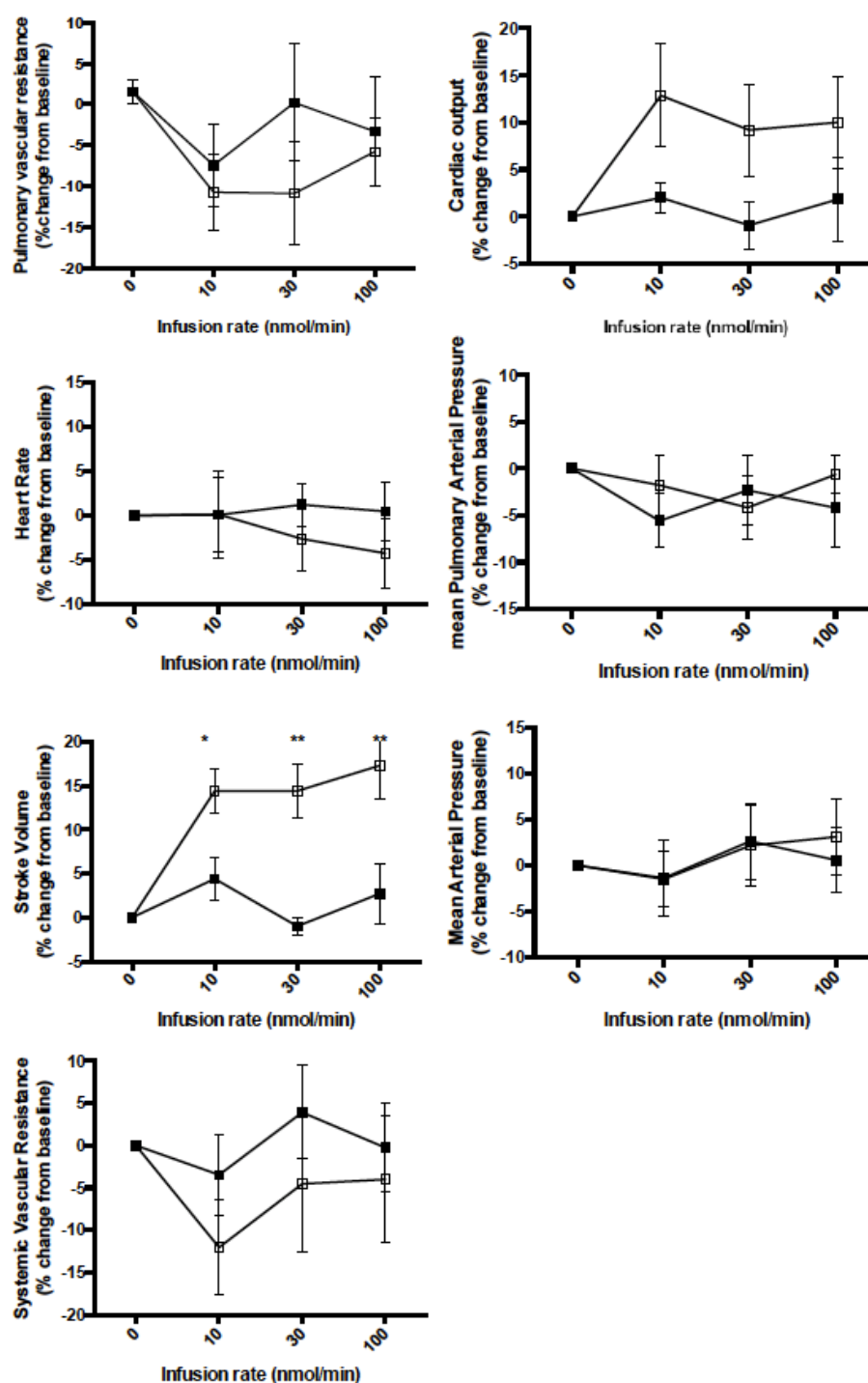
Figure 3-6 Haemodynamics in males with PAH
Percentage change from baseline in pulmonary vascular resistance, cardiac output, heart rate, mean pulmonary artery pressure, stroke volume, mean arterial pressure and systemic vascular resistance during infusion of (Pyr[1])apelin-13 (□) or matched saline placebo (■) in male patients with pulmonary arterial hypertension. *p<0.05, **p<0.01, 2-way ANOVA with posthoc bonferroni tests.

3.1.5.3 Results: Analysed by gender

As the younger age group were more likely to be female (70% of the group), I analysed all the results by gender to see if this may be a significant finding. Table 3-1 shows that there were 12 females included in the study and only 7 males. In comparison to saline placebo, (Pyr[1])apelin-13 infusion reduced PVR in females (Figure 3-5, $p<0.001$) but not males. In females (Pyr[1])apelin-13 infusion increased CO ($p<0.0001$) and SV ($p<0.001$) and did not affect heart rate (HR) or mPAP. In males there was a significant increase in SV but no significant change in CO or PVR. There was a trend towards a reduction in HR, seen in the male group, which was not significant, but this may have had an effect on the CO and PVR not reaching significance. It is difficult to interpret these results as there were so few males included in analysis and I suspect if there were higher numbers we would find no difference between the gender groups.

There was no significant change in systemic vascular resistance for either group.

The only other clinical difference between the two gender groups is that the males were more likely to be treatment naïve, although a similar percentage of males and females were treated with phosphodiesterase-5 inhibitors (Table 3-1).

3.1.6 Post-Hoc Analysis: Results analysed by subjects pre-study treatment

3.1.6.1 Haemodynamics in patients treated with PDE5 inhibitors
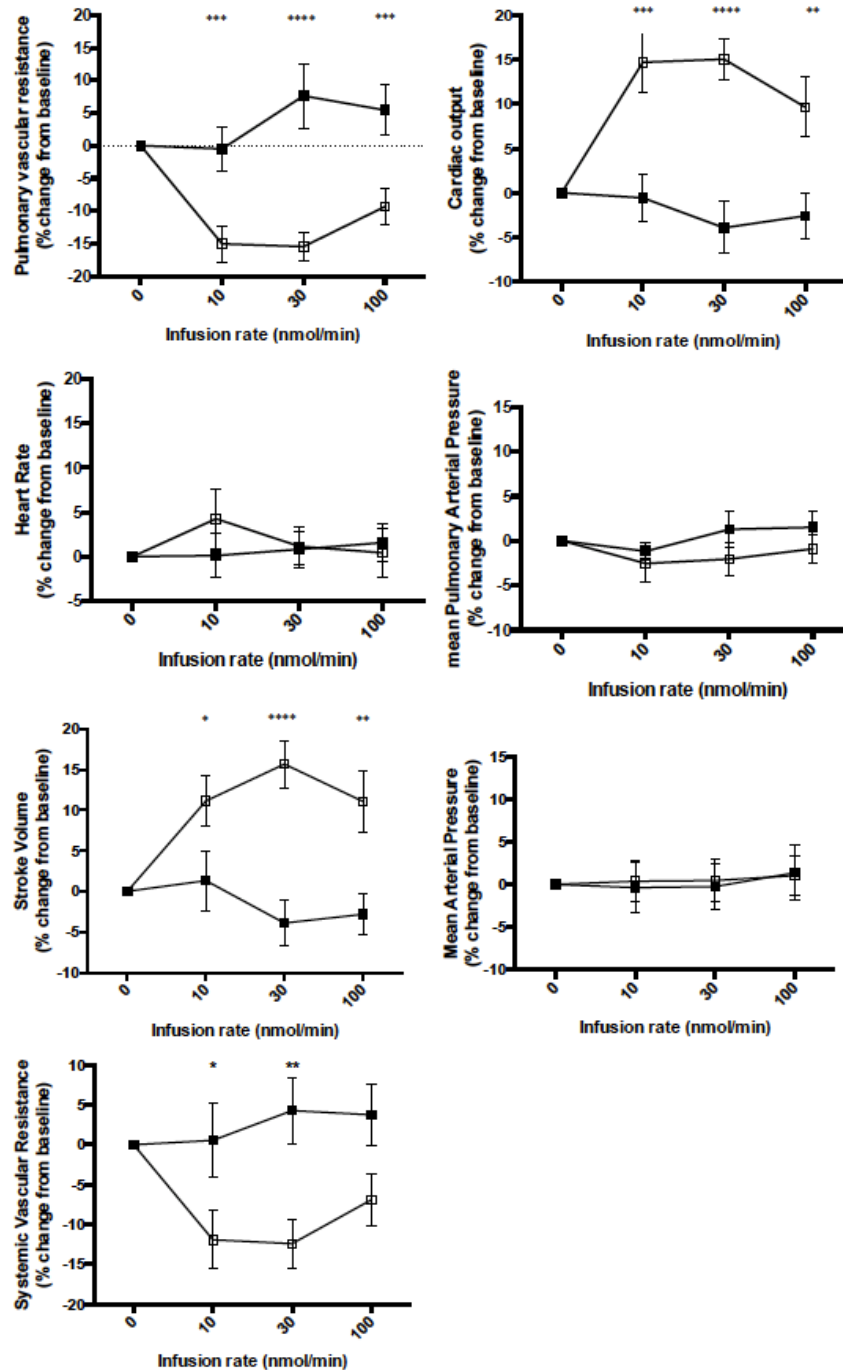
Figure 3-7 Haemodynamics in patients treated with PDE5 inhibitors
Percentage change from baseline in pulmonary vascular resistance, cardiac output, heart rate, mean pulmonary artery pressure, stroke volume, mean arterial pressure and systemic vascular resistance during infusion of (Pyr[1])apelin-13 (□) or matched saline placebo (■) in patients with pulmonary arterial hypertension who are on treatment with phosphodiesterase-5 inhibitors. *p<0.05, ** p<0.01, ***p<0.001, **** p<0.0001 2-way ANOVA with posthoc bonferroni tests.

3.1.6.2 Haemodynamics in patients not treated with PDE5 inhibitors
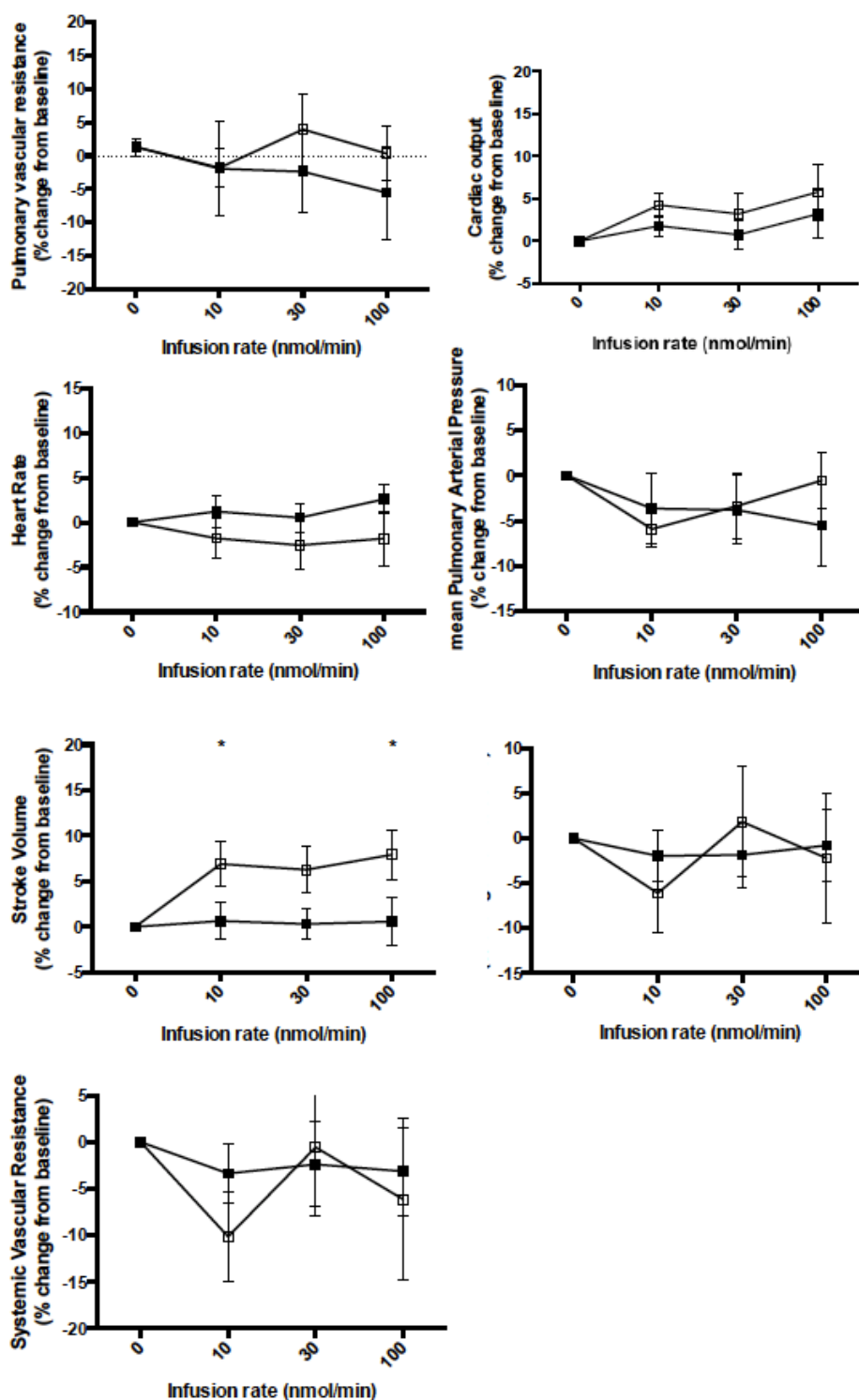
Figure 3-8 Pulmonary Vascular Resistance in patients not treated with PDE5 inhibitors
Percentage change from baseline in pulmonary vascular resistance, cardiac output, heart rate, mean pulmonary artery pressure, stroke volume, mean arterial pressure and systemic vascular resistance during infusion of (Pyr[1])apelin-13 (□) or matched saline placebo (■) in patients with pulmonary arterial hypertension who are not on treatment with phosphodiesterase-5 inhibitors. *$p<0.05$, 2-way ANOVA with posthoc bonferroni tests.

3.1.6.3 Haemodynamics in patients on any treatment
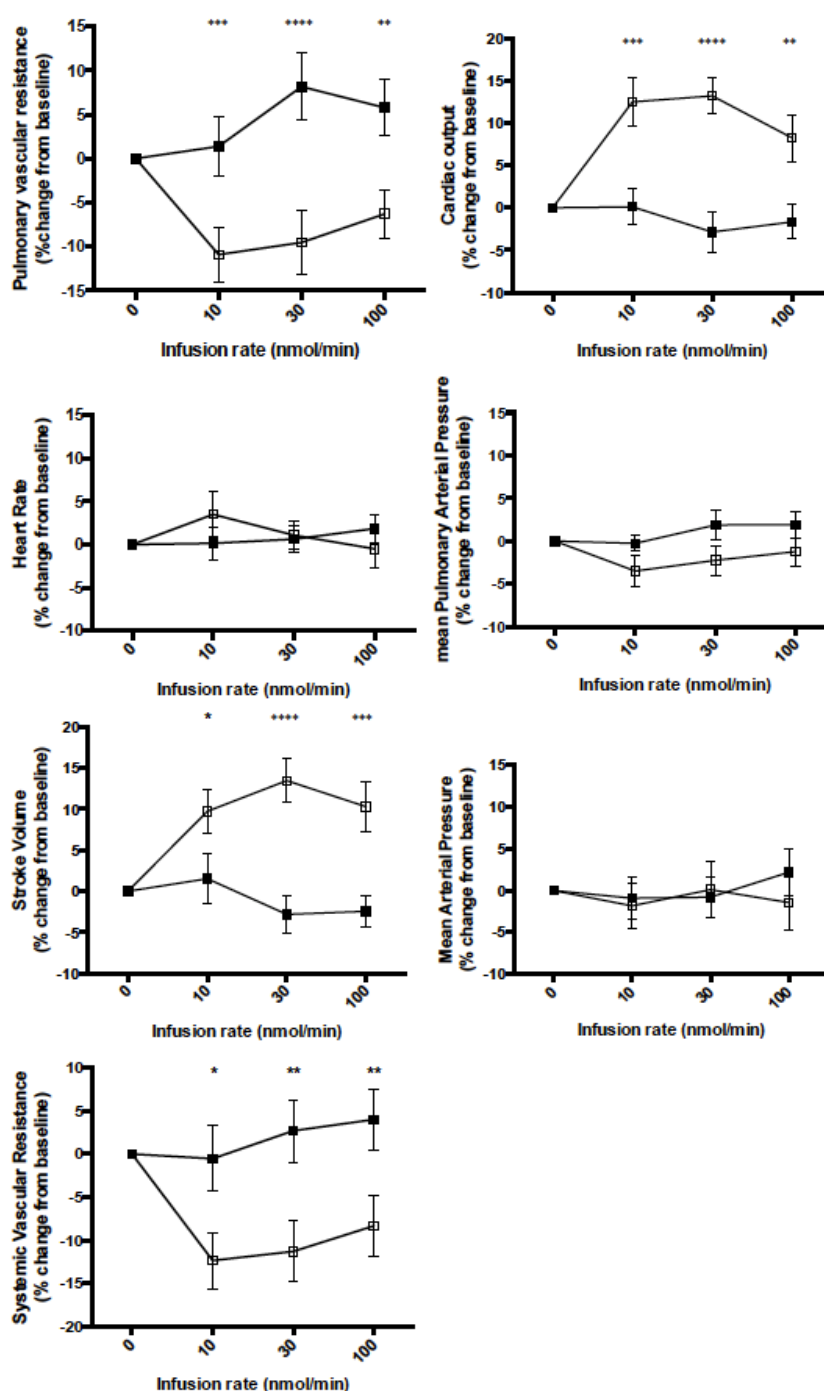
Figure 3-9 Haemodynamics in patients on any treatment
Percentage change from baseline in pulmonary vascular resistance, cardiac output, heart rate, mean pulmonary artery pressure, stroke volume, mean arterial pressure and systemic vascular resistance during infusion of (Pyr[1])apelin-13 (☐) or matched saline placebo (■) in patients with pulmonary arterial hypertension who are established on any treatment. *p<0.05, **p<0.01, *** p<0.001, ****p<0.0001, 2-way ANOVA with posthoc bonferroni test.

3.1.6.4 Haemodynamics in treatment naïve patients
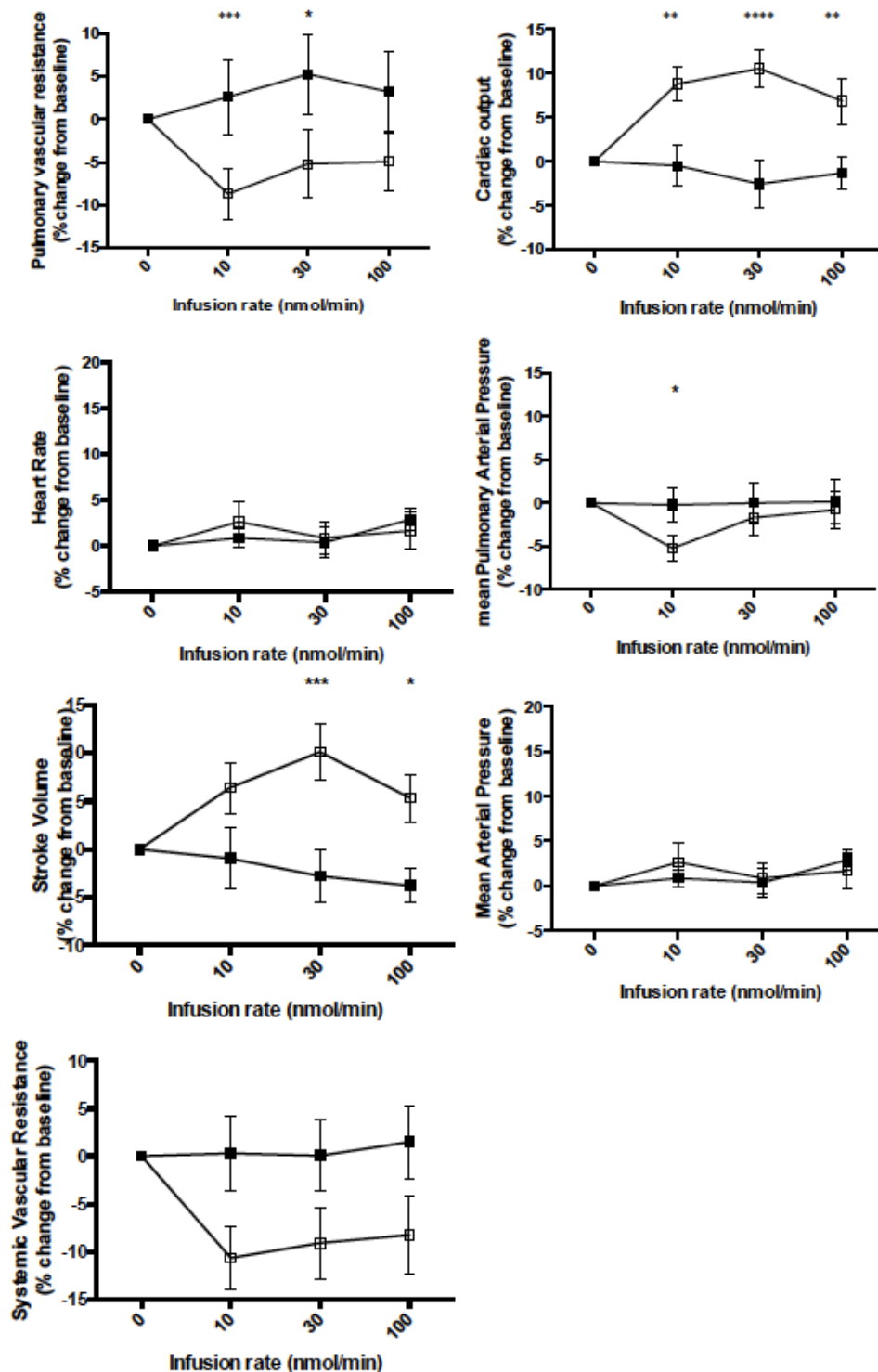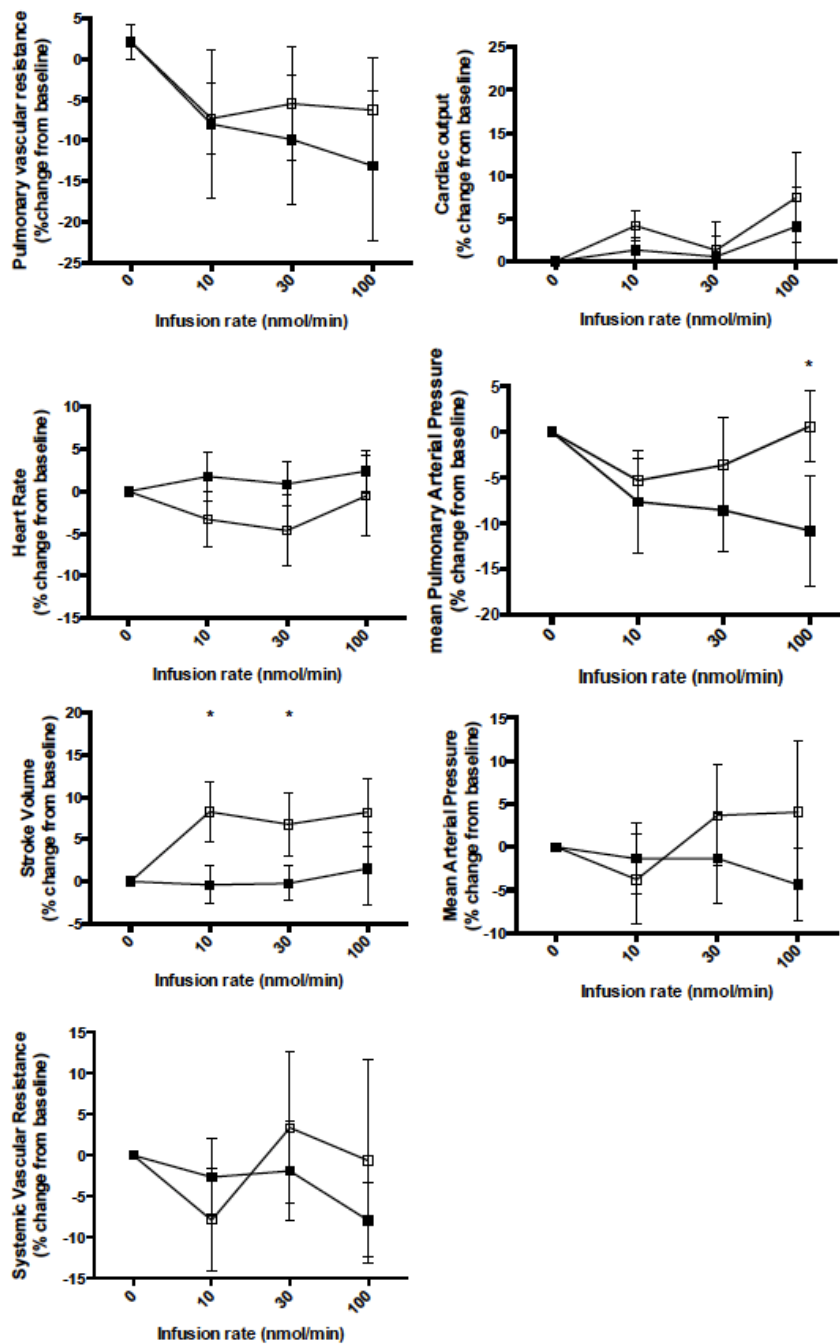

Figure 3-10 Haemodynamics in treatment naïve patients
Percentage change from baseline in pulmonary vascular resistance, cardiac output, heart rate, mean pulmonary artery pressure, stroke volume, mean arterial pressure and systemic vascular resistance during infusion of (Pyr[1])apelin-13 (□) or matched saline placebo (■) in patients with pulmonary arterial hypertension who are treatment naïve. *$p < 0.05$, 2-way ANOVA with posthoc bonferroni tests.

3.1.6.5 Results: Analysed by pre-study treatment

In comparison to saline placebo, (Pyr[1])apelin-13 infusion reduced PVR (Figure 3-9, $p<0.0001$) in subjects who were on treatment but not on those who were treatment naïve (Figure 3-10). When analysed further this effect seemed to be due to phosphodiesterase-5 inhibitors. As in the previous analysis this reduction in PVR was seen with and increase in CO ($p<0.0001$), increase in SV ($p<0.0001$) and no change in HR or mPAP. There was also a reduction SVR ($p<0.01$) (Figure 3-7).

11 out of 19 subjects were on phosphodiesterase-5 inhibitors (58%). Only 26% of subjects were treatment naïve (Table 3-1). Of the subjects not treated with phosphodiesterase-5 inhibitors and those who were treatment naïve, there was a significant increase in SV (Figure 3-10, $p<0.05$). It may be that higher numbers would show a significant change in PVR and CO in these groups.

3.1.7 Results: Blood assay

In a subset of patients (n=8) we measured serum apelin concentrations pre and post infusion of both (Pyr[1])apelin-13 infusion and saline placebo. There was no detectable basal levels but following infusion with (Pyr[1])apelin-13 there was a significant increase in apelin levels (Figure 3-11).
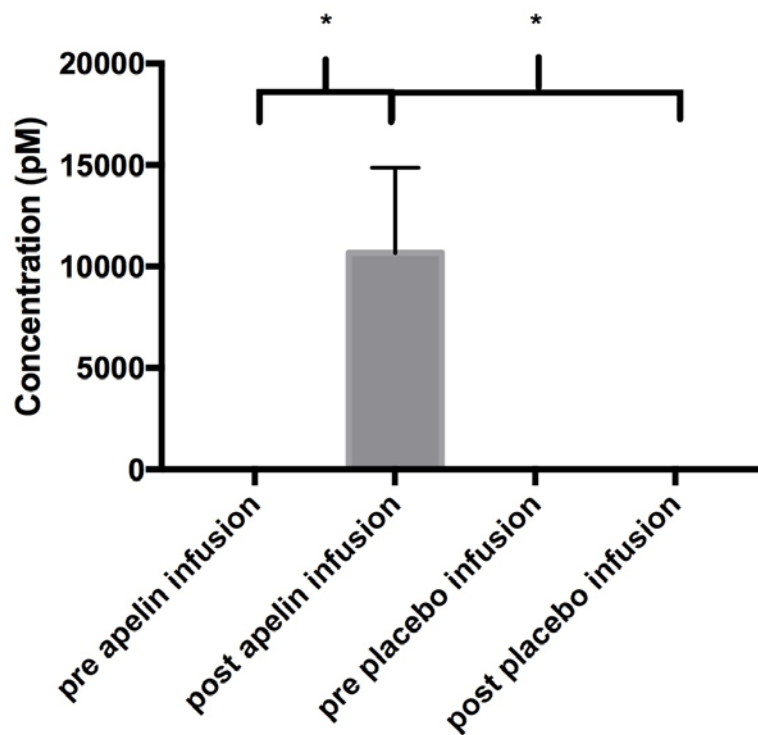
Figure 3-11 Apelin serum concentrations.
Serum apelin concentration (pM) before and after infusion of (Pyr^1)apelin-13 or matched saline placebo in patients with pulmonary arterial hypertension. *p<0.05, paired T test.

3.1.8 Conclusion

This study has shown for the first time that acute apelin infusion reduces PVR and increase CO and SV in patients with PAH. Due to its acute haemodynamic effects and antiremodelling effects the apelin-APJ pathway may be an exciting potential therapeutic target for the treatment of pulmonary arterial hypertension. Further studies are required to determine the longterm effects of APJ agonism in PAH and development of an oral preparation that can easily deliver prolonged APJ agonism is needed

3.2 The remaining 2 groups

3.2.1 PH due to Left Heart Disease

The grant procured provided funding for a clinical fellow to be based in Edinburgh to recruit for this arm of the study. Dr Colin Stirrat went on to be employed in this role, carried out the studies and analysed the data.

The Scottish Pulmonary Vascular Unit was based in the National Services Division at the Golden Jubilee National Hospital which also included the Scottish Advanced Heart Failure Service. Patients around Scotland with advanced heart failure who are suitable for consideration of cardiac transplant are assessed and treated here. This allowed the opportunity to recruit patients in Glasgow to this arm of the study.

I recruited 2 patients from Glasgow to this arm of the study.

There were some problems encountered during recruitment for this arm of the study. As an example the first subjects identified and assessed as possible study subjects were:

1. 12/7/12 – Study cancelled due to patient developing AKI and hypotension (systolic blood pressure 60 mmHg)

2. 14/7/12 – Study postponed due to patient developing pneumonia. Patient later recruited as subject 202 on 27/11/12.

3. 22/8/12 – Patient agreed to participate in study but repeat right heart catheterisation (done routinely in transplant candidates) demonstrated that previously elevated mean pulmonary artery pressure had improved after treatment optimisation and no longer meets study recruitment requirements.

4. 11/9/12 – Study cancelled as subject hypotensive (systolic BP 65 mmHg).

5. 19/9/12 – Study 201 carried out.

6. 26/9/12 – Subject agreed to participate in study but had an ischaemic cerebrovascular infarct prior to the study being carried out.

7. 15/10/12 - Patient agreed to participate in study but repeat right heart catheterisation demonstrated that previously elevated mean pulmonary artery pressure had improved after treatment optimisation and no longer meets study recruitment requirements.

8. 7/11/12 - Patient agreed to participate in study but repeat right heart catheterisation demonstrated that previously elevated mean pulmonary artery pressure had improved after treatment optimisation and no longer meets study recruitment requirements.

9. 13/11/15 - Patient agreed to participate in study but repeat right heart catheterisation demonstrated that previously elevated mean pulmonary artery pressure had improved after treatment optimisation and no longer meets study recruitment requirements.

10. 27/11/15 - Study 202 carried out.

I think the problems recruiting to this arm of the study at the Glasgow site was that the heart failure population tended to be younger and more unwell as they were being assessed for transplant. As can be seen, when these patients are optimally treated, their right heart pressures tend to improve and it may be the subset of patients with pulmonary vascular remodelling and the more severe phenotype (See chapter 1.1.2.2) are very rare.

The other patients recruited for this arm of the study were done so in a more conventional group of heart failure patients at the Edinburgh site by Dr Colin Stirrat, whom also collated and analysed the data. In total 14 subjects were recruited to this group (with 4 of these undergoing the exercise protocol). Unfortunately the results did not show any significant difference (Figure 3-11) in haemodynamic effects between (Pyr[1])apelin-13 infusion and saline placebo in this group. He will write up the results for his own thesis.
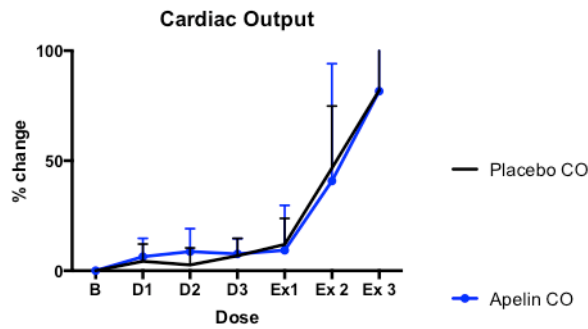
Figure 3-12 Cardiac Output in patients with left heart failure
Percentage change from baseline in cardiac output during infusion of (Pyr[1])apelin-13 (blue circles) or matched saline placebo (Black line) in patients with left heart disease.

3.2.2 Healthy Volunteers

These subjects were recruited by Dr Colin Stirrat at the Edinburgh recruitment site who managed to recruit 7 healthy volunteers. All 7 subjects underwent the full study protocol including the exercise component. Unfortunately the results did not show any significant difference (Figure 3-12) in haemodynamic effects between (Pyr[1])apelin-13 infusion and saline placebo in this group. Dr Colin Stirrat will write up the results of this group for his own thesis.
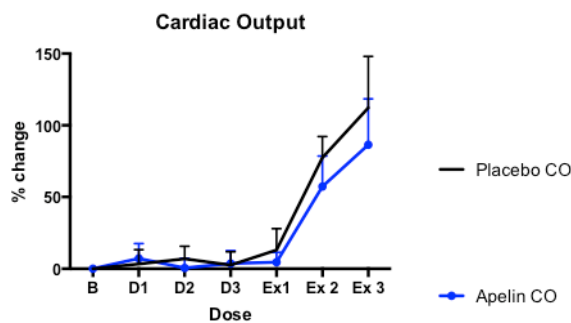
Figure 3-13 Cardiac Output in healthy volunteers
Percentage change from baseline in cardiac output during infusion of (Pyr[1])apelin-13 (blue circles) or matched saline placebo (Black line) in healthy volunteers.

4 Results: The role of apelin in pulmonary vascular remodelling

4.1 Basic science results

As a small additional study to the clinical trial, I worked in the SPVU laboratory to determine some of the effects apelin has on pulmonary artery fibroblasts. As previously discussed, there are extensive structural changes within the pulmonary vasculature by remodelling of the 3 layers of the vessel wall (intima, media and adventitia) which results in luminal obliteration and increased pulmonary vascular resistance. Our lab has a long history of studying the importance of the pulmonary artery fibroblast in this process[83-85]. There is also emerging evidence that fibroblasts are critical regulators of vascular wall function and release inflammatory mediators which may play an important role in the development of pulmonary hypertension[86].

4.1.1 Anti-proliferative effect of apelin on pulmonary artery fibroblasts

As has previously been demonstrated, pulmonary artery fibroblasts proliferate in cell culture in response to increasing doses of fetal calf serum. If the pulmonary artery fibroblasts are incubated in a hypoxic incubator (5% oxygen) for 24 hours, this prompted an increase in proliferation (columns 1 and 2 in Figure 4-1). This is data our lab has shown previously[87]. The lab went on to show that p38 MAP kinases are activated by acute hypoxia in pulmonary artery fibroblasts as well as in fibroblasts derived from rats exposed to chronic hypoxia. We believe that p38 MAP kinase activity is an important pathway for the proliferation of pulmonary artery fibroblasts and plays an important role in pulmonary artery remodelling[84].

I postulated that apelin may have an antiproliferative effect on pulmonary artery fibroblasts. Figure 4-1 demonstrates that apelin inhibited the proliferative response to hypoxia in the pulmonary artery fibroblast.
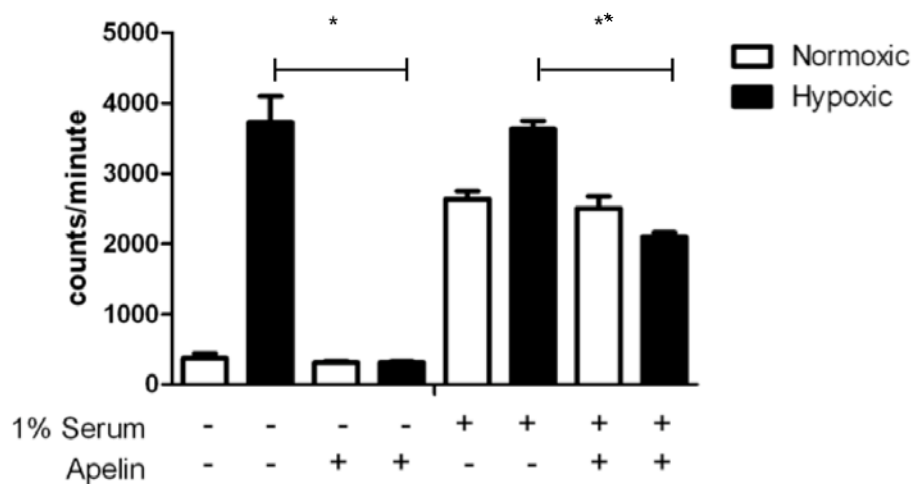
Figure 4-1 Apelin inhibits the proliferative response to hypoxia in the pulmonary artery fibroblast

[³H] thymidine proliferation assay of rat pulmonary artery fibroblasts grown in both serum starved and 1% FCS conditions +/- apelin. The cells were then incubated in normoxic or hypoxic incubators for 24hours. Hypoxia causes increased proliferation of the cells. Incubation with apelin attenuated this proliferation. n=2. * $p < 0.001$ ** $p < 0.05$

4.1.2 Cell migration

In pulmonary hypertension, pulmonary artery fibroblasts migrate from the adventitia into the media layer as part of the pulmonary vascular remodelling process. Using a Cytoselect migration assay, which allows migration through a semi-permeable membrane we showed that pulmonary artery fibroblasts showed increased cell motility in response to acute hypoxia (Figure 4-2). When the experiment was repeated in the presence of apelin, this increase in cell migration was prevented ($p < 0.005$).
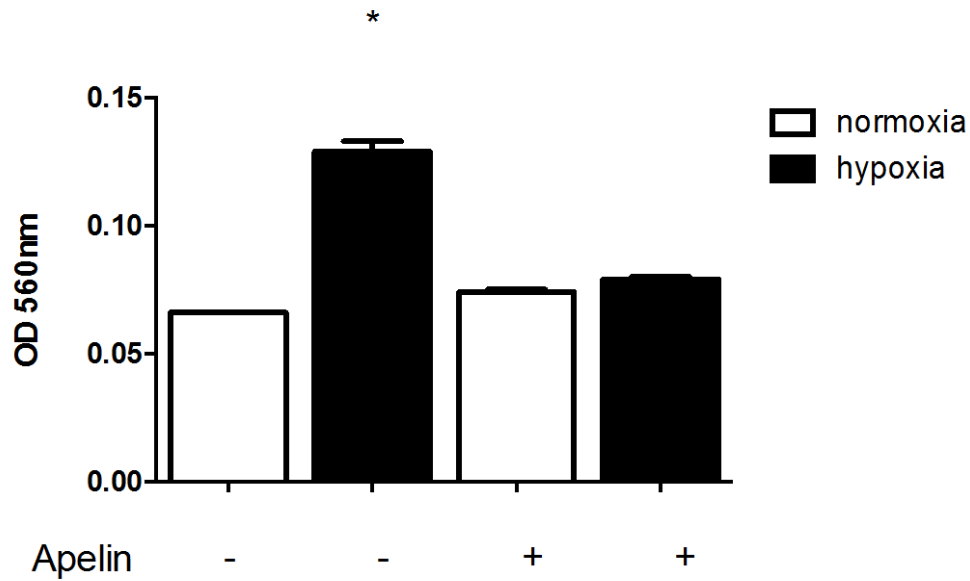
Figure 4-2 Cell migration of pulmonary artery fibroblasts when exposed to hypoxia is reduced by apelin
Rat pulmonary artery fibroblasts show increased migration when exposed to hypoxia using the cytoselect migration assay. This migration was attenuated when the cells were incubated with apelin. n=2. * p<0.005

4.1.3 Immunoblotting

First we looked to see with immunoblotting whether the apelin receptor, APJ was present in rat pulmonary artery fibroblast cells (Figure 4-3).
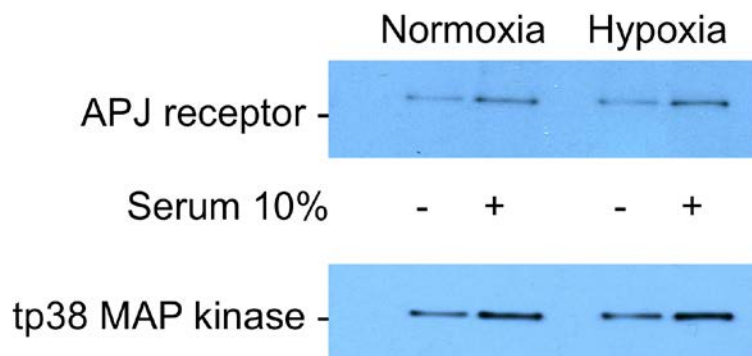
Figure 4-3 APJ receptor presence in rat pulmonary artery
Western blot analysis of APJ receptor presence in rat pulmonary artery fibroblasts. Rat pulmonary artery fibroblasts were cultured in normoxic and hypoxic chambers. (Total p38 MAP kinase as control).

As our lab has previously shown, proliferation of pulmonary artery fibroblasts in hypoxia may be due to activation of the p38 MAP kinase pathway. As apelin prevented the hypoxic proliferation of pulmonary artery fibroblasts, I postulated that apelin may be inhibiting the activation of this pathway. To examine the activity of p38 MAP kinases, I carried out western blot analysis of total p38 MAP kinase as a control and the active phosphorylated p38 MAP kinase in response to hypoxia with and without incubation with apelin.
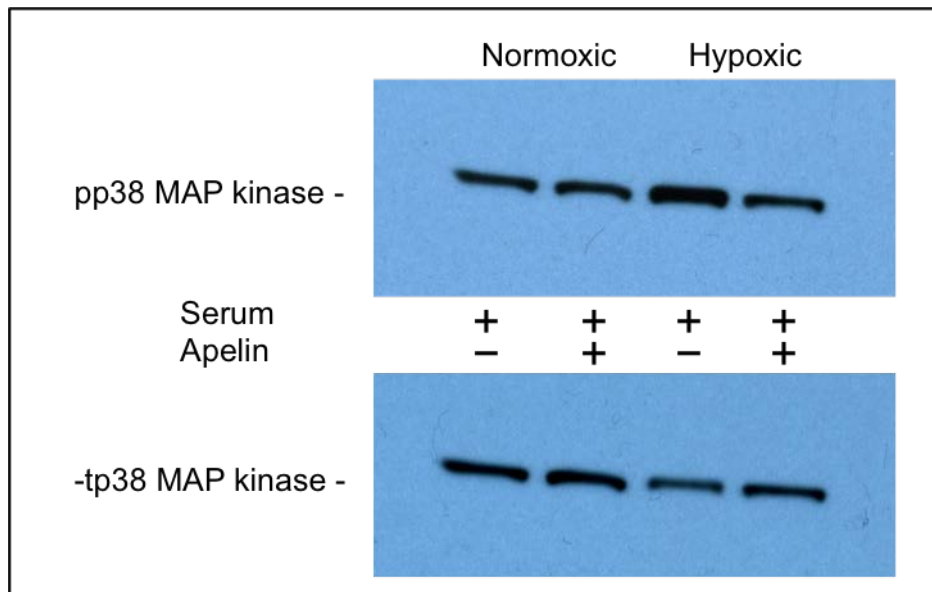
Figure 4-4 Increased phosphorylation of p38 AMP kinase to hypoxia was reduced by apelin
Rat pulmonary artery fibroblasts were cultured +/- FCS, +/- apelin. Hypoxia has previously been shown to increase the phosphorylation of p38 MAP kinase. Incubation with apelin reduced this effect.

By immunoblotting we showed that active phosphorylated p38 MAP kinase increased in response to hypoxia as expected. However, when pulmonary artery fibroblasts were exposed to hypoxia in the presence of apelin this increase in phosphorylated p38 MAP kinase was reduced (Figure 4-4). When the blot is analysed by densitometry (Figure 4-5) this confirms that there is a significant reduction in p38 MAP kinase activity ($p < 0.05$).
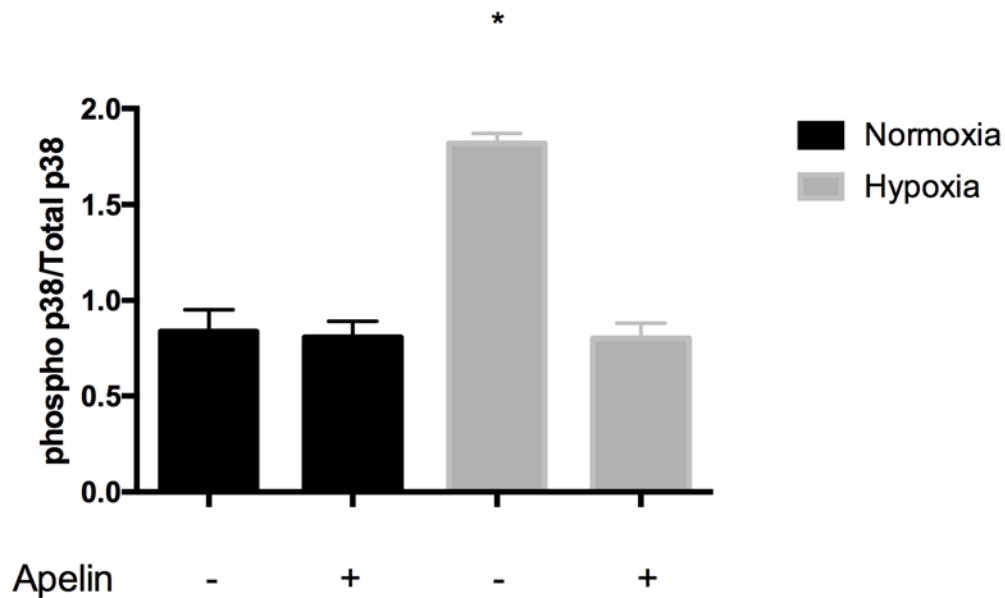
Figure 4-5 Increased phosphorylation of p38 AMP kinase to hypoxia was reduced by apelin
Densitometry of the protein bands on the western blot film were carried out to show the relative difference between phosphorylation of p38 MAP kinase when exposed to hypoxia +/- apelin. Apelin significantly reduced the phosphorylation seen in hypoxia. *$p < 0.05$

4.1.4 Conclusion

We have shown that rat pulmonary adventitial fibroblasts express the apelin receptor – APJ. Apelin prevents the hyperproliferative and cell migratory response to hypoxia seen in rat pulmonary artery fibroblasts. Apelin prevents the phosphorylation of p38 MAP kinase in response to hypoxia and so its actions may in part involve activity through this pathway.

5 Final Discussion

For the first time, this study demonstrates that acute intravenous (Pyr[1])apelin-13 infusion causes a fall in pulmonary vascular resistance with an increase in cardiac output and stroke volume in patients with pulmonary arterial hypertension. The effect of chronic apelin agonism and its potential role in the treatment of PAH needs further exploration.

Pulmonary Vascular resistance is derived from:

$$PVR = \frac{80 \times (mPAP - PAWP)}{Cardiac\ Output}$$

mPAP and PAWP did not change with (Pyr[1])apelin-13 infusion (Figure 3-1 and PAWP not shown). It is therefore the change in cardiac output which leads to the improvement in pulmonary vascular resistance.

Cardiac output itself is calculated from:

$$Cardiac\ Output = Stroke\ Volume \times Heart\ Rate$$

As we saw in Figure 3-1, heart rate was not affected by (Pyr[1])apelin-13 infusion but stroke volume was.

Stroke Volume is derived from:

$$Stroke\ Volume = End\ Diastolic\ Volume - End\ Systolic\ Volume$$

The factors which increase stroke volume include;

1. Increased preload (an increased venous return or fast filling time can increase the end diastolic volume and therefore the stroke volume).

2. Increased contractility (decreases end systolic volume and thereby increases stroke volume).

3. Reduced afterload (reduced vascular resistance decreases end systolic volume and so increases stroke volume).

The current recommendations for treatment goals as set out at the fifth world symposium on pulmonary hypertension advise normalization of RV function as a treatment goal which is defined as RAP < 8 mmHg and CI >2.5 l/min/m[25,88]. By infusing (Pyr[1])apelin-13 into patients with PAH we have managed to improve CO as recommended. This suggests that apelin exerts an acute inotropic effect in PAH patients.

Apelin itself may not currently represent a viable therapeutic intervention for PAH as its only method of delivery is systemic, however this study has demonstrated that activation of the apelin-APJ pathway leads to a significant improvement in PVR and CO even in patients established on current PAH therapies. Development of drugs which cause APJ agonism need to be developed as this would be an exciting approach to treatment of PAH.

This study was designed as an invasive study as this is the gold standard method in measuring pulmonary haemodynamics and the most accurate method for determining haemodynamic values. The study design was a within subject comparison crossover study in order to gain as accurate results as possible. The study was designed to measure both resting and exercise haemodynamics because patients with PAH are more symptomatic during activity and I hoped to demonstrate that apelin has therapeutic benefit during exercise. Interestingly the study demonstrated changes in pulmonary haemodynamics at rest but, surprisingly, exercise did not further augment these changes. This may be because of the large changes seen in haemodynamics caused by exercise alone. It may be that longer term apelin infusions leads to an improvement in exercise pulmonary haemodynamics and this may be worth examining in future studies.

The apelin system may have major relevance for patients with PAH. Dysfunction of BMP signalling is an important pathway in the pathogenesis of PAH[61] and it appears to markedly influence apelin production as a potential mediator of the pathogenesis of PAH. In a chronic hypoxic rodent model of right ventricular

failure[89], apelin has inotropic effects and treating rats with monocrotaline-induced pulmonary hypertension with apelin infusion led to improved RV mass and haemodynamic variables[68]. In human studies, apelin expression is reduced in the pulmonary artery endothelial cells (PAEC) of patients with idiopathic pulmonary arterial hypertension (IPAH)[65]. Reducing apelin in human PAEC by small interfering RNA (siRNA) impairs PAEC survival and promotes pulmonary artery smooth muscle cell proliferation[65]. Furthermore, Apelin can directly suppress vascular smooth muscle cell proliferation in response to growth factors, and is pro-apoptotic[65]. Plasma apelin concentrations are reduced in patients with IPAH[46].

Professor Newby provided the first evidence that apelin has vasoactive actions in humans. He showed that apelin causes vasodilatation *in vivo* in the human forearm circulation of healthy volunteers through a predominantly nitric oxide-dependent mechanisms, and that this effect is preserved in patients with heart failure[58,59]. Furthermore, apelin is a direct coronary vasodilator and increases myocardial contractility in humans. It causes a reduction in both peak and end-diastolic left ventricular pressures[59].

Included in this study were patients who were taking phosphodiesterase-5 (PDE5) inhibitors. It is known that in ex vivo myography studies, inhibition of nitric oxide (NO) attenuated vasorelaxation in human mesenteric arteries[48]. As Professor Newby demonstrated[58], the effects of apelin are partially attenuated with a "NO clamp" (NO synthase inhibitor) suggesting that apelin causes arterial vasodilation in a nitric oxide dependent manner. PDE5 inhibitors also act on the NO pathway by preventing degradation of cGMP. I felt it was important to include this group for recruitment in this study to determine if there was any synergistic vasodilatory effect when combining two drugs that act on the NO-cGMP pathway. I also felt it was important to assess what effect this combination of treatment would have on SVR from a safety perspective. Interestingly, on post-hoc analysis, the group of patients established on PDE5 inhibitors had a significant improvement in PVR, CO and SV while the group who were not on treatment with PDE5 inhibitors showed no significant effect. This study was not powered to investigate this effect but it would be important for future studies to explore this effect further.

There is a lot of interest currently in upfront combination treatment for PAH and whether this has longterm beneficial effects in patients with PAH by the establishment of early control and preservation of RV function. This has to be weighed up however with the potential for drug to drug interactions and potential adverse effects. This study did not demonstrate any adverse effects but this would need to be explored further in longterm studies. The group of patients on PDE5 inhibitors did have a more significant reduction in SVR. This was asymptomatic in this study cohort who were supine on the catheterisation table and it may be that this change in SVR may have a more significant effect in longer term studies. There was also no significant change in in MAP, possibly explaining the lack of symptoms and potentially meaning the reduced SVR would have the benefit of improving tissue and organ perfusion via increased blood flow to them.

As reported in the post-hoc analysis data the younger patients had a significant improvement in PVR and CO that the older patients didn't. Again the study was not powered to analyse this effect. The demographic data demonstrate that the younger age group have a higher RVP, higher mPAP, lower CO, lower SV and higher PVR at baseline. The younger age group appear to have haemodynamically more severe PAH meaning they may have more to gain from the apelin infusion. It is also noted that 90% of the younger group are on PDE5 inhibitors while only 22% of the older group were on PDE5 inhibitors so the effect demonstrated may be reflecting the synergist effect of these two drugs rather than a difference between the patients. This makes it hard to draw conclusions about the difference in response between the age groups.

This study examines the short term effects of apelin administration and longer studies would have to be carried out to determine longterm haemodynamic benefits as well as longterm adverse effects. Professor Newby has previously determined the effect of prolonged systemic infusion of apelin in man. During 6 hour infusions of apelin in healthy volunteers and patients with heart failure the apelin was well tolerated and its inotropic actions were maintained with a sustained increase in cardiac index and left ventricular ejection fraction[60]. Furthermore, they monitored pulmonary artery blood flow during the first hour of apelin infusion using echocardiography and found this to be increased with

apelin infusion versus saline placebo. This suggests that apelin will have sustained benefits on pulmonary vascular haemodynamics

Apelin has also been shown in preclinical studies to target pulmonary vascular remodelling[65] so not only does it have beneficial haemodynamic effects in the human pulmonary vasculature but it may also prevent the pulmonary vasculature remodelling seen in PAH.

It is surprising that the haemodynamic effects apelin exerts in the PAH population is not demonstrated in the healthy volunteer group or the group 2 PH group. This may be due to the underlying pulmonary vascular changes seen in patients with PAH compared to the other 2 groups. The group 2 PH group in particular will have to be explored further to clarify if they had 'reactive' changes in the pulmonary vasculature or whether they have the more severe phenotype described in chapter 1 as it would be interesting if possible to do subanalysis between these 2 groups and see if the more severe phenotypes had a similar response to apelin as the patients with PAH. This would suggest that the damaged pulmonary vasculature was in some way being 'restored' by the apelin and exerting the haemodynamic changes seen.

(Pyr^1)apelin-13 peptide is unlikely to represent a viable long-term therapeutic intervention for PAH as it would require continuous intravenous systemic infusion. However our study has demonstrated that activation of the apelin-APJ pathway led to an important improvement in pulmonary vascular resistance and cardiac output even in patients established on current PAH therapies. Development of orally active drugs that can cause long-term apelin agonism would be an exciting approach to the treatment of PAH. To make apelin agonism a viable potential treatment for patients with PAH, further drug development is required to develop an oral or inhaled preparation that can be easily delivered to enable prolonged apelin agonism , in order to avoid the difficulties of long-term infusion treatments well demonstrated with prostacyclin. There are already synthetic agonists of the apelin receptor showing some promise in research[90].

5.1 Limitations

The main limitations of the clinical study are its short infusion time. A longer study will be able to provide more information on longer term effects of apelin and in particular may provide more information on potential adverse effects such as hypotension. A further limitation of the clinical study is the fact that the studies were carried out in the supine position at rest. This was necessary to carry out the right heart catheterisation but doesn't represent real life activity for these patients.

There are limitations in the conclusions that can be drawn from the post hoc analysis as the study was not powered to assess these smaller groups.

5.2 Ideas for further research

With regards to the clinical study, ideally future research will be around developing an oral agonist for the APJ receptor which will have a longer duration of action allowing longer term studies of apelin agonism that can provide data regarding long term haemodynamic modifications, exercise tolerance changes and mortality information. In the meantime, a potential way to examine prolonged effects of apelin infusion in patients with PAH would be to carry out a similar study to what professor Newby and his colleagues did looking at a 6 hour infusion in healthy volunteers and patients with chronic heart failure[60]. It may be less feasible to do this during right heart catheterisation, measuring pulmonary haemodynamics, but would be useful in exploring adverse effects such as symptomatic hypotension which may be a problem in patients with PAH. Newby et al showed that there was a reduction in peripheral vascular resistance during the first hour in healthy volunteers but that this was not sustained and that mean arterial pressure was unchanged throughout the 6 hour infusion. However in patients with chronic heart failure there was a maintained reduction in both mean arterial pressure and peripheral vascular resistance throughout the 6 hour infusion period.

It would also be interesting to carry out a clinical study looking in particular at comparing patients who are and aren't on treatment with PDE5 inhibitors to see

if there is a statistically significant difference in the haemodynamic changes seen with apelin in these 2 groups.

Further laboratory work would be helpful in establishing mechanisms for the possible anti-remodelling effects and the mechanistic pathways underlying this. Work that may help establish reasons behind the reduced proliferation of rat pulmonary artery fibroblasts to hypoxia seen when incubated with apelin would include looking at the supernatant from both sides of the study to see if there are any particular mediators involved. The mediators released may provide insight into which cell signalling pathways may be involved and this may guide further laboratory experiments. Similarly looking at the supernatant from the migration studies may also provide insight into mechanisms for anti-remodelling.

Further work could also look at the combination of PDE5 inhibition and apelin to see if there is enhancement of the reduced proliferation and migration seen with hypoxia.

Additionally, looking at carrying out these lab experiments in human pulmonary artery fibroblasts would be important as there can be significant inter-species differences.

6 Final Comment

The work done for this thesis has shown for the first time that acute apelin infusion causes a fall in pulmonary vascular resistance in humans with pulmonary arterial hypertension. There is also an increase in cardiac output and stroke volume.

As well as the haemodynamic effects of apelin in PAH, we have shown that apelin reduces the hypoxic proliferation and migration of rat pulmonary artery fibroblasts and that these effects may be exerted through inhibition of the p38 MAP kinase pathway. This would suggest that apelin may have an anti-remodelling role in PAH as well as effects on pulmonary haemodynamics.

Apelin agonism may represent a future treatment for PAH. The effect of chronic apelin agonism and its potential role in treatment of PAH needs further exploration.

Appendices

Appendix A
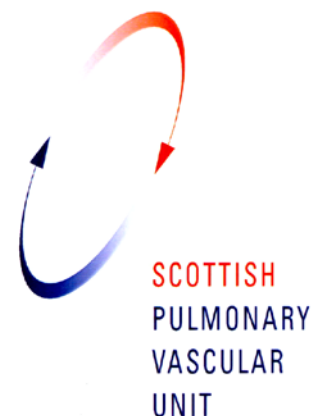
Date:

Dear

STUDY: Haemodynamic Effects of Apelin in Pulmonary Hypertension.

CONTACT: Dr Lauren Brash
            Scottish Pulmonary Vascular Unit
            Golden Jubilee National Hospital
            Agamemnon Street
            Clydebank
            G81 4DY

            Tel: 0141 951 5497 or 07738 569980
            Email: laurenbrash@nhs.net

We would like to invite you to take part in a research study. Before you decide to accept, it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully. Part 1 explains to you about the purpose of the study and what will happen to you if you decide to take part. Part 2 gives you more information on the conduct of the study. A member of the research team will go through the information sheet with you and answer any questions that you may have. You can discuss the information with others if you wish. This could be friends, family, nursing staff, your GP or anyone else you would like to talk to.

PART 1

What is the purpose of this study?
Blood is pumped by the right side of the heart to the lungs, where the blood cells pick up oxygen from the air we breathe. The blood then travels back to the left side of the heart which pumps the oxygen enriched blood around our body (see figure 1). The oxygen is used by our body's cells for energy and without it we couldn't survive.
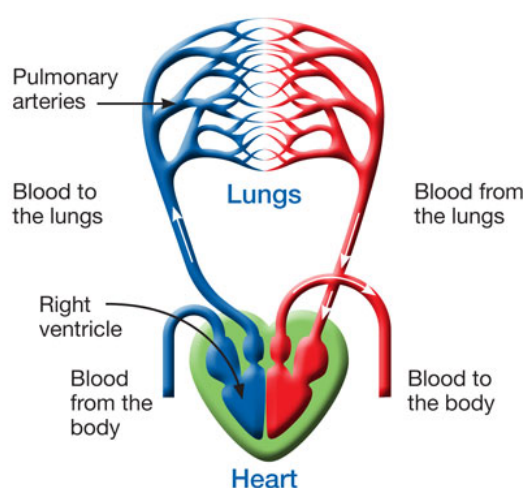
Figure 1: The heart and Circulatory System. Reproduced with permission from www.phassociation.uk.com

Pulmonary arterial hypertension (PAH) is a rare disease caused by damage to the pulmonary arteries (these are the blue blood vessels carrying blood *to* the lungs

in figure 1). This damage causes thickened, narrowed blood vessel walls (see figure 2). When a drainpipe in a sink becomes clogged up, the flow of water from the sink becomes sluggish and slow. The same happens with the blood flow through the narrowed pulmonary arteries. The right ventricle is the muscle on
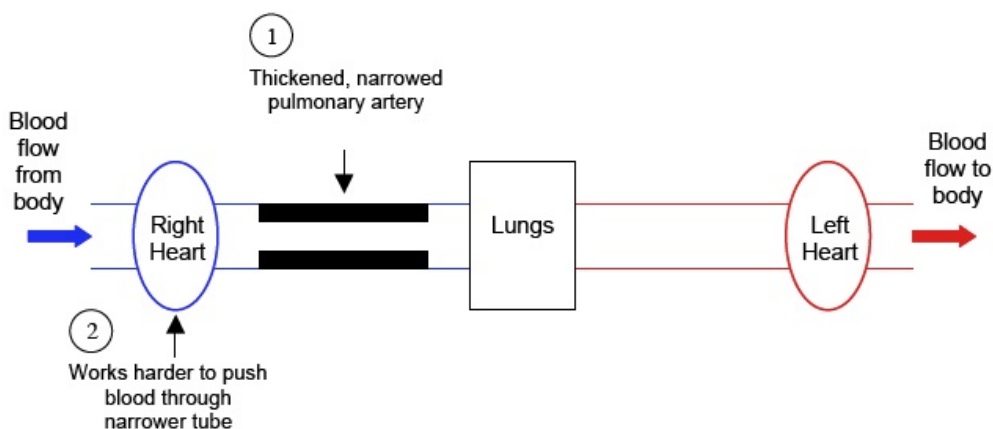
Figure 2: Schematic drawing of heart and lung circulation in PAH.

the right side of the heart which pumps blood through the pulmonary arteries to the lungs. The right ventricle works harder to get the blood flowing through these narrowed pulmonary arteries. Initially this increase in work compensates for the damage to the pulmonary arteries but over time the right heart is put under strain and struggles to pump the blood efficiently. This leads to symptoms of breathlessness, fatigue and fluid retention. If untreated these symptoms will get worse over time. There are a number of treatments available for PAH, which help with the symptoms, but currently no cure. Research is essential to improve our knowledge of this condition and help develop new treatments.

There has been research carried out on a protein found in the heart and lungs called apelin. This protein has been found to be less active in people who have conditions such as PAH and heart failure. There have been studies carried out involving the administration of apelin to both healthy volunteers and to patients with heart failure. These studies were carried out in Edinburgh by members of our research team. These studies showed that apelin increases blood flow in the general circulation as well as helping the heart to pump stronger. The effect of apelin in the pulmonary circulation is unknown.

The purpose of this study is to assess what effects the study drug, Apelin, has on the pulmonary arteries and specifically to see if it helps widen the pulmonary artery, thereby improving the flow of blood to the lungs and reducing the strain on the right side of the heart.

Why have I been chosen?
You have been invited to participate because you either have suspected or confirmed PAH. We plan to look at the effects of apelin on the pulmonary circulation in 3 patient groups; people who have PAH, people who have pulmonary hypertension due to heart failure and healthy people with normal heart and lungs. It is important to see if apelin has any effects on the pulmonary circulation and to see if this effect is lost or maintained in people with PAH or heart failure. In total we will ask 63 people to participate in this study from 3 different sites in the UK.

Do I have to take part?
No. It is entirely your decision about whether to take part or not. If you decide to take part, you will be given a copy of this patient information sheet and be asked to sign a consent form. If you decide to take part in this study you are free to withdraw at anytime without having to give a reason. If you decide not to take part or you withdraw at any point in the study the standard of care you will receive will not be affected.

What will happen to me if I take part?

You will be asked to give consent:
If you decide to take part in the study you will first be asked to sign the consent form. Be sure that you read all the information you are provided with carefully so that you understand what taking part in this study will involve.

You will have a right heart catheterisation procedure:
On the day of the study you will have a test called a right heart catheterisation. PAH causes raised blood pressure in the lung blood vessels. It is possible to estimate the pressure using non-invasive methods such as an echocardiogram (which uses sound waves to visualise the heart) but this method is much less accurate. Right heart catheterisation is considered the only way to definitively diagnose PAH.

This procedure involves having an injection of local anaesthetic to numb the skin over one of the large veins. This will most likely be one of the large veins in the neck but it may be one in the groin. Once the skin is numb, a plastic tube is placed in the vein through a small incision. This allows a catheter to be fed through to the heart and the lung blood vessels. X-ray pictures are taken to check the position of the catheter. Various measurements including pressure can be made through special sensors in the catheter. These pressures tell us about how high the blood pressure in the lungs is as well as how well the heart is pumping.

Everybody with PAH will have a right heart catheterisation to make the diagnosis. If you choose to take part in this study we will first carry out the routine diagnostic catheterisation. If this confirms that you have PAH, the procedure will continue while you receive the study drug.

It may be that you already have a diagnosis of PAH and are already on treatment. If this is the case you will have had a right heart catheterisation when you were diagnosed. We do not routinely repeat right heart catheterisations and although this test will give us more up to date information on the severity of your disease, it is unlikely that this will lead to an alteration in your management.

You will be given the study treatment:
You will be given either apelin treatment or placebo treatment in random order via a drip while the pressure measurements are repeated. A placebo is a 'dummy' treatment which looks like the active apelin treatment but contains no active ingredient. There will then be a half an hour rest period before you are given the second treatment. This will be apelin if you received placebo initially or placebo if you previously received apelin. Each treatment period will last for

15 minutes. This is known as a 'cross-over' study, meaning you will receive both treatments and your body's responses will be recorded and compared to see if one treatment is better than the other. Both yourself and the doctor taking the measurements will not be told which order you receive the treatments. This is known as a 'double-blinded' study, meaning you are both 'blinded' to the treatment. It is done to prevent bias when recording the results. The study will be unblinded when it is time to analyse the results.

You will have blood tests taken:
Before the right heart catheterisation is started, you will have a drip placed in your arm. This will allow blood samples to be taken during the study period. This is to look at apelin levels as well as NT-proBNP, which is a hormone which is secreted by the heart in response to excessive stretching of the heart muscles (when they are under strain). NT-proBNP is typically elevated in people who have either heart failure or PAH.

You will be asked to exercise:
After the initial measurements are made during the right heart catheterisation, you will be asked to carry out some exercise if you are able. This will be in the form of cycling while lying flat on the examination couch. You will be asked to cycle for a maximum of 5 minutes with no apelin treatment and then for a further 5 minutes with apelin treatment. Each exercise period will be separated by a 1 hour rest period in which the other data already discussed is collected. Many people with PAH have no symptoms at rest and only have symptoms while they are walking or carrying out other activities. It will be useful to look at how apelin affects the lung circulation while under the stress of exercise.

How long will the study take?:
The addition of the study to your diagnostic catheterisation will increase the duration of the procedure from around 45 minutes to just over 2 hours. The study itself will take place on 1 day.

You will be started on treatment for PAH:
Once the study is complete, if you are a new diagnosis of PAH, you will be started on appropriate therapy. Your specialist team will decide what this should be. Current medical treatment for PAH comprises of general strategies such as diuretics, digitalis, anticoagulants, and oxygen as well as specialised PAH specific medication like Phosphodiesterase Inhibitors (e.g. Sildenafil), Endothelin Receptor Antagonists (e.g. Bosentan and Ambrisentan) and Prostacyclin Analogues (e.g. Epoprostenol and Iloprost). Your decision regarding participation in this study will not affect the choice your team make about which treatment to start you on. You can discuss these treatments further with your PAH specialist. You will return to clinic for follow up of your PAH but will require no further visits for the study.

If you have a known diagnosis of PAH, you should already be established on appropriate treatment. It is unlikely that this will be changed. You can discuss this further with your specialist doctor.

It is important that you know that this study is being carried out to assess the potential effects of apelin on the pulmonary arteries. We do not know for sure that it will have any beneficial effect. If it does have a beneficial effect, this effect will be short-lived and will not give you any lasting benefit. This is

because apelin is broken down and cleared from the body quickly. If this study shows that apelin has beneficial effects on the pulmonary arteries, further research and trials will need to be carried out before apelin can be developed into a practical treatment for PAH.

What if you are on warfarin?:
You may be on warfarin for your pulmonary hypertension. To be safe to undergo the study test you will be asked to stop your warfarin. In general patients with pulmonary hypertension are advised to be on warfarin because it is believed that those who are treated with warfarin survive longer than those who are not. There has been no randomised controlled trial to confirm this belief yet. Warfarin can make you more prone to bleeding and patients who have a significant contraindication (ie. have bleeding or falls risk) will not be treated with warfarin.

The reason for stopping warfarin is that the right heart catheterisation is an invasive procedure involving a needle being passed into the large vein in your neck. If you remain on warfarin during the procedure, there is an increased risk of serious bleeding. There is very little research to suggest that stopping warfarin for a short period of time will cause you any problems.

Once the study has been completed you will have to restart the warfarin. It can take a few days before the INR reaches the target range of 2 – 3. The study team will advise you on what dose to take. Your GP will be notified that you will be off warfarin temporarily. On the rare occasion that the study is delayed or cancelled, you should restart your warfarin. A member of the study team will contact you and advise you on what dose to take.

Please let the study team know if you are on warfarin for any other reason as you may not be eligible for the study. If you have any questions, feel free to contact the study team using the contact details on the first page.

Please feel free to ask your study team any questions regarding any of this information.

What does taking part in the study mean to me?
If you are a new patient, undergoing diagnostic investigations, you will require a right heart catheterisation as part of these investigations. If you decide to take part in this study, the difference to routine care will be: The duration of the right heart catheterisation will be more than twice as long; you will be given an experimental treatment; and you will be asked to give additional blood samples. The risks are explained in more detail below.

Expenses and Payments
You will not receive any payment for this study but any expenses you incur such as travel will be compensated. You will find out more about this by discussing with your research team.

Additional information for women of child bearing potential.
There is no information on what effect apelin could have on an unborn child, therefore, if you are a woman of child bearing potential, you will be asked to have a pregnancy test prior to inclusion in the study. Also if you have recently

given birth and are breast feeding your child, you would be excluded from taking part in this study. Please inform the study doctor if this is the case.

What are the possible disadvantages and risks of taking part?
Many of these procedures are necessary for the diagnosis of PAH but have been adapted for the purposes of the study. The potential risks are discussed below:

Blood tests: It is likely that you have had blood tests carried out before. The main adverse effects can be discomfort, bruising, bleeding and less commonly infection. The maximum amount of blood collected will be 120 mls. This is around a quarter of what is taken during blood donation. This will be quickly replaced by your body. If you have recently donated blood, please inform your study doctor.

Right heart catheterisation: Possible complications of this procedure include: bruising; bleeding, infection; severe cardiac arrhythmia (e.g. fast irregular heart beats); damage to the heart wall and heart valves; rupture of the pulmonary artery; thrombosis (formation of new blood clots); pneumothorax (collapsed lung); and rarely, death. The risk of serious complications is small in experienced centres like the one your study will be carried out in. A multicentre survey found the risk of such complications to be 1.1%. Your doctor will discuss the benefits and risks of right heart catheterisation in greater detail. You should be aware that you are entitled to withdraw from the study at any time.

Radiation: During the right heart catheterisation, x-ray screening is used to check the position of the catheter. X-rays are a form of radiation which can be harmful. The dose of radiation you will receive during this study is equivalent to 11 months background radiation. Background radiation is the radiation we are exposed to all the time from natural sources.

Apelin: The study treatment, apelin, has not been used in patients with PAH before. It has, however been used in healthy people and in people who have heart failure. There have been over 130 people who have been treated with apelin in short studies. The doses used in this study are the same as in those previous studies. There were no serious adverse effects in the previous studies. However this does not rule out the possibility of side effects that we are not yet aware of, as the study treatment remains experimental. Throughout the study you will be continuously monitored to assess for adverse effects.

Travel: You may have some distance to travel to get to the hospital for the study. If you require help with transport please let your study doctor know and arrangements can be made for this. If you require compensation for travel costs please discuss this with your study doctor.

What if the tests reveal abnormal/unexpected findings?
If the tests show any unexpected results, we will not proceed with the study. Your doctors will explain the results of the tests to you and explain why this excludes you from the study. Your doctors will also explain what your treatment options are and refer you to other specialists, if this is appropriate.

What are the possible benefits of taking part?
The study treatment will be given only for the duration of the catheter test. There is not yet enough information to use this as a treatment for people with

either PAH or heart failure. By participating in this study, important information regarding the effects of apelin on the lung circulation will be gathered which may help give us new insights into developing treatments for these conditions in the future.

What happens when the research study stops?
At the end of your study your specialist team will discuss the results of the diagnostic catheterisation test and will discuss the treatment options with you. Current medical treatment for PAH comprises of general strategies such as diuretics, digitalis, anticoagulants, and oxygen as well as specialised PAH specific medication like Phosphodiesterase Inhibitors (e.g. Sildenafil), Endothelin Receptor Antagonists (e.g. Bosentan and Ambrisentan) and Prostacyclin Analogues (e.g. Epoprostenol and Iloprost). When the whole study is complete and the results are analysed, we will circulate a newsletter to let you know about the study's progress. We will ask for your consent to do this.

What if there is a problem?
Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. See details in part 2.

Will my taking part in this study be kept confidential?
All information regarding your participation in this study will be kept confidential. We will however ask for you permission to inform your GP about your participation in this study.

If the information in part 1 has interested you and you are considering participation, please read the information in part 2 before making your decision.

PART 2

What will happen if I don't want to carry on with the study?
If you decide to take part in this study, you are free to withdraw at anytime without having to give a reason. If you decide to withdraw at any point in the study, the standard of care you will receive from your specialist PAH team will not be affected.

What if there is a problem?
If you have any concerns or complaints about any aspect of this study you should ask to speak to Prof Peacock the chief investigator of this study. If you remain unhappy and wish to complain formally you can do this through the NHS complaints procedure. Details can be obtained from your hospital.

Will my taking part in this study be kept confidential?
If you join the study, some parts of your medical records and the data collected for the study may be looked at by authorised persons from the study sponsors to ensure that the study is being carried out correctly. These people will have a duty of confidentiality to you as a research participant and we will do our best to meet this need. All information which is collected about you during the

course of this research will be kept strictly confidential, and any information which leaves the hospital will have your name and address removed so that you cannot be recognised.

Informing your GP of your participation in this study.
We would like to ask your permission to inform your GP about your participation in this study. If you agree please initial the relevant box on the consent page and we will send the information to your GP.

What will happen to samples I give?
Samples will be stored securely in a laboratory until the time that they will be analysed. The samples will be stored in an anonymised way. If you would like to know anything more about how the samples are analysed, please ask your study doctor. You can, at any time, get in touch with your doctor to ask for the samples to be destroyed. There will be no genetic analysis of your samples.

What will happen to the results of the study?
The results of the study will be analysed and published in a scientific journal, as well as being presented at scientific meetings. Your personal details will be encoded to ensure that your identity will remain confidential.

Who is organising and funding the study?
The Golden Jubilee National Hospital is the sponsor for the study. The British Heart Foundation has provided a grant to fund this study.

Who has reviewed this study?
The details of the study have been reviewed and approved by the West of Scotland Research and Ethics Committee.

Contact for further information:

Researcher:
Dr Lauren Brash
Scottish Pulmonary Vascular Unit
Golden Jubilee National hospital
Agamemnon Street
Clydebank
Glasgow
G81 4DY
Tel: 0141 951 5497 or 07738 569980
Email: laurenbrash@nhs.net

If you would like any further information on this study, please don't hesitate to get in touch.

Yours Sincerely,

Professor Andrew J Peacock.

CONSENT FORM

TITLE OF STUDY: The Effects of Apelin in Pulmonary Hypertension

CHIEF INVESTIGATOR: **Professor Andrew J Peacock**, Scottish Pulmonary Vascular Unit, Golden Jubilee National Hospital, Agamemnon Street, Clydebank. G81 4DY.

CONTACT: **Dr Lauren Brash**, Scottish Pulmonary Vascular Unit, Golden Jubilee National Hospital, Agamemnon Street, Clydebank. G81 4DY.
Tel: 0141 951 5497 or 07738 569980 Email: laurenbrash@nhs.net

STUDY No.:

CENTRE: Glasgow

Please initial relevant box

		Yes	No
1	I confirm that I have read and understand the patient information sheet version 5 dated 24th January 2013 for the above study. I have had opportunity to consider the information, ask questions and have had these answered satisfactorily.		
2	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care and legal rights being affected.		
3	I understand that relevant sections of my medical notes and data collected during the study may be viewed by individuals involved in the study. I give permission for these individuals to have access to my records.		
4	I agree to participate in this study as described in the patient information sheet.		
5	I agree to blood sampling as detailed in the patient information sheet.		
6	I agree to my GP being informed of my participation in the study.		
7	I would like to receive a newsletter when the study is complete.		
8	I agree to take part in the above study.		

Patient:

_____ _____ _____
Name of Patient   Signature   Date

To be completed by individual taking consent:

I confirm that I have explained and discussed with the patient the nature,
purpose, requirements and risks of the study. I confirm that the patient
has understood the information presented to them and is capable of giving
informed consent.

_____ _____ _____
Name of Researcher   Signature   Date

One copy for investigators file.
One copy for the patient.

Appendix B   RESTING PROTOCOL PRESCRIPTION

Patient name:	
Hospital number:	
Trial number:	

Required by:	
Call when ready:	

RESTING SYRINGE 1:

Date:	Start time:	Concentration: **10 nanomol/ml**	Flow rate setting: 1 ml/min	Total Volume infused: ………………of 15 mLs	Prescribed by: …………………………	Administered by: …………………………

RESTING SYRINGE 2:

Date:	Start time:	Concentration: **30 nanomol/ml**	Flow rate setting: 1 ml/min	Total Volume infused: ……………….of 15 mLs	Prescribed by: …………………………	Administered by: …………………………

RESTING SYRINGE 3:

Date:	Start time:	Concentration: **100 nanomol/ml**	Flow rate setting: 1 ml/min	Total Volume infused: ……………of 13.5 mLs	Prescribed by: …………………………	Administered by: …………………………

WASHOUT PERIOD

Study name	The Effects of Apelin in Pulmonary Hypertension		Prescription Sheet – APELIN-13 GOLDEN JUBILEE
Version	3	Date	29th February 2012

RESTING SYRINGE 4:

Date:	Start time:	Concentration:	Flow rate setting:	Total Volume infused:	Prescribed by:	Administered by:
		10 nanomol/ml	1 ml/min	……………of 15 mLs …………………………	……………………………	……………………………

RESTING SYRINGE 5:

Date:	Start time:	Concentration:	Flow rate setting:	Total Volume infused:	Prescribed by:	Administered by:
		30 nanomol/ml	1 ml/min	……………of 15 mLs …………………………	……………………………	……………………………

RESTING SYRINGE 6:

Date:	Start time:	Concentration:	Flow rate setting:	Total Volume infused:	Prescribed by:	Administered by:
		100 nanomol/ml	1 ml/min	……………of 13.5 mLs …………………………	……………………………	……………………………

Study name	The Effects of Apelin in Pulmonary Hypertension		Prescription Sheet – APELIN-13 GOLDEN JUBILEE
Version	3	Date	29th February 2012

EXERCISE PROTOCOL PRESCRIPTION

Patient name:	
Hospital number:	
Trial number:	

Required by:	
Call when ready:	

EXERCISE SYRINGE 1:						
Date:	Start time:	Concentration: **10 nanomol/ml**	Flow rate setting: 1 ml/min	Total Volume infused: ………………of 15 mLs	Prescribed by: …………………………	Administered by: …………………………

EXERCISE SYRINGE 2:						
Date:	Start time:	Concentration: **30 nanomol/ml**	Flow rate setting: 1 ml/min	Total Volume infused: ………………of 15 mLs	Prescribed by: …………………………	Administered by: …………………………

EXERCISE SYRINGE 3:						
Date:	Start time:	Concentration: **100 nanomol/ml**	Flow rate setting: 1 ml/min	Total Volume infused: ……………of 26.7 mLs	Prescribed by: …………………………	Administered by: …………………………

WASHOUT PERIOD

Study name	The Effects of Apelin in Pulmonary Hypertension		Prescription Sheet – APELIN-13 GOLDEN JUBILEE
Version	3	Date	29th February 2012

EXERCISE SYRINGE 4:

Date:	Start time:	Concentration: **10 nanomol/ml**	Flow rate setting: 1 ml/min	Total Volume infused: ………………….of 15 mLs	Prescribed by: ………………………….	Administered by: ………………………….

EXERCISE SYRINGE 5:

Date:	Start time:	Concentration: **30 nanomol/ml**	Flow rate setting: 1 ml/min	Total Volume infused: ……………….of 15 mLs	Prescribed by: ………………………….	Administered by: ………………………….

EXERCISE SYRINGE 6:

Date:	Start time:	Concentration: **100 nanomol/ml**	Flow rate setting: 1 ml/min	Total Volume infused: ……………of 26.7 mLs	Prescribed by: ………………………….	Administered by: ………………………….

Study name	The Effects of Apelin in Pulmonary Hypertension		Prescription Sheet – APELIN-13 GOLDEN JUBILEE
Version	3	Date	29th February 2012

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The Effects of Apelin in Pulmonary Hypertension – Infusion Preparation Guide : Resting Protocol

This document describes the process of preparing the infusions for the study: 'The effects of Apelin in Pulmonary Hypertension.' If there are any questions please contact the Chief Investigator.

Contact:

Dr Lauren Brash
(on behalf of Prof. A Peacock)
Scottish Pulmonary Vascular Unit
Golden Jubilee National Hospital
Agamemnon Street
Clydebank
Glasgow
G81 4DY

Tel: 0141 951 5497
Mob: 07738 569980
Email: laurenbrash@nhs.net

Apelin Infusions
Equipment Required:
3x vials Bachem Apelin-13   50 mls 0.9% sodium Chloride   - Graseby 2000 syringe pump (or equivalent. Check what size syringes are compatible) – Set to deliver 1ml/minute.   3x IV extension set   - leur lock syringes – 5mls/10mls/20mls/30mls/50mls   - hypodermic needles   - Sterile disposable drape   - Sterile gloves   - Red combi syringe stoppers (or equivalent)   - Pre-prepared 'Exercise' syringe labels   - Someone to prepare vials and someone to check preparation

Instructions	
Primary Stock Solution:	300 nanomol/ml

1. Lay disposable sterile drape over clean work surface, clean hands and use sterile gloves to prepare Apelin infusion.

2. Draw up 6.5 mls of 0.9% sodium chloride in 10 mls Syringe.
3. Distribute the 6.5 mls between the 3 vials of lyophilised Apelin. (2.2 mls in 2 vials, 2.1 mls in the third.)
4. Aspirate the solution from all 5 vials back into the syringe.
5. This syringe should be labelled 'PRIMARY STOCK SOLUTION' and contains 6.5 mls of Apelin-13 at a concentration of 300 nanomol/ml.

First dilution step:	******SECOND INFUSION******	30 nanomol/ml

1. Using 20 -50 mls syringe (if this is compatible with syringe pump) aspirate 13.5 mls of 0.9% sodium chloride.
2. Using 5 mls syringe aspirate 1.5 mls of 'PRIMARY STOCK SOLUTION' and add this to 20 mls syringe containing 13.5 mls of 0.9% Sodium Chloride.
3. Put combi stopper (red cap or equivalent) onto the tip of the syringe and ensure solution is thoroughly mixed.
4. This syringe now contains 15mls of Apelin-13 at a concentration of 30 nanomol/ml.
5. Label this syringe with prepared sticker 'RESTING APELIN SYRINGE 2'
6. Attach IV extension set to tip of syringe and manually prime the line ensuring there is no loss of Apelin.
7. 'RESTING APELIN SYRINGE 2' is ready for use.

Secondary Stock Solution:	100 nanomol/ml

1. Using a 20 - 50 mls syringe (if this is compatible with syringe pump) aspirate 10 mls of 0.9% sodium chloride.
2. Add the remaining 'PRIMARY STOCK SOLUTION' to the 10 mls. There should be 5 mls. The final volume will be 15 mls.
3. Put combi stopper (red cap or equivalent) onto the tip of the syringe and ensure solution is thoroughly mixed.
4. This syringe should be labelled 'SECONDARY STOCK SOLUTION' and contains 15 mls of Apelin-13 at a concentration of 100 nanomol/ml.

Second dilution step:	******FIRST INFUSION******	10 nanomol/ml

1. Using a 20 - 50 mls syringe (if this is compatible with syringe pump) aspirate 13.5 mls of 0.9% sodium chloride.
2. Using 5 mls syringe aspirate 1.5 mls of 'SECONDARY STOCK SOLUTION' and add this to 20 mls syringe containing 13.5 mls of 0.9% Sodium Chloride.
3. Put combi stopper (red cap or equivalent) onto the tip of the syringe and ensure solution is thoroughly mixed.
4. This syringe now contains 15mls of Apelin-13 at a concentration of 10 nanomol/ml.
5. Label this syringe with prepared sticker 'RESTING APELIN SYRINGE 1'

6. Attach IV extension set to tip of syringe and manually prime the line ensuring there is no loss of Apelin.
7. 'RESTING APELIN SYRINGE 1' is ready for use.

Final step:	******THIRD INFUSION******	100 nanomol/ml

1. The 20 - 50 mls syringe labelled 'SECONDARY STOCK SOLUTION' should now contain 13.5 mls of Apelin-13 at a concentration of 100 nanomol/ml.
2. Label this syringe with prepared sticker 'RESTING APELIN SYRINGE 3'
3. Attach IV extension set to tip of syringe and manually prime the line ensuring there is no loss of Apelin.
4. 'RESTING APELIN SYRINGE 3' is ready for use.

Final Product:

1. <u>'RESTING APELIN SYRINGE 1'</u>. Syringe containing 15 mls of Apelin-13 at a concentration of **10 nanomol/ml.**

2. <u>'RESTING APELIN SYRINGE 2'</u>. Syringe containing 15 mls of Apelin-13 at a concentration of **30 nanomol/ml.**

3. <u>'RESTING APELIN SYRINGE 3'</u>. Syringe containing 13.5 mls of Apelin-13 at a concentration of **100 nanomol/ml**

Saline Infusions
Equipment Required:

- 50 mls 0.9% sodium Chloride
- 3x IV extension set
- leur lock syringes – 5mls/20mls/30mls/50mls
- hypodermic needles
- Sterile disposable drape
- Sterile gloves
- Red combi syringe stoppers (or equivalent)
- Pre-prepared 'Exercise' syringe labels

Placebo Infusion 1:

1. Aspirate 15mls of 0.9% sodium chloride into a 20 - 50 mls syringe (if this is compatible with syringe pump).
2. Label this syringe with prepared sticker 'RESTING SALINE SYRINGE 1'

3. Attach IV extension set to tip of syringe and manually prime the line ensuring there is no loss of Saline.

Placebo Infusion 2:

1. Aspirate 15mls of 0.9% sodium chloride into a 20 - 50 mls syringe (if this is compatible with syringe pump).
2. Label this syringe with prepared sticker 'RESTING SALINE SYRINGE 2'
3. Attach IV extension set to tip of syringe and manually prime the line ensuring there is no loss of Saline.

Placebo Infusion 3:

1. Aspirate 13.5 mls of 0.9% sodium chloride into a 20 - 50 mls syringe (if this is compatible with syringe pump).
2. Label this syringe with prepared sticker 'RESTING SALINE SYRINGE 3'
3. Attach IV extension set to tip of syringe and manually prime the line ensuring there is no loss of Saline.

Final Product:

1. 'RESTING SALINE SYRINGE 1' Syringe containing 15mls of 0.9% sodium chloride.
2. 'RESTING SALINE SYRINGE 2' Syringe containing 15mls of 0.9% sodium chloride.
3. 'RESTING SALINE SYRINGE 3' Syringe containing 13.5 mls of 0.9% sodium chloride.

Infusion protocol

- Someone who is not carrying out the right heart catheter and who is not recording the data will need to be unblinded to the study.
- Prior to the procedure they should contact Val Pollock via the protocol to establish the order of infusion.
- This will either be 'Apelin then Saline placebo' or 'Saline placebo then Apelin'.
- They will then start each infusion when required.
- If the order is 'Apelin then Saline placebo' they will deliver the syringes in the following order:
    - 'RESTING APELIN SYRINGE 1'
    - 'RESTING APELIN SYRINGE 2'
    - 'RESTING APELIN SYRINGE 3'
    - Then 30 minute washout period before:

- - ‘RESTING SALINE SYRINGE 1’
  - ‘RESTING SALINE SYRINGE 2’
  - ‘RESTING SALINE SYRINGE 3’
- If the order is 'Saline placebo then Apelin' they will deliver the syringes in the following order:
  - ‘RESTING SALINE SYRINGE 1’
  - ‘RESTING SALINE SYRINGE 2’
  - ‘RESTING SALINE SYRINGE 3’
  - Then 30 minute washout period before:
  - ‘RESTING APELIN SYRINGE 1’
  - ‘RESTING APELIN SYRINGE 2’
  - ‘RESTING APELIN SYRINGE 3’

The Effects of Apelin in Pulmonary Hypertension – Infusion Preparation Guide : Exercise Protocol

This document describes the process of preparing the infusions for the study: 'The effects of Apelin in Pulmonary Hypertension.' If there are any questions please contact the Chief Investigator.

Contact:

Dr Lauren Brash
(on behalf of Prof. A Peacock)
Scottish Pulmonary Vascular Unit
Golden Jubilee National Hospital
Agamemnon Street
Clydebank
Glasgow
G81 4DY

Tel: 0141 951 5497
Mob: 07738 569980
Email: laurenbrash@nhs.net

Apelin Infusions
Equipment Required:

- 5x vials Bachem Apelin-13
- 50 mls 0.9% sodium Chloride
- Graseby 2000 syringe pump (or equivalent. Check what size syringes are compatible) – Set to deliver 1ml/minute.
- 3x IV extension set
- leur lock syringes – 5mls/20mls/30mls/50mls
- hypodermic needles
- Sterile disposable drape
- Sterile gloves
- Red combi syringe stoppers (or equivalent)
- Pre-prepared 'Exercise' syringe labels
- Someone to prepare vials and someone to check preparation

Instructions	
Primary Stock Solution:	**300 nanomol/ml**

6. Lay disposable sterile drape over clean work surface, clean hands and use sterile gloves to prepare Apelin infusion.

7. Draw up 10.9 mls of 0.9% sodium chloride in 20 mls Syringe.
8. Distribute the 10.9 mls between the 5 vials of lyophilised Apelin. (2.2 mls in 4 vials, 2.1 mls in the fifth.)
9. Aspirate the solution from all 5 vials back into syringe.
10. This syringe should be labelled 'PRIMARY STOCK SOLUTION' and contains 10.9 mls of Apelin-13 at a concentration of 300 nanomol/ml.

First dilution step:	******SECOND INFUSION******	30 nanomol/ml

8. Using 20 - 50 mls syringe (if this is compatible with syringe pump) aspirate 13.5 mls of 0.9% sodium chloride.
9. Using 5 mls syringe aspirate 1.5 mls of 'PRIMARY STOCK SOLUTION' and add this to 20 mls syringe containing 13.5 mls of 0.9% Sodium Chloride.
10. Put combi stopper (red cap or equivalent) onto the tip of the syringe and ensure solution is thoroughly mixed.
11. This syringe now contains 15mls of Apelin-13 at a concentration of 30 nanomol/ml.
12. Label this syringe with prepared sticker 'EXERCISE APELIN SYRINGE 2'
13. Attach IV extension set to tip of syringe and manually prime the line ensuring there is no loss of Apelin.
14. 'EXERCISE APELIN SYRINGE 2' is ready for use.

Secondary Stock Solution:	100 nanomol/ml

5. Using a 30 – 50 mls syringe (if this is compatible with syringe pump) aspirate 18.8 mls of 0.9% sodium chloride.
6. Add the remaining 'PRIMARY STOCK SOLUTION' to the 18.8 mls. There should be 9.4 mls. The final volume will be 28.2 mls.
7. Put combi stopper (red cap or equivalent) onto the tip of the syringe and ensure solution is thoroughly mixed.
8. This syringe should be labelled 'SECONDARY STOCK SOLUTION' and contains 28.2 mls of Apelin-13 at a concentration of 100 nanomol/ml.

Second dilution step:	******FIRST INFUSION******	10 nanomol/ml

8. Using 20 - 50 mls syringe (if this is compatible with syringe pump) aspirate 13.5 mls of 0.9% sodium chloride.
9. Using 5 mls syringe aspirate 1.5 mls of 'SECONDARY STOCK SOLUTION' and add this to 20 mls syringe containing 13.5 mls of 0.9% Sodium Chloride.
10. Put combi stopper (red cap or equivalent) onto the tip of the syringe and ensure solution is thoroughly mixed.
11. This syringe now contains 15mls of Apelin-13 at a concentration of 10 nanomol/ml.
12. Label this syringe with prepared sticker 'EXERCISE APELIN SYRINGE 1'

13. Attach IV extension set to tip of syringe and manually prime the line ensuring there is no loss of Apelin.
14. 'EXERCISE APELIN SYRINGE 1' is ready for use.

Final step:	******THIRD INFUSION******	100 nanomol/ml

5. The 30 - 50 mls syringe labelled 'SECONDARY STOCK SOLUTION' should now contain 26.7 mls of Apelin-13 at a concentration of 100 nanomol/ml.
6. Label this syringe with prepared sticker 'EXERCISE APELIN SYRINGE 3'
7. Attach IV extension set to tip of syringe and manually prime the line ensuring there is no loss of Apelin.
8. 'EXERCISE APELIN SYRINGE 3' is ready for use.

Final Product:

4. <u>'EXERCISE APELIN SYRINGE 1'</u>. Syringe containing 15 mls of Apelin-13 at a concentration of **10 nanomol/ml.**

5. <u>'EXERCISE APELIN SYRINGE 2'</u>. Syringe containing 15 mls of Apelin-13 at a concentration of **30 nanomol/ml.**

6. <u>'EXERCISE APELIN SYRINGE 3'</u>. Syringe containing 26.7 mls of Apelin-13 at a concentration of **100 nanomol/ml**

Saline Infusions
Equipment Required:

- 50 mls 0.9% sodium Chloride
- 3x IV extension set
- leur lock syringes – 5mls/20mls/30mls/50mls
- hypodermic needles
- Sterile disposable drape
- Sterile gloves
- Red combi syringe stoppers (or equivalent)
- Pre-prepared 'Exercise' syringe labels

Placebo Infusion 1:

4. Aspirate 15mls of 0.9% sodium chloride into a 20 - 50 mls syringe (if this is

compatible with syringe pump).
5. Label this syringe with prepared sticker 'EXERCISE SALINE SYRINGE 1'

Placebo Infusion 2:

4. Aspirate 15mls of 0.9% sodium chloride into a 20 - 50 mls syringe (if this is compatible with syringe pump).
5. Label this syringe with prepared sticker 'EXERCISE SALINE SYRINGE 2'

Placebo Infusion 3:

4. Aspirate 26.7 mls of 0.9% sodium chloride into a 30 - 50 mls syringe (if this is compatible with syringe pump).
5. Label this syringe with prepared sticker 'EXERCISE SALINE SYRINGE 3'

Final Product:

4. 'EXERCISE SALINE SYRINGE 1' Syringe containing 15mls of 0.9% sodium chloride.
5. 'EXERCISE SALINE SYRINGE 2' Syringe containing 15mls of 0.9% sodium chloride.
6. 'EXERCISE SALINE SYRINGE 3' Syringe containing 26.7mls of 0.9% sodium chloride.

Infusion protocol

- Someone who is not carrying out the right heart catheter and who is not recording the data will need to be unblinded to the study.
- Prior to the procedure they should contact Val Pollock via the protocol to establish the order of infusion.
- This will either be 'Apelin then Saline placebo' or 'Saline placebo then Apelin'.
- They will then start each infusion when required.
- If the order is 'Apelin then Saline placebo' they will deliver the syringes in the following order:
  - 'EXERCISE APELIN SYRINGE 1'
  - 'EXERCISE APELIN SYRINGE 2'
  - 'EXERCISE APELIN SYRINGE 3' (with exercise)
  - Then 30 minute washout period before:

Study name	The Effects of Apelin in Pulmonary Hypertension		Infusion Preparation Guide: Exercise protocol
Version	2	Date	25th Feb 2013

- - ‘EXERCISE SALINE SYRINGE 1’
  - ‘EXERCISE SALINE SYRINGE 2’
  - ‘EXERCISE SALINE SYRINGE 3’ (with exercise)
- If the order is ‘Saline placebo then Apelin’ they will deliver the syringes in the following order:
  - ‘EXERCISE SALINE SYRINGE 1’
  - ‘EXERCISE SALINE SYRINGE 2’
  - ‘EXERCISE SALINE SYRINGE 3’ (with exercise)
  - Then 30 minute washout period before:
  - ‘EXERCISE APELIN SYRINGE 1’
  - ‘EXERCISE APELIN SYRINGE 2’
  - ‘EXERCISE APELIN SYRINGE 3’ (with exercise)

Study Title: Acute Pulmonary Haemodynamic Effects of Apelin in Pulmonary Hypertension

Investigator: Prof. A Peacock   **PI:**   **Centre**:

Drug: (Pyr[1])-Apelin-13 (human) Acetate Clinalfa basic	Molecular Weight: 1533.8 g/mol	Weight: 1.0 mg/vial	Lyophilised	Certificate of Analysis enclosed

		Apelin-13 received	Apelin-13 dispensed		Apelin-13 destroyed	Apelin-13 in Stock		
		No. of Vials	Patient ID	No. of Vials	No. of Vials	Total No. of Vials		
Date	Batch No.		N/A if not applicable				Name/ Signature	Comments

Apelin Data Collection Proforma
Appendix C
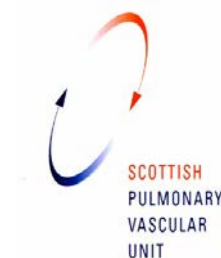
Patient Study Number:

Date:	Approach:
Study centre:	
Operators:	

Age:	Gender	Hb (g/dl)
Body surface area (m^2): 0.00	Height (m):	Weight (kg):
Measured VO2 (mL/min):	Estimated VO2:	BMI (kg/m²):

		Baseline Data		Inhaled NO			
Time							
Load		Unloaded	Unloaded	Unloaded	Unloaded	Unloaded	Unloaded
Watts							
Infusion rate (nmol/min)		No Infusion	No Infusion	No Infusion	No Infusion	No Infusion	No Infusion
Blood Sampling							
FiO2							
SaO2							
Heart Rate							
Systemic BP	Syst						
	Diast						
	Mean						
SVR (dyns)							
SVR (WU)							
RAP (mean)							
RVP (systolic)							
RVEDP							
Pulmonary artery pressure	Syst						
	Diast						
	Mean						
PAWP							

Transpulm gradient							
Fick CO (Q systemic)							
Cardiac output (L/min)							
Cardiac index							
PVR (Wood units)							
PVR Index							
TPR							
Stroke Volume (ml)							
Stroke Volume Index							
Capacitance index							
SvO2 (main PA)							

Study Name	The effects of Pulmonary Hypertension		
Version	5	Date	18th October 2012

Apelin Data Collection Proforma

SCOTTISH PULMONARY VASCULAR UNIT
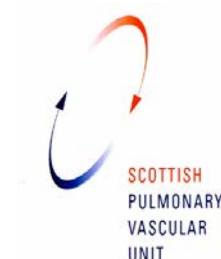
		Resting				Exercise		
		Infusion A						
Time								
Load		Unloaded	Unloaded	Unloaded	Unloaded	Legs Raised	20%	40%
Watts								
Infusion rate (nmol/min)		0	10	30	100	100	100	100
Blood Sampling								
FiO2								
SaO2								
Heart Rate								
Systemic BP	Syst							
	Diast							
	Mean							
SVR (dyns)								
SVR (WU)								
RAP (mean)								
RVP (systolic)								
RVEDP								
Pulmonary artery pressure	Syst							
	Diast							
	Mean							
PAWP								
Transpulm gradient								
Fick CO (Q systemic)								
Cardiac output (L/min)								
Cardiac index								
PVR (Wood units)								
PVR Index								
TPR								
Stroke Volume (ml)								
Stroke Volume Index								
Capacitance index								
SvO2 (main PA)								
Comments								

Study Name	The effects of Apelin in Pulmonary Hypertension	Data collection
Version	5	Date	18th October 2012

Apelin Data Collection Proforma

	Washout - 30 Minutes					Resting Data
Time						
Load	Unloaded	Unloaded	Unloaded	Unloaded	Unloaded	Unloaded
Watts						
Infusion rate (nmol/min)	0	0	0	0	0	0
Blood Sampling						
FiO2						
SaO2						
Heart Rate						
Systemic BP Syst						
Systemic BP Diast						
Systemic BP Mean						
SVR (dyns)						
SVR (WU)						
RAP (mean)						
RVP (systolic)						
RVEDP						
Pulmonary artery pressure Syst						
Pulmonary artery pressure Diast						
Pulmonary artery pressure Mean						
PAWP						
Transpulm gradient						
Fick CO (Q systemic)						
Cardiac output (L/min)						
Cardiac index						
PVR (Wood units)						
PVR Index						
TPR						
Stroke Volume (ml)						
Stroke Volume Index						
Capacitance index						
SvO2 (main PA)						
Comments						

Study Name	The effects of Apelin in Pulmonary Hypertension	Data collection
Version	5	Date	18th October 2012

Apelin Data Collection Proforma

		Resting				Exercise		
		Infusion B						
Time								
Load		Unloaded	Unloaded	Unloaded	Unloaded	Legs Raised	20%	40%
Watts								
Infusion rate (nmol/min)		0	10	30	100	100	100	100
Blood Sampling								
FiO2								
SaO2								
Heart Rate								
Systemic BP	Syst							
	Diast							
	Mean							
SVR (dyns)								
SVR (WU)								
RAP (mean)								
RVP (systolic)								
RVEDP								
Pulmonary artery pressure	Syst							
	Diast							
	Mean							
PAWP								
Transpulm gradient								
Fick CO (Q systemic)								
Cardiac output (L/min)								
Cardiac index								
PVR (Wood units)								
PVR Index								
TPR								
Stroke Volume (ml)								
Stroke Volume Index								
Capacitance index								
SvO2 (main PA)								
Comments								

Study Name	The effects of Apelin in Pulmonary Hypertension	Data collection
Version	5	Date	18th October 2012

SCOTTISH PULMONARY VASCULAR UNIT

Apelin Data Collection Proforma

		Extra Data Sheet					
Time							
Load							
Watts							
Infusion rate (nmol/min)							
Blood Sampling							
FiO2							
SaO2							
Heart Rate							
Systemic BP	Syst						
	Diast						
	Mean						
SVR (dyns)							
SVR (WU)							
RAP (mean)							
RVP (systolic)							
RVEDP							
Pulmonary artery pressure	Syst						
	Diast						
	Mean						
PAWP							
Transpulm gradient							
Fick CO (Q systemic)							
Cardiac output (L/min)							
Cardiac index							
PVR (Wood units)							
PVR Index							
TPR							
Stroke Volume (ml)							
Stroke Volume Index							
Capacitance index							
SvO2 (main PA)							
Comments							

Study Name	The effects of Apelin in Pulmonary Hypertension		Data collection	
Version	5	Date	18th October 2012	

Appendix D

Haemodynamic Effects of Apelin in Pulmonary Hypertension

Protocol Manual

Pulmonary Arterial Hypertension Cohort

Study Contacts and References

Contact	Role	Address/Number
Prof AJ Peacock	Chief Investigator - Glasgow	Scottish Pulmonary Vascular Unit Golden Jubilee National Hospital Agamemnon Street Clydebank Glasgow G81 4DY Tel: 0141 951 5497 Fax: 0141 951 5948 Email: apeacock@udcf.gla.ac.uk
Dr Lauren Brash **CONTACT IF ANY PROBLEMS**	Research Fellow - Glasgow	Scottish Pulmonary Vascular Unit Golden Jubilee National Hospital Agamemnon Street Clydebank Glasgow G81 4DY Tel: 0141 951 5497 Mob: 07738 569980 Email: laurenbrash@nhs.net
Prof DE Newby	Principal Investigator - Edinburgh	Centre for Cardiovascular Science Queen's Medical Research Institute 47 Little France Crescent Edinburgh EH16 4TJ Tel:0131 242 6422 Email: d.e.newby@ed.ac.uk
Dr Luke Howard	Principal Investigator - London	National Pulmonary Hypertension Service - London, Department of Cardiac Sciences, Hammersmith Hospital, Imperial College Healthcare NHS Trust, Du Cane Road, London W12 0HS Tel: 020 8383 3171 Tel (pa): 020 8383 2330 Fax: 020 8383 2331 Email: l.howard@imperial.ac.uk
Dr Naomi Loyse	Clinical Trials Coordinator - London	Wellcome Trust - McMichael Clinical Research Facility (MWTCRF) Imperial College Healthcare NHS Trust Hammersmith Hospital DuCane Road W12 0HS

		T: 020 838 36199 F: 020 338 31763 Email: n.loyse@imperial.ac.uk
Mr AG Brennan	Radiation protection adviser	Health Physics West House (Ground Floor) Gartnavel Royal Hospital 1055 Great Western Road Glasgow G12 0XH Tel: 0141 211 3428 Fax: 0141 211 6761 Email: andy.brennan@ggc.scot.nhs.uk
Victoria McNulty **Fiona Shankland**	Pharmacy - Glasgow	Clinical Trials Pharmacist, Pharmacy Department, Golden Jubilee National Hospital, Agamemnon Street, Clydebank, Dumbartonshire. G81 4DY Tel: 0141 951 5814 Email: victoria.mcnulty@gjnh.scot.nhs.uk Fiona.shankland@gjnh.scot.nhs.uk
Melanie Campbell **Ilyas Ali** **Regina Storch**	Pharmacy - London	Clinical Trials, Pharmacy Department, Area A, Hammersmith Hospital, Du cane Road, London. W12 0HS. Tel: 020 331 34333 Email:melanie.campbell3@imperial.nhs.uk Ilyas.ali2@imperial.nhs.uk Regina.storch@imperial.nhs.uk
Val Pollock	Clinical Trials Nurse – Glasgow **Randomisation contact**	Scottish Pulmonary Vascular Unit Golden Jubilee National Hospital Agamemnon Street Clydebank Glasgow G81 4DY Tel: 0141 951 5485 Email: val.pollock@gjnh.scot.nhs.uk

Centre/Organisation	Reference number	Contact
West of Scotland REC 4	11/AL/0014	**Evelyn Jackson** West of Scotland REC 4 Ground Floor Tennent Building Western Infirmary 38 Church Street Glasgow G11 6NT Tel: 0141 211 1722 Fax: 0141 211 1847 Email: evelyn.jackson@ggc.scot.nhs.uk
Golden Jubilee National Hospital R&D and Sponsor	11/CARD/04	**Dr Catherine Sinclair** Research and Development Manager Beardmore Centre for Health Science National Waiting Times Centre Board Beardmore Street Clydebank G81 4SA Tel: 0141 951 5440 Email: Catherine.Sinclair@gjnh.scot.nhs.uk
		Stella Barr Centre Administrator Beardmore Centre for Health Science National Waiting Times Centre Board Beardmore Street Clydebank G81 4SA Tel:0141 951 5190 Email: stella.barr@gjnh.scot.nhs.uk
NHS Lothian R&D	2011/R/Car/11	**Elizabeth Brownsell** Research Governance Officer Research & Development Department Room E1.12, QMRI Building Royal Infirmary of Edinburgh 47 Little France Crescent Old Dalkeith Road

		Edinburgh EH16 4SA Tel: 0131 242 3359 Email:Elizabeth.brownsell@luth.scot.nhs.uk
Imperial College London	JROHH0283	**Veena Bedekar** Contracts Negotiator Joint Research Office Imperial College London & Imperial College Healthcare NHS Trust Hammersmith Hospital 2nd Floor, Commonwealth Building Du Cane Road London W12 0NN Tel: 020 8383 4578 Email: v.bedekar@imperial.ac.uk
		Becky Ward Research Governance Manager AHSC Joint Research Office Imperial College London and Imperial College Healthcare NHS Trust St Mary's Hospital Faculty of Medicine Room GM14 Ground Floor Mezzanine Floor Praed Street Wing London W12 1PG Tel: 020 3312 2242 Fax: 020 3312 2244 Email: becky.ward@imperial.ac.uk
		Nicola Maycock Research Facilitator AHSC Joint Research Office Imperial College London and Imperial College Healthcare NHS Trust St. Mary's Hospital, Faculty of Medicine Room GM14 Ground Mezzanine Floor Praed Street Wing W2 1PG

		Tel: 020 3312 2240 Fax: 020 3312 2244 Email: nicola.maycock@imperial.nhs.uk
NHS Research Scotland Permissions Coordinating Centre (NRS Permisions CC)	NRS11/RM30	**Stewart Morgan** Senior NRS Administrator NHS Research Scotland Coordinating Centre (NRS Permissions CC) Research & Development Office, Foresterhill House Annexe, Foresterhill, Aberdeen AB25 2ZB Tel: 01224 552690 Email: stewart.morgan@nhs.net
National Institute for Health and Research (NIHR) Coordinated System for gaining NHS Permissions (CSP) reference www.crncc.nihr.ac.uk	63167	**Jillian Martin** RM&G Manager C&L CLRN Tel: 01772 524944 Email: jillian.martin@lthtr.nhs.uk
		Jane Darnbrough Research Approvals Facilitator NIHR Clinical Research Network Tel: 020 3328 6722 Fax: 020 7636 5138 Email: jane.e.darnbrough@nihr.ac.uk
www.clinicaltrials.gov	NCT01457170	Updated by Lauren Brash/Sponsor
UK Clinical Research Network (UKCRN) Study Portfolio	In progress	Updated by Sponsor
BHF Project Grant no.	PG/11/113/29280	

Contents

1. Identifying Study participants

1.1 Screening

Patients will be identified as potential study participants when attending clinic for review or when admitted to the ward for diagnostic investigation of probable idiopathic pulmonary arterial hypertension (IPAH).

The recruitment checklist will be used to identify if patient meets inclusion/exclusion criteria. They will need to have had previous echocardiogram, bloods, pulmonary function tests and CT scan to establish whether they meet recruitment criteria.

If attending for diagnostic investigations they will be recruited and consented on the proviso that during the diagnostic right heart catheterisation (RHC) they will meet all the inclusion criteria thus allowing continuation into the study protocol.

1.2 Consenting

If the patient meets all the inclusion criteria (or all inclusion criteria other than impending RHC criteria) the patient will be offered participation into trial. They should be given a patient information sheet and allowed at least 24 hours to consider the information prior to consent being obtained. Patients who are on warfarin may give verbal consent over the phone to stop warfarin. They should have read the patient information sheet and have had a member of the study team explain the consequences of stopping warfarin.

1.3 Cardiopulmonary exercise testing

In the lead up to the study, the participant should undergo an erect maximal incremental cardiopulmonary exercise test (CPET) to establish their maximum workload. This will allow calculation of the workload required during the exercise component of the study protocol. If the subject is unsuitable for the exercise component of the study this step can be omitted.

1.4 Randomisation

The IPAH participants will be allocated a study number from 301 to 321. Each study number has been randomly allocated an infusion order. They will either receive Apelin as Infusion A then Placbo as Infusion B or vice versa. Participants for the IPAH cohort will be recruited at two sites; Hammersmith and Glasgow. There is one contact who will allocate the study number and randomisation order for recruits at both sites. This is Val Pollock, clinical trials nurse at Glasgow. Once a participant has been recruited, a member of the study team should contact Val to receive their study number and Infusion order. Contact should be made at least a few days before the study is planned via email if possible. The person who collects this information will be unblinded and should not be involved in collecting data.

Val Pollock
Scottish Pulmonary Vascular Unit
Golden Jubilee National Hospital
Agamemnon Street
Clydebank
Glasgow
G81 4DY

Tel: 0141 951 5485
Email: val.pollock@gjnh.scot.nhs.uk

1.5   Intravenous access

Study participants will require a peripheral intravenous cannula (preferably 20G or 18G) to allow administration of the infusions. This should not be in the same arm as the sphygmomanometer cuff that will be used during RHC to measure systemic BP.

1.6   Introducer sheath

A sheath will be inserted as is usual procedure for RHC. In order to allow the possibility of the exercise component of the protocol to be carried out, this should preferably be in the internal jugular vein. And the femoral veins should be avoided.

2. Apelin

2.1   Ordering/Transport

The Apelin is provided by Bachem Clinalfa basic and is approved for use in clinical trials. Each vial contains 1mg. Depending on whether exercise is planned or not each study will require either 5 or 3 vials.

Bachem have agreed to deliver the Apelin in 3 batches. Each batch is transported in -20C conditions. There is a temperature logger with each delivery and this must be downloaded and returned to Bachem who will analyse the data and notify the units regarding whether the delivery is safe for use. It can be arranged for Apelin to be delivered between sites if necessary. When a delivery is received it should be logged in the drug accountability document.

If stocks are running low please get in touch with Dr Lauren Brash to order more Apelin.

There is a Certificate of Analysis and transmissible spongi-form encephalopathy (TSE) certificate with each delivery.

2.2   Storage

Apelin should be stored at -20°C when it arrives in pharmacy until it is used.

2.3   Preparation/Labelling

The Apelin should be collected from pharmacy at least 30 minutes before use, to allow time for it to defrost at room temperature.

5 vials will be required if exercise is planned. 3 vials will be required if no exercise is planned. The Apelin can be requested using the Apelin request form and will be logged in the Drug Accountability document.

Please follow the appropriate Infusion Preparation document for further guidance on preparing the infusions.

Labels will be provided for each infusion. If more labels are required, please contact Dr Lauren Brash

2.4   Infusion

Infusion of both Apelin and 0.9% sodium chloride placebo should be via a Graseby Pump (or equivalent) set at 1ml/min. The infusion line will need to be primed manually with 1 mL of the solution. The pump automatically stops with 1 mL left to infuse. Therefore the infusion time of each syringe is 2 minutes less than expected.

An unblinded person at each site will have received the randomisation order and will label the 6 syringes as infusion A (10, 30, 100 nanomol/mL) or Infusion B (10, 30, 100 nanomol/mL).

Infusion A should be given in sequential order. There will then be a 30 minute washout period before Infusion B is delivered in sequential order.

3. Data Collection

3.1   Clinical enrolment proforma

There is a clinical enrolment proforma and a data collection proforma.

Once a patient has been identified as a potential study participant, the clinical enrolment proforma should be completed. This will document the relevant inclusion and exclusion variables to ensure they are appropriate for the study.

3.2   Data collection proforma

All data during the cath lab study should be collected on the provided data collection proforma.

The person carrying out the RHC and collecting the data should be blinded to the infusion order. (ie whether Infusion A is placebo or Apelin.)

Initially diagnostic RHC data will be collected including heart rate (HR), systemic blood pressure (BP), right atrial pressure (RAP), right ventricular pressure (RVP), right ventricular end-diastolic pressure (RVEDP), pulmonary artery pressure (PAP), pulmonary artery wedge pressure (PAWP) and cardiac outputs. Nitric oxide should be given as per standard protocol if this is a diagnostic assessment.

3.3   Order of data collection

Infusion A at 10 nanomol/mL should be put in the Grasby Pump set to deliver the infusion at 1 mL/min. A timer should be set and data collection should start at 5 mins.

Data to be collected is:

   mean PAP, PAWP, CO x2, mean PAP, HR *then* **manual systemic BP**
   (so that cuff doesn't cause increase in any other pressure measurements)

This should be repeated with Infusion A at 30 nanomol/mL.

Then repeated with Infusion A at 100 nanomol/mL.

After the resting data is collected the third infusion should be paused to allow time to get the supine ergometer into position on the procedure table. Once this is in position with the participants feet strapped into the pedals the third infusion should be restarted. Initally the infusion should be delivered while the participant rests with their feet elevated in the pedals. The same data (above in bold) should be collected at 5 minutes.
The participant should then start cycling at 20% of their maximal erect CPET workload. After 2 minutes of cycling further data should be collected (the participant should continue exercise while this is collected).
The workload should be increased to 40% of their maximal erect CPET workload and after a further 2 minute of cycling further data should be collected.

A 30 minute washout/rest period is then required before carrying out the same protocol for Infusion B.

4. Blood Samples

Blood samples should be collected just prior to starting both infusion A and Infusion B and immediately post exercise (or if no exercise, at the end of the infusion period) for each infusion.

There should be 1 x EDTA bottle (4ml) and 2 x serum separating bottles (5ml) collected at each time point. The EDTA bottles with the blood should be placed immediately on ice on withdrawal from the vein. The SST bottles with bloods will be allowed to stand at room temperature for 30mins – 60mins before processing. Both samples should be processed within 1hour after collection.The samples should be taken via the 3 way tap on the side arm of the IJV sheath.

The EDTA samples should be spun in a centrifuge at 4000rpm for 15 mins at 4°C. The SST samples should be spun at the same centrifuge setting as the EDTA but at room temperature (between 18°C -20°C). Each plasma Sample (EDTA) and serum samples (SST) should then be aliquoted in two 1.5ml eppendorfs storage tubes and then stored at -80°C until the end of the study.

The collection tubes should be labelled:

Study No:…. Date….. Pre-Inf A. (EDTA)
Study No:…. Date….. Pre-Inf A. (SST)
Study No:…. Date….. Pre-Inf A. SST

Study No:…. Date….. Post-Inf A.(EDTA)
Study No:…. Date….. Post-Inf A. (SST_)
Study No:…. Date….. Post-Inf A. (SST)

The same should be done for the samples collected during Infusion B.

The Eppendorf tubes should be labelled as above however there should be two 1.5 ml for each colletion tube at each time-point for infusion A and infusion B.

5. Unblinding Procedure

The principal Investigator at each site will determine whether there is any reason for a particular study to be unblinded. If they establish this to be the case, they can approach the unblinded person on site who can provide the infusion details. Any unblinding should be reported to the Chief Investigator with details of the reason for unblinding as soon as possible.

6. Progress Reporting

In order to provide the study sponsor with recruitment updates, Dr Lauren Brash will email each centre at the end of each month to confirm recruitment numbers. If there have been and serious adverse events (SAEs) these will need to be reported also.

The information the sponsor requires is:

Total recruitment numbers for the month
Patient study ID numbers for the month
Dates of recruitment
SAE reports

Appendix E (Clinical Services Division)

DEPARTMENT OF CLINICAL PHYSICS AND BIO-ENGINEERING
Health Physics Service
West House (Ground Floor)

Gartnavel Royal Hospital	**Telephone**: 0141 211 3428
1055 Great Western Road	**Fax**: 0141 211 6761
Glasgow G12 OXH	**E-mail**: andy.brennan@ggc.scot.nhs.uk

memorandum

To: Prof A Peacock
Scottish Pulmonary Vascular Unit,
GJNH

From: Mr A G Brennan

Date: 10 Dec 2010

Subject: ACUTE PULMONARY HAEMODYNAMIC EFFECTS OF APELIN IN PATIENTS WITH PULMONARY HYPERTENSION
Study:Apelin PH Protocol 23-11-10

1. <u>Radiation Dose Assessment</u>

A radiation dose assessment for the proposed examination protocol is tabulated below. My interpretation of the supplied data (following discussion with Dr Lauren Brash) is that these patients are expected to have 1 Rt Heart Cath Procedure during a Diagnostic Coronary Angiogram from their involvement in this study. I've used Mean recorded patient dosimetry data for Right Heart Cath procedures performed by Dr M Johnson in the Cath Lab. The local DRL for a diagnostic coronary angiogram is $36Gycm^2$ & 5.6 minutes fluoroscopy (3rd Quartile).

The Total Research Protocol Dose is 7.8 mSv, i.e. the expected dose for x1 CA & x1 Rt Heart Cath at GJNH site.

Radiological Protocol	Examination	DAP (Gycm^2)	Total Effective Dose (mSv) (NRPB-W4)	Additional Lifetime Fatal Cancer Risk	Risk
Normal	1 CA	22.1	5.7	1 in 3480	Intermediate
Expected	1 CA & 1 Rt Heart Cath	30.1	7.8	1 in 2560	Intermediate
Additional	1 Rt Heart Cath	8	2.1	1 in 9600	Intermediate

2. <u>Risk Estimate</u>
2.1 mSv effective dose is equivalent to 11 months background radiation and represents an additional risk of lifetime fatal cancer of 1 in 9600. In terms of comparative lifetime risk of death, it is similar to the lifetime risk of death from work in a service industry, and is in the Intermediate risk category for bio-medical research (ICRP Publication 62, 1991).

A G Brennan

AA

Radiation Protection Adviser
& Consultant Medical Physicist

c.c.   Dr Lauren Brash
       Dr Grant Baxter
       Miss Lynn Dallas

Appendix F

Study Title: Acute Pulmonary Haemodynamic Effects of Apelin in Pulmonary Hypertension

Main REC: West of Scotland REC 4   **REC reference number:** 11/AL/0014
Chief Investigator: Prof. A Peacock

	Centre	Principal Investigator

Study Participant

Patient No.	Date of Birth	Gender	Date of RHC

Event Information

Event	Date/Time of onset	Action Taken	Outcome	Date/time of resolution	Relationship to study procedure
		None ☐ Terminated prematurely ☐	Unknown ☐ Resolved ☐ Resolved with sequelae ☐ Ongoing ☐ Fatal ☐		Not reasonably attributable ☐ Possibly attributable ☐ Reasonably attributable ☐ N/A ☐

Minor Event ☐

Serious Event: (Report to Chief Investigator ASAP)

Death ☐
Life Threatening ☐
Hospitalisation/prolongation of existing ☐

Persistent/significant disability or incapacity ☐
Congenital anomaly or birth defect ☐

Appendix F

Concomitant Medication (attach additional sheets if required)		
Medication name	Dose	Route

Adverse Event – Details (attach additional sheets if required)

Adverse Event – Details Continued…	

Reporting Investigator				
Name (printed)	Address	Contact number/email	Signature	Date

Please send completed forms to the Chief Investigator via:

Contact: Dr Lauren Brash
Scottish Pulmonary Vascular Unit
Golden jubilee National Hospital
Agamemnon Street
Clydebank
Glasgow
G81 4DY

Tel: 07738 569980
Fax: 0141 951 5948
Email: laurenbrash@nhs.net

List of References

1. Morrell, N. W. *et al.* Cellular and Molecular Basis of Pulmonary Arterial Hypertension. *Journal of the American College of Cardiology* **54**, S20–S31 (2009).
2. Badesch, D. B. *et al.* Diagnosis and Assessment of Pulmonary Arterial Hypertension. *Journal of the American College of Cardiology* **54**, S55–S66 (2009).
3. Hoeper, M. M. *et al.* Definitions and diagnosis of pulmonary hypertension. *Journal of the American College of Cardiology* **62**, D42–50 (2013).
4. Galie, N. *et al.* 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). *European Heart Journal* **37**, 67–119 (2016).
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