Abstract

Pulmonary arterial hypertension (PAH) is a rare, but fatal disease of the pulmonary arteries that evades early diagnosis. Although modern targeted therapies have improved symptoms and outcomes in PAH, lung transplantation remains the only cure. Identification of new biomarkers of PAH would facilitate early diagnosis and enable prompt targeted therapy that could halt the dire outcome.

Immune markers of PAH are under intense scrutiny given the substantial evidence implicating inflammatory processes in PAH pathogenesis. T-lymphocytes and NK cells are known to play important roles in animal models of PAH. Decreased circulating cytotoxic CD8+ T lymphocytes and natural killer (NK) cells are reported in IPAH patients but the effect of their depletion on disease outcome is unknown. Elevated cytokine levels are reported to predict adverse outcome in IPAH patients but the underlying mechanism for this remains unknown.

Non-immune markers of PAH such as plasma Brain Natriuretic Peptide (BNP), cardiac troponins and endothelin have been under investigation for some time. Plasma BNP is currently the favoured prognostic biomarker and is the only marker recommended by international guidelines.

Method: This prospective study analysed the lymphocyte and cytokine profiles of patients with idiopathic PAH (IPAH), connective tissue disease associated PAH (CTD-APAH) and matched healthy controls. CD4+ (Helper T-cell marker), CD8+ (Cytotoxic T-cell marker), CD56/CD16 (NK cell marker) and CD19+ (mature B-cell marker) cell numbers were assessed together with the immunofluorescence levels of 507 inflammatory cytokines using a commercially available cytokine array. Lymphocyte counts and cytokine profiles were then correlated with clinical outcome.
and with the plasma BNP. Finally, the utility of cardiac troponin I and endothelin-1 as prognostic markers was explored.

Results: Fourteen PAH patients (9 IPAH, 5 CTD) were recruited. Three patients were deceased at one year follow up; all had elevated BNP levels, elevated CD4:CD8 ratios and deficiencies of NK cells and cytotoxic CD8+ T-lymphocytes at recruitment. Patients with normal lymphocyte profiles at recruitment were all alive a year later and none on the active transplant list. Deficiency of NK and CD8+ cells was associated with increased interleukin (IL)-23 and decreased IL-21 immunofluorescence levels. As univariate markers of survival cytotoxic CD8+ T-cell and NK cell counts (and the associated cytokine profile) appear to be more powerful predictors of short term survival than the currently recommended biomarker, BNP. Cardiac troponin I was an insensitive prognostic marker in both IPAH and CTD-APA patients. Endothelin-1 levels were predictive of adverse outcome in IPAH patients but not CTD-APA.

Conclusion: NK cell and cytotoxic CD8+ T-cell depletion in IPAH and CTD-APA patients is associated with elevated BNP levels and an increased risk of death. An altered cytokine profile (elevated IL-23 and depleted IL-21 immunofluorescence levels) is associated with combined deficiency of NK and CD8+ cells. Further research is required to elucidate the mechanism of these findings and could lead to new NK and CD8+ directed immunotherapy.
Preface

Acknowledgements

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I would like to dedicate this research to: my children Eleri Rhianwen, Thomas Llewelyn and to their new sibling “et al” (who will arrive in the next few days) and also to Tadcu and Mamgu who will always remain dear to me.
Publications

The work presented in this thesis, has in part, been published and presented at conferences.

Papers.

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Presentation.

Oral Presentation to the College of Intensive Care Medicine of New Zealand, Hawkes Bay February 2010.

Poster presentation at the Thoracic Society of Australia and New Zealand, Brisbane April 2010.

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List of abbreviations

Activin A Receptor type 1 (ACVR1)

Arterial blood gas (ABG)

**Bone morphogenetic protein 2 (BMP-2)**

Bone morphogenetic protein 4 (BMP-4)

Bone morphogenetic protein receptor type 2 gene (BMPR-2)

Bone morphogenetic protein receptor type II (BMPR-II) encoded by BMPR-2

**Brain Natriuretic Peptide (BNP)**

Cardiac troponin I (cTnI)

Cardiac troponin T (cTnT)

Cardiac output (CO)

Cluster of differentiation (CD)

Connective tissue disease (CTD)

Connective tissue disease associated PAH (CTD-APAH)

Coronary artery bypass graft (CABG)

Diffusion lung capacity for carbon monoxide (DLCO)

Endothelin-1 (ET-1)

Endothelin-3 (ET-3)

Enzyme linked immunosorbent assay (ELISA)

Forkhead box (Fox)

Idiopathic PAH (IPAH)

Interleukin (IL)
List of abbreviations continued

Human immunodeficiency virus (HIV)

Mean pulmonary arterial pressure (mPAP)

Natural killer cell (NK cell); CD56+ CD3-

Natural killer T cell (NKT cell); CD56+, CD3+

New York Heart Association (NYHA) functional class

N terminal fragment NT-proBNP (NT-proBNP)

Matrix metalloproteinase (MMP)

Monocyte chemotactic protein (MCP)

Pulmonary arterial hypertension (PAH)

Pulmonary arterial pressure (PAP)

Pulmonary hypertension (PH)

Pulmonary vascular resistance (PVR)

Pulmonary wedge pressure (PWP)

Programmed death 1 (PD-1)

Right heart catheterisation (RHC)

Right ventricular systolic pressure (RVSP)

6 Minute walk distance (6MWD)

Systemic sclerosis (SSc)

T-helper cells (Th) cells (CD4+)

T-cytotoxic cells (Tc) (CD8+)

T-regulatory (Treg) cells (CD4+ CD25hiFoxP3CD127low)
CHAPTER 1

General introduction
1.1 **Pulmonary arterial hypertension (PAH)**

Pulmonary arterial hypertension is a rare, but fatal disease of the pulmonary arteries. It is characterised by vascular proliferation and remodeling accompanied by progressive increases in pulmonary vascular resistance culminating in right ventricular failure and death.\(^{1,2}\) Despite significant cardiovascular changes the early symptoms and signs of PAH are subtle and patients are frequently only diagnosed when presenting with incapacitating breathlessness and signs of severe right heart failure. Symptoms are often attributed to deconditioning or other commoner cardio-respiratory conditions leading to delayed diagnosis.

The diagnosis of PAH is confirmed by right heart catheterisation (RHC) and is defined by the presence of pre-capillary pulmonary hypertension (PH): mPAP $\geq$ 25mmHg; a pulmonary wedge pressure (PWP) $< 15$mmHg; pulmonary vascular resistance (PVR) $> 3$ Wood units; normal or reduced cardiac output at rest and absence of all other causes of pre-capillary PH, Table 1.1 and Figure 1.1.\(^1\) The severity of PAH is established using a combination of RHC measurements and functional assessments such as the 6 minute walk distance (6MWD) and New York Heart Association (NYHA) functional class.

PAH has been subdivided into five aetiological groups: idiopathic PAH (IPAH), heritable PAH, drug and toxin induced PAH, persistent pulmonary hypertension (PH) of the newborn and PH associated with conditions such as connective tissue disease (CTD-APA), Table 1.2.\(^{1,3}\) The French National Registry reported the aetiology of 674 PAH patients: IPAH (39%), heritable PAH (9%), anorexigen-induced (9.5%) and associated with CTD (15.3%), congenital heart disease (11.3%), portal hypertension
Table 1.1: Haemodynamic Definitions of Pulmonary Hypertension

(Adapted from the European Guidelines 2009\textsuperscript{1})

<table>
<thead>
<tr>
<th>Definition</th>
<th>Characteristics</th>
<th>Clinical groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulmonary hypertension (PH)</td>
<td>Mean PAP≥ 25mmHg</td>
<td>All</td>
</tr>
<tr>
<td>Pre-capillary PH</td>
<td>Mean PAP≥ 25mmHg</td>
<td>1. Pulmonary arterial hypertension</td>
</tr>
<tr>
<td></td>
<td>PWP≤ 15mmHg</td>
<td>2. PH due to lung disease</td>
</tr>
<tr>
<td></td>
<td>CO normal or reduced</td>
<td>3. Chronic thromboembolic PH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. PH unclear/multifactorial cause</td>
</tr>
<tr>
<td>Post-capillary PH</td>
<td>Mean PAP≥ 25mmHg</td>
<td>PH secondary to left heart disease</td>
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<tr>
<td></td>
<td>PWP&gt; 15mmHg</td>
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<tr>
<td></td>
<td>CO normal or reduced</td>
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</table>

Pulmonary hypertension (PH), PAP = pulmonary arterial pressure, PWP = pulmonary wedge pressure, CO = cardiac output.
Figure 1.1: Diagnostic algorithm for PAH patients in New Zealand

(From European Guidelines and New Zealand Review article)\textsuperscript{1,4}

\textbf{ALK-1} = actin-receptor-like kinase; \textbf{ANA} = antinuclear antibodies; \textbf{BMPR2} = bone morphogenetic protein receptor 2; \textbf{CHD} = congenital heart disease; \textbf{CMR} = cardiac magnetic resonance; \textbf{CTD} = connective tissue disease; \textbf{CTEPH} = chronic thromboembolic pulmonary hypertension; \textbf{Group} = clinical group (1-5); \textbf{HHT} = hereditary haemorrhagic telangiectasia; \textbf{HIV} = human immunodeficiency virus; \textbf{HRCT} = high-resolution computed tomography; \textbf{LFT} = liver function tests; \textbf{mPAP} = mean pulmonary arterial pressure; \textbf{PAH} = pulmonary arterial hypertension; \textbf{PCH} = pulmonary capillary haemangiomatosis; \textbf{PFH} = pulmonary function test; \textbf{PH} = pulmonary hypertension; \textbf{PVOD} = pulmonary veno-occlusive disease; \textbf{PWP} = pulmonary wedge pressure; \textbf{RHC} = right heart catheterisation; \textbf{TTE} = transthoracic echocardiography; \textbf{U&L} = ultrasonography; \textbf{V/Q} scan = ventilation/perfusion lung scan.
Table 1.2: Clinical classification of Pulmonary Hypertension (PH)

(Adapted from European and American guidelines 2009\textsuperscript{1,3})

**Group 1: Pulmonary arterial hypertension (PAH)**

1.1 Idiopathic PAH (IPAH)

1.2 Heritable PAH

1.3 Drugs and toxin induced

1.4 Associated with

   **1.41 Connective tissue disease (CTD-APAHA)**

   1.42 HIV infection

   1.42 Portal hypertension

1.43 Congenital heart disease

1.44 Schistosomiosis

1.45 Chronic haemolytic anaemia

1.5 Persistent PH of the newborn

**Group 1**: Pulmonary veno-occlusive disease and/or pulmonary capillary haemangiomatosis

**Group 2**: PH secondary to left heart disease

**Group 3**: PH secondary to lung disease and/or hypoxia

**Group 4**: Chronic thromboembolic PH

**Group 5**: PH unclear and/or multifactorial mechanism
(10.4%) and HIV (6.2%).\textsuperscript{8} The prevalence of PAH in New Zealand is estimated at 40 cases per million population similar to that reported in European studies.\textsuperscript{4,5,6}

Untreated, IPAH has an estimated median survival from diagnosis of 2.8 years with 1-, 3- and 5-year survival rates of 68%, 48%, and 35%.\textsuperscript{7,8} The REVEAL study of 2716 patients (from all PAH sub-groups) receiving modern day therapies reported 1-, 3-, 5- and 7-year survival rates from diagnostic RHC of 85%, 68%, 57% and 49% respectively.\textsuperscript{9,10} Sub group analysis showed CTD-APAH patients had lower 1- and 3-year survival rates than IPAH patients 86% v 93% (p=0.0001) and 67% v 73% (p=0.03) respectively.\textsuperscript{11}

The algorithm for treating PAH patients is shown in Figure 1.2.\textsuperscript{4} Lung transplantation is the only known cure, a devastating prospect for the majority of patients as many die waiting for that scarcely available compatible donor organ. Even when lung transplantation is feasible the long-term survival rates are poor with 1-, 5- and 10 year survival rates of 78%, 51% and 18% respectively.\textsuperscript{12} Modern therapies have improved the symptoms and quality of life of PAH patients, however, morbidity and mortality rates remain unacceptably high. To a large degree, these high morbidity and mortality rates reflect delayed diagnosis and the lack of adequate early screening or diagnostic tests.

There is a desperate need to enhance our understanding of the pathogenesis of PAH and identify non-invasive biomarkers of disease which could improve the diagnostic process and allow earlier targeted therapies.
At the time of conducting this study many pharmaceutical agents including intravenous prostacyclin and 'Triple therapy' (ERA+prostanoids+PDE-5 inhibitors) were not available in clinical practice in New Zealand due restricted funding from the Pharmaceutical Management Agency (PHARMAC).
Although the pathology of PAH is well characterised, with aetiological sub-groups sharing almost identical pathologies (medial hypertrophy, intimal proliferative and fibrotic changes, adventitial thickening and marked perivascular inflammatory infiltrates)\textsuperscript{13-16} the pathogenesis of PAH is complex and poorly understood.

Germline mutations in the bone morphogenetic protein receptor type 2 (BMPR2) are detected in up to 40% of IPAH and 74% of heritable PAH but none are identified in patients with CTD-APAH.\textsuperscript{17,18} This finding together with the relatively low penetrance in family members with BMPR-2 mutations support a multi-hit hypothesis, in which vascular abnormalities are triggered by accumulation of genetic, inflammatory and/or environmental insults in a susceptible person.

Patients with infections associated with lymphocyte depletion have an increased prevalence of PAH.\textsuperscript{18} Lymphopoiesis is regulated by bone morphogenetic proteins-2 and -4 the lung specific ligand’s for the receptor encoded by BMPR-2.\textsuperscript{19} Inflammatory cytokines such as interleukin (IL)-6 involved in BMPR-2 signaling become dysregulated in patients harboring BMPR-2 mutations and may increase their susceptibility to an inflammatory insult.\textsuperscript{17}

To identify new biomarkers of PAH, this study will focus on the role played by the immune system in the pathogenesis of PAH, in particular lymphocytes and inflammatory related cytokines. Their utility as immune biomarkers will then be compared with the previously validated biomarker, plasma Brain Natriuretic peptide (BNP).
1.2 Biomarkers

1.2.1 What is an ideal biomarker in PAH?

Right heart catheterisation is the gold standard diagnostic tool in PAH. However, this procedure is invasive and costly and therefore an impractical and risky procedure for repeated assessments such as monitoring the response to therapies. An ideal biomarker would be sensitive, reproducible, non-invasive and easy to perform, correlate with haemodynamic measurements and parallel the impact of therapy.

The REVEAL study (2716 PAH patients, all aetiological groups) and the French National registry (354 patients with heritable, anorexigen-induced or IPAH) have identified that female sex, NYHA functional class I and II and greater 6MWD are associated with a good clinical outcome. In the REVEAL study renal insufficiency, CTD-APAH, NYHA class III, mean right atrial pressure, resting systolic blood pressure and heart rate, percent predicted carbon monoxide diffusing capacity, pericardial effusion (on echocardiography) and plasma BNP all predicted mortality at 1 year. While these are powerful clinical predictors, they are also obvious signs of advanced disease. A biomarker identifying patients with a poor prognosis in PAH prior to developing advanced illness would clinically be more useful.

Plasma BNP is the only peripheral blood biomarker currently recommended for routine use by international PAH guidelines and was the standard of care at Christchurch Hospital PAH clinic during study design. Nagaya et al was the first to demonstrate that PAH patients with a baseline median BNP level ≥ 150pg/ml had a worse outcome than those with a plasma level < 150pg/ml, and those with persistent elevation of BNP (>180pg/ml) at 3 month follow-up had a worse
outcome. Recently, plasma BNP analysis conducted in 1340 patients (REVEAL study) showed that BNP levels ≥ 180pg/ml are associated with an increased risk of death at 1 year, whereas, BNP levels < 50pg/ml were associated with increased survival at 1 year.\(^{13}\)

1.2.2 Immune biomarkers of PAH

Increasing evidence implicates the immune system in PAH pathogenesis.\(^{17,18}\) Inflammatory cells (such as lymphocytes, mast cells and dendritic cells) are found surrounding the plexiform lesions in diseased pulmonary arteries from patients with IPAH and CTD-APAH, suggesting a common immune pathophysiology.\(^{13-15}\) Circulating levels of C-reactive protein (CRP) levels are increased in PAH patients compared with normal controls and levels correlate with PAH severity and patient survival confirming that immune mediators may be useful prognostic markers in PAH.\(^{23}\)

1.2.2.1 Lymphocytes as prognostic biomarkers

Lymphocyte depletion is known to be an adverse prognostic marker in chronic viral infections (such as HIV and hepatitis C) and cancer.\(^{24,25}\) Although lymphocyte depletion is already described in PAH patients, the clinical impact of their depletion has not been reported.\(^{26,27}\) This study will characterise the lymphocyte profile of PAH patients and correlate findings with clinical outcome measures, such as transplant free survival in chapter 2.
1.2.2.2 Inflammatory related cytokines as prognostic biomarkers

Increased circulating levels of inflammatory cytokines are described in patients with idiopathic and associated PAH. \(^{28-31}\) Elevated plasma levels of five inflammatory cytokines (IL-2, IL-6, IL-8, IL-10 and IL-12p70) from a total of eleven cytokines studied were recently reported to predict survival in PAH patients.\(^ {31}\) Using a commercially available cytokine array which can simultaneously assess the expression of 507 inflammatory related proteins (such as cytokines, chemokines and growth factors) this study will determine the protein profile of PAH patients and then correlate findings with clinical outcome measures in chapter 3.

1.2.3 Non-immune biomarkers

Plasma endothelin-1 (ET-1) was shown to be significantly higher in patients with IPAH than in normal subjects but the prognostic significance is unknown. \(^ {32}\) Persistent elevation of cardiac troponin T (cTnT) was observed in 4 out of 56 patients with stable PH who suffered premature death, but the sensitivity of the test was felt to be low. \(^ {33}\) Literature on the higher sensitivity assay cardiac troponin I (cTnI) in PAH is sparse and its utility as a prognostic marker unknown.

This study will investigate the plasma levels of cTnI and ET-1 in IPAH and CTD-APAH patients and correlate findings with clinical outcome and plasma BNP in chapter 4.
1.3 Study aims

1.3.1 Lymphocyte markers of PAH

1.3.1.1 Determine the peripheral lymphocyte profile of human PAH patients in comparison to normal controls

1.3.1.2 Correlate lymphocyte profile with clinical outcome, defined as transplant free survival at one year

1.3.1.3 Compare the utility of lymphocyte markers with the previously validated biomarker BNP

1.3.2 Inflammatory related cytokines as markers in PAH

1.3.2.1 Determine the differential expression of 507 inflammatory related cytokines in the peripheral blood of patients with PAH and normal controls using a cytokine array and then:

1.3.2.2 Correlate cytokine profile with clinical outcome

1.3.2.3 Correlate cytokine profile with lymphocyte profiles

1.3.3 Non-Immune markers of PAH

1.3.3.1 Investigate the utility of BNP as a prognostic marker in PAH

1.3.3.2 Investigate the utility of cTnI as a prognostic marker in PAH

1.3.3.3 Investigate the role of ET-1 as a prognostic marker in PAH
CHAPTER 2

Lymphocytes and PAH
2.1 Introduction

Considerable evidence implicates inflammation in the pathogenesis of PAH.\textsuperscript{17,18} Lymphocytes, mast cells, macrophages and dendritic cells have been found surrounding plexiform lesions in the diseased pulmonary arteries of IPAH and CTD-APA patients.\textsuperscript{13-15} Explanted lungs from all PAH subgroups show evidence of inflammation within the pulmonary arteries which correlate with the extent of vascular remodelling, suggesting a common immunepathophysiology.\textsuperscript{16} Patients with systemic inflammatory conditions such as connective tissue disease and infections (such as Schistosomiasis, HIV, human herpes virus 8 and hepatitis C) have a higher prevalence of PAH.\textsuperscript{17-19} A higher prevalence of PAH is also noted in patients who have undergone splenectomy, even after trauma.\textsuperscript{17-19}

2.1.1 Overview of Lymphocytes

Lymphocytes arise from a common lymphoid progenitor cell developing along three separate lineages into B-lymphocytes, T-lymphocytes and natural killer (NK) cells, Figure 2.1. T-lymphocyte development occurs in the thymus gland whilst B-lymphocyte and the majority of NK cell development occurs in bone marrow.\textsuperscript{34}

2.1.1.1 T-lymphocytes

T-lymphocytes play a key role in cell-mediated immunity. Several T-lymphocyte subsets are described, of which three are important in PAH: CD4+ T-helper cells (Th) and CD8+ cytotoxic T (Tc) cells and T-regulatory (Treg) cells.
Figure 2.1: Lymphopoiesis

(Adapted from a picture supplied by South Western Medical centre, Dallas, USA)

Cluster of differentiation (CD); Forkhead box (Fox); NK cell = natural killer cell (CD56+/CD3-); NKT cell = natural killer T cell (CD56+/CD3+); IEL = intraepithelial lymphocyte; Treg cell = T-regulatory cell (CD4+ CD25hi FoxP3 CD127low).
**CD4+ Th cells** become activated when peptide antigens are presented to them by major histocompatibility (MHC) II molecules on antigen presenting cells. Th cells can stimulate B-cell differentiation, macrophage activation and Tc cells to bind to MHC class I molecules and kill infected cells. CD4+Th cells differentiate further into Th1 and Th2 (and other subtypes) which secrete cytokines that regulate or assist in the active immune response. 30,34

**CD8+ Tc cells** express T-cell receptors that recognize intracellular antigens (such as viruses) bound to MHC class I molecules. If the T-cell receptor is specific for the antigen, it binds to the MHC class I molecule and antigen complex (helped along by CD8) and the T lymphocyte destroys the cell. In this way CD8+ Tc cells destroy virally infected cells and tumour cells. 30,34

**CD4+Treg cells** are important in balancing Th1 and Th2 cell responses, maintaining self-tolerance and controlling autoimmunity. 28,34

### 2.1.1.2 B-lymphocytes

When the B cell receptor detects foreign antigen in the body, the B cell proliferates and secretes a free form of those receptors (antibodies) with identical binding sites as the ones on the original cell surface. After activation the cell differentiates into plasma cells which produce large amounts of antibodies (but are short lived and undergo apoptosis once the inciting antigen is removed) and memory B cells that are specific to the antigen encountered during the primary immune response (and are long lived). These memory cells are crucial to the adaptive
immune system and are able to mount a more efficient and powerful antibody response on future encounters with the antigen.  

2.1.1.3 Natural killer cells

NK cells are granular lymphocytes which play a key role in immune defense against viral and bacterial infections and malignancies. The majority of circulating human NK cells are CD56dim NK cells (low density expression of CD56 and high expression of CD16) and have a cytolytic role whereas 10% are CD56bright NK cells (high expression of CD56 low or zero expression of CD16) and have an immunoregulatory role. Recent advances in NK cell biology have shown that natural killer (NK) cells are important in both innate and adaptive immunity, and there is also accumulating evidence of NK cell memory.
2.1.2 Lymphocytes and PAH

The role played by lymphocytes in PAH pathogenesis is becoming clearer. Lymphocytes are the predominant cell type surrounding plexiform lesions within diseased pulmonary arteries of explanted lungs from PAH patients (of all sub groups). PAH is known to complicate infections associated with lymphocyte depletion such as HIV and chronic hepatitis C. Rats deficient in T-lymphocytes show increased propensity to develop PAH but when injected with endothelial progenitor cells are protected from developing PAH. This protective mechanism was shown to be dependent on the presence of natural killer (NK) cells suggesting that T-lymphocytes and NK cells play an important role in animal models of PAH.

Their role in human PAH is under intense scrutiny: Ulrich et al showed that IPAH patients have decreased circulating cytotoxic CD8+ T-lymphocytes, increased T-regulatory cells and that their B-lymphocytes have a distinct RNA profile. We followed with a report of two PAH patients sharing identical immune profiles (decreased NK cells and cytotoxic CD8+T-lymphocytes) who died prematurely. Recently, decreased circulating CD56dim NK cells (the NK cell subtype with a cytolytic role) was reported in patients with heritable PAH and IPAH. The clinical significance of T-lymphocyte and NK cell deficiency in human PAH, however, is unknown.

In this study we analysed the peripheral lymphocyte profile of PAH patients in comparison to normal controls, and then correlated changes with clinical outcomes including a primary endpoint of one year transplant free survival.
2.2 Specific aims

2.2.1 Determine the peripheral lymphocyte profile of human PAH patients in comparison to normal controls

2.2.2 Correlate lymphocyte profile with clinical outcome, defined as transplant free survival at one year

2.2.3 Compare the utility of lymphocyte markers with the previously validated biomarker BNP.

2.3 Materials and methods

2.3.1 Subjects

PAH patients were recruited from the PAH clinic at Christchurch Hospital, New Zealand which serves a population of one million. PAH was defined as pre-capillary pulmonary hypertension (resting mean pulmonary arterial pressure >25mmHg, wedge pressure <15mmHg) in the absence of other causes of PH. Patients with IPAH or CTD-APAH aetiologies were included as they share similar immune pathologies and systemic sclerosis-APAH is the prototypic syndrome in which to study inflammatory processes involved in PAH pathogenesis. Eligible patients were recruited following diagnostic RHC and matched for gender, ethnicity and age (+/-3 years) with healthy volunteers. Patients were recruited at differing times during their disease course with a median time range from diagnostic RHC to study recruitment of 8 months (0-22). Healthy controls were all NYHA I, on no medication and did not undergo RHC. Recruitment occurred from March 2008 until March 2010 during which time every single eligible patient referred to our regional
centre with this rare condition was included. The study was approved by the Upper South A Regional ethics committee (URA/08/02/006) and all participants gave informed written consent.

2.3.2 Study design

This prospective study analysed the peripheral lymphocyte profile of IPAH, CTD-APAH patients and matched healthy controls at recruitment. Lymphocyte subset deficiencies were then correlated with clinical outcome and the previously validated prognostic marker, plasma BNP.¹

At recruitment basic demographics were collected together with aetiology (IPAH or CTD-APAH), medication, NYHA functional class, haemodynamic measurements from RHC performed closest to recruitment date, 6MWD, arterial blood gas (ABG), diffusion lung capacity for carbon monoxide (DLCO), and plasma BNP level.

A peripheral venous blood sample (approximately 40ml) was collected from each participant at recruitment; lymphocyte surface marker, full blood count and plasma BNP analysis was performed by Canterbury District Health Board Laboratories using standard protocols. Remaining blood samples were used to prepare FTA cards (for future genotyping) and following centrifugation, serum and plasma samples were extracted, aliquoted and stored at -80 degrees Celsius.

**Lymphocyte surface marker analysis:** Antibody mixes CD14, CD4, CD45, CD3, CD8 and CD14, CD56/CD16, CD45, CD3, CD19 were added to two separate tubes. A 100µl of whole blood was added to each tube, vortexed and incubated (protected from light) for at least 15 minutes. 1mL of lyse/fix solution (2.5ml of fixative to 100ml
of VersaLyse) was added to each tube, vortexed and incubated (protected from light) for 10 minutes. Flow cytometric analysis was performed on a FC500 Flow Cytometry System using Cytomix RXP Analysis Software (all samples and equipment, Beckman Coulter, USA). The NK cell marker used for the initial two patients was CD16 whereas a combined CD56/CD16 marker was used for all other participants.

The ARCHITECT BNP assay was performed for the quantitative determination of human BNP in plasma according to manufacturer’s instructions (Abbott Diagnostics, USA). Coefficient of variation within run was 3.5% at 30pmol/L.

All patients were reviewed at yearly intervals following recruitment. Data recorded included: functional assessment (6MWD, NYHA), clinical outcome (mortality, lung transplant) and laboratory investigations (DLCO, PaO₂, BNP).

2.3.3 Statistics

Data are presented as median and significance is determined using Mann Whitney or Chi square tests. Survival data is summarised as Kaplan Meier curves and compared between groups using log-rank tests. Areas under the ROC curves were calculated using the standard non-parametric method for 3 year survival. Associations between lymphocyte counts, BNP and functional status were tested using Wilcoxon rank and Spearmans correlation coefficients. All p-values reported were two-sided; values p<0.05 were considered statistically significant. SPSS 19.0 software package (SPSS Inc. USA) was used for statistical analyses while graphs were prepared using Prism 6 (GraphPad, USA).
2.4 Results

A total of fourteen patients (9 IPAH, 5 CTD-APAH) were recruited, twelve were Caucasian. Three CTD-APAH patients had limited systemic sclerosis, one diffuse systemic sclerosis and another systemic lupus erythematosis/systemic sclerosis overlap syndrome. Patients were followed for a minimum of twelve months, 3 died prior to one year follow-up, Table 1.

2.4.1 PAH patients compared with normal controls

Peripheral blood lymphocyte subsets and BNP levels recorded at recruitment for PAH patients and normal controls are illustrated in Figure 2.2. Five PAH patients had elevated CD4:CD8 ratios (>3.6:1), four patients had depleted cytotoxic CD8+ cells (< 200 cells/μL), four patients had depleted NK cell numbers (< 90 cells/μL) and one patient had depleted CD4+ helper T cells (< 300 cells/μL) according to the Christchurch Hospital reference range, Figure 2.2.

2.4.2 Transplant free survival at 1yr according to baseline lymphocyte counts

The primary end point of one year transplant free survival according to lymphocyte counts and BNP levels at recruitment is shown in figure 2.3.

A total of three patients died: two CTD-APAH patients (5 and 10 months post recruitment) and one IPAH patient (8 months post recruitment, following lung transplantation). All three deceased patients had elevated CD4:CD8 ratios depleted cytotoxic CD8+ cells, depleted NK cells and elevated BNP levels at recruitment. One IPAH patient, on the active transplant list had NK cell depletion, a high CD4:CD8 ratio
### Table 2.1: Baseline characteristics according to transplant free survival at one year

<table>
<thead>
<tr>
<th></th>
<th>Normal Controls N=14</th>
<th>ALL PAH N=14</th>
<th>PAH ALIVE N=11</th>
<th>PAH DEAD* N=3</th>
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<tr>
<td><strong>Age (years)</strong></td>
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<td>55</td>
<td>43</td>
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<tr>
<td><strong>Female Gender</strong></td>
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<td>12</td>
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<td>3</td>
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<td><strong>Aetiology (IPAH:CTD APAH)</strong></td>
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<td>8:3</td>
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<td><strong>Months following diagnosis of PAH</strong></td>
<td>8 (0-22)</td>
<td>9 (0-22)</td>
<td>2 (0-12)</td>
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<td><strong>NYHA functional class - (no.)</strong></td>
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<tr>
<td>I</td>
<td>3</td>
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</tr>
<tr>
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<td>4</td>
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</tr>
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<td>7</td>
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<td>Pulmonary arterial pressure (mmHg)</td>
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<td>53</td>
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<tr>
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<td>1</td>
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<td></td>
</tr>
</tbody>
</table>

Date of diagnosis = date of initial right heart catheterisation study confirming PAH; * includes one patient who died following lung transplantation; NYHA=New York Heart Association Classification. Data is presented as median values. Significance was determined using Mann Whitney or Chi square tests. Comparisons (Normal v All PAH; PAH alive v PAH deceased) revealed no statistically significant differences.
Figure 2.2: Graphs showing the distribution of lymphocyte counts and BNP levels in the peripheral blood of PAH patients and healthy volunteers at recruitment.

Christchurch Hospital index and reference range: CD8+ (Cytotoxic T /Suppressor cell marker) = 200-900 cells/µL; CD4 (T Helper/Inducer cell marker) = 300-1400 cells/µL; CD4:CD8 ratio = 1.0-3.6:1; CD56+ and/or CD16+ (Natural killer cell marker) = 90-600 cells/µL; CD19 (Mature B cell marker) = 100-500 cells/µL; BNP <100 pg/ml.

- Idiopathic PAH ▲ Connective tissue disease associated PAH ■ Normal control
Figure 2.3: Graphs showing the 1 year transplant free survival according to peripheral blood lymphocyte counts and BNP levels at recruitment.

Christchurch Hospital reference range: CD4:CD8 ratio=1.0-3.6:1; CD8+ (Cytotoxic T/Suppressor cell marker) = 200-900cells/μL; CD4+ (T helper/Inducer cell marker) = 300-1400cells/μL; CD56+ and or CD16+ (Natural killer cell marker) = 90-600cells/μL; CD19 (Mature B cell marker) = 100-500 cells/μL; BNP <100 pg/ml.

● IPAH ▲ CTD-APAH ■ Normal controls * Active transplant list
(low-normal CD8+ count) and elevated BNP level. A CTD-APAH patient with a low CD4+ count and low BNP level was alive at one year follow up. Patients with lymphocyte counts within the normal range were all alive at 1 year and none had been placed on the transplant list.

2.4.3 Transplant free survival at 3yr according to baseline lymphocyte counts

Thirty six months post recruitment: All 5 CTD-APAH patients were deceased; 2 died within 10 months of recruitment (CD8+ and NK cell deficiency, high CD4:CD8 ratio, high BNP), 2 died at 21 and 30 months (normal lymphocyte count, normal BNP) and one at 27 months (low CD4 count, normal BNP). Three IPAH patients were deceased; one at 6 months (CD8+ and NK cell deficiency, high CD4:CD8 ratio, high BNP), one at 34 months after declining lung transplantation (NK cell deficiency, high CD4:CD8 ratio, low-normal CD8+ count, high BNP) and one at 35 months (normal lymphocyte count, high BNP). All other IPAH patients were alive and none on the active transplant list.

Figure 2.4 shows that a CD4:CD8 ratio > 3.6:1, a cytotoxic CD8+ T-cell count < 200 cells/μl, or a natural killer cell count < 90 cells/μl is associated with an increased risk of death. There was little association with CD4+ (helper T cells), CD19+ (mature B cells) or the favoured prognostic marker BNP.\(^1\)
Figure 2.4: Kaplan Meier curves showing 3 year transplant free survival according to whether CD8+ counts (<200 cells/μL), CD4:CD8 ratio (>3.6:1), NK cell counts (< 90 cells/μL) and BNP level (≥100 pg/ml).

Cut offs determined by the Christchurch Hospital reference range: CD4:CD8 ratio 1.0-3.6:1; CD8 (cytotoxic T / suppressor cell marker) = 200-900cells/μL; CD56+ and/or CD16+ (natural killer cell marker) = 90-600cells/μL; BNP <100 pg/ml. P-values from log-rank tests. The areas under the ROC curve for the prediction of 3 year survival are: CD4:CD8 (0.708), CD8+ (0.854), CD56+ (0.729) and BNP (0.531).
2.4.4 Transplant free survival according to IPAH or CTD-APAH aetiology

The increased risk of death associated with CTD-APAH aetiology is shown in Figure 2.5 (a). Figure 2.5 (b) shows the survival time since PAH diagnosis and demonstrates that the decreased survival of CTD-APAH patients is not related to the length of time since diagnosis.

2.4.5 Lymphocyte counts and BNP levels correlated with functional status

The association between lymphocyte counts, BNP levels and functional status (NYHA and 6MWD) are shown in Figure 2.6 and Figure 2.7 respectively. Decreasing CD8+ cell counts are associated with an increasing NYHA class (p=0.01) and decreasing 6MWD (p=0.029).

2.4.6 Lymphocyte counts correlated with BNP levels

The association between CD8+ counts, NK cell counts, CD4:CD8 ratio and BNP levels are shown in figure 2.8. Depleted CD8+ and NK cell counts and elevated CD4:CD8 ratios are associated with elevated BNP levels.
Figure 2.5 (a): Kaplan-Meier curves demonstrating the transplant free survival at 36 months post recruitment according to whether patients had idiopathic or CTD-associated PAH.

Figure 2.5 (b): Kaplan-Meier curves demonstrating the transplant free survival following diagnosis of PAH (IPA or CTD-APA) recorded 3 years following study recruitment. Significance was determined using log-rank analysis.
Significance was established using Wilcoxon rank tests. Significant associations are highlighted on the graphs, all other comparisons were non-significant.
Figure 2.7: Graphs showing the association of lymphocyte counts (and plasma BNP levels) with the 6 minute walk distance recorded at recruitment.

Significance was determined using Spearman’s correlation coefficients.
Figure 2.8 Graphs showing the association between lymphocyte counts and BNP levels.

○ IPAH ▲ CTD-APAH
2.5 Discussion

This study shows that depletion of natural killer cells and cytotoxic CD8+ T-lymphocytes occur in patients with IPAH and CTD-APAH and are associated with an increased risk of death and elevated BNP levels. As univariate markers of survival at 1 and 3 years, CD8+ and NK cell counts and CD4:CD8 ratios appear more powerful than the currently favoured prognostic marker BNP. Although lymphocyte depletion has been reported previously in PAH patients this is the first study to show the impact of cytotoxic CD8+ and NK cell depletion on clinical outcome in both aetiological sub-groups.

NK cells and CD8+ T-lymphocytes originate from a common lymphoid progenitor cell, share parallel regulatory pathways and primarily develop into “professional killers” of cells corrupted with infection or tumours. Combined deficiencies of NK and CD8+ T-cells are reported in interleukin-15 deficient mice and infants with life threatening Varicella but have not previously been described in PAH. Here we show that combined deficiency of NK and CD8+ cells in IPAH and CTD-APAH patients was associated with death within 10 months of recruitment. The fact that lymphocyte depletion is present in both aetiologies adds to the evidence that IPAH and CTD-APAH patients share a common immune pathophysiology.

CD8+ lymphocyte depletion is a recognized adverse prognostic marker in cancer and chronic infections such as hepatitis C and HIV. In this study, CD8+ lymphocyte depletion was independently associated with an increased risk of death and values correlated with 6MWD and NYHA classification. Two other groups have reported alterations in the CD8+ compartment in PAH but neither reported the clinical effect of lymphocyte deficiencies. Ulrich et al found a decreased
percentage of cytotoxic CD8+ T-lymphocytes in IPAH patients (N=30) compared with healthy controls (p=0.02). Austin et al found alterations in CD8+ subtypes but no significant difference in total CD8+ T lymphocytes between IPAH patients (N=18) and controls (similar to our findings).

These results suggest that CD8+ T-lymphocyte depletion is present in some but not all PAH patients. Possibly CD8+T-cell depletion in PAH develops as the disease process deteriorates analogous to the “hierarchical CD8+T-cell exhaustion” described in chronic viral models. It may also be a reflection of the smaller number of patients (of mixed aetiology) in our study. In Ulrich’s study the NYHA class and survival of patients was not reported, however, 68% were receiving intravenous prostacyclin compared to 21% receiving nebulised prostacyclin in our study. This suggests that a higher proportion of patients in Ulrich’s study had advanced PAH (compared to ours) at the time of lymphocyte analysis which may explain the significantly decreased CD8+ cell counts in PAH vs Normal controls in their study.

Phenotypic and functional analysis of CD8+ cells from patients as they progress through the various stages of PAH may unravel the mechanistic role of cytotoxic CD8+ cells in PAH pathogenesis.

Several human NK subtypes are now recognised with roles in immunity (both innate and adaptive) and immunoregulation. NK cell depletion is described in fatal viral infections, chronic hepatitis C, coronary artery disease (reversed after successful CABG) and cancer (particularly with advanced disease). Ormiston et al report a decreased absolute number of CD56+dim NK cells (but not total NK cells) in 11 patients with idiopathic and heritable PAH, with demonstrated impairment of cytolytic function (linked to reduced expression of activating receptors) when
compared with healthy controls. The clinical significance of this altered NK cell phenotype and function is currently unknown. CD56$^{\text{dim}}$ and CD56$^{\text{bright}}$ cell ratios are altered in a number of other condition (such as chronic hepatitis C and systemic lupus erythematosis) but the clinical consequence is unclear. Although total NK cell counts between PAH patients and normal controls were not significantly different in our study all four patients with total NK cell depletion died prematurely, the first time that NK depletion has been shown to be associated with an increased risk of death. It would be interesting to establish whether the CD56$^{\text{dim}}$: CD56$^{\text{bright}}$ ratio was altered in these patients, however, this was not possible due to the combined NK cell marker (CD56/CD16) used in our hospital assay. Incidentally, the dim and bright NK subtypes in Ormiston’s study were CD56+ but were not assessed for CD16. The clinical fate of patients with depleted CD56$^{\text{dim}}$ cells remains to be defined.

It is unclear whether CD8+ and NK cell depletion in PAH are a consequence of or a predisposing factor in this disease. A similar lymphocyte profile is observed in chronic viral infections (such as HIV and hepatitis C) and cancer, suggesting there may be a common pathological mechanism.

Elegant research has led to the characterization of “exhausted” lymphocytes (in cancer and chronic infection) and the discovery of therapies to reverse their demise. Exhausted lymphocytes exhibit intrinsic abnormalities including the step wise up-regulation of inhibitory cell surface receptors, such as programmed death-1 (PD-1), as the disease process deteriorates. PD-1 is up-regulated on “exhausted” HIV specific CD8+T cells and is associated with an adverse outcome; blocking the PD-1 receptor reverses CD8+ T cell exhaustion and decreases HIV viral
Similarly blocking the PD-1 receptor on “exhausted” NK cells in patients with multiple myeloma restores their natural ability to kill myeloma cells. It will be interesting to explore the level of PD-1 expression on NK and CD8+ cells in PAH patients and whether it correlates with disease progression (and lymphocyte depletion).

Extrinsic factors regulating lymphocyte exhaustion include immunoregulatory cells. Depletion of Foxp3+CD4+Treg cells or regulatory NK cells in mouse models of chronic infection leads to improved CD8+ function and viral control. In IPAH patients elevated Foxp3+CD4+Treg cell numbers in association with depleted CD8+ cells have been reported, but the mechanism for these lymphocyte alterations or their effect on clinical outcome (or NK cells) is unknown.

Other potential mechanisms include redistribution of circulating NK and CD8+ cells to diseased pulmonary arteries, an unidentified infective organism, a defect in the common progenitor cell or the downstream pathways regulating lymphocyte differentiation and survival.

This study is limited by the small number of patients enrolled (from a single PAH clinic in New Zealand), the mixed aetiology of the patients, lack of serial lymphocyte analysis, confounding factors (such as variable drug therapy and different stages of disease at recruitment) and lack of functional assays.

Nevertheless, deficiency of NK and cytotoxic CD8+ cells in PAH indicate a significantly higher risk of mortality mirroring that reported in cancer and infectious disease. This observation has great importance in that it raises the possibility of developing lymphocyte directed therapy for PAH, as is under investigation for some other life-threatening diseases.
CHAPTER 3

Inflammatory related cytokines and PAH
3.1 Introduction

Considerable evidence supports the role of inflammatory cytokines in the pathogenesis of PAH. Rodents develop severe PH if injected with monocrotaline (a plant-derived alkaloid) which induces endothelial injury followed by intense perivascular inflammation. Macrophages, immature dendritic cells and lymphocytes are found within inflamed pulmonary arteries; a process associated with elevated serum and pulmonary cytokine levels. IL-1 is excessively produced in the lungs of rats with monocrotaline induced PH. Following repeated injections with an IL-1 receptor antagonist a reduction in the level of PH is found. Rodents exposed to chronic hypoxia develop increased lung permeability, recruitment of alveolar macrophages and up-regulation of inflammatory cytokines and their receptors. Progressive pulmonary vascular remodelling ensues, associated with accumulation of perivascular inflammatory cells and resulting in mild to moderate PH. IL-6 deficient mice are protected from hypoxia-driven experimental PH.

In humans increased circulating inflammatory cytokines are reported in PAH patients from all aetiological sub-groups. In IPAH patients, increased circulating levels of macrophage inflammatory protein (MIP)-1-alpha, monocyte chemo attractant protein(MCP)-1, P-selectin, Tumour Necrosis Factor alpha (TNF-α), IL-1 and IL-6 are found. In patients with Systemic Sclerosis-APAH, serum levels of soluble intercellular adhesion molecule (ICAM-1), vascular adhesion molecule (VCAM)-1, P-selectin and platelet endothelial cell adhesion molecule (PECAM)-1 (markers of endothelial activation or injury) were higher than in healthy controls at baseline and fell to normal values after 12 months of bosentan therapy. A reduction of ICAM-1 and of IL-6 plasma levels in PAH patients after bosentan
treatment correlated with haemodynamic improvements. Elevated plasma levels of five inflammatory cytokines IL-2, IL-6, IL-8, IL-10 and IL-12p70 were shown to predict survival in IPAH patients (a total of eleven cytokines were studied). 31

Modern technological advances have resulted in a commercially available antibody array (RayBio Biotin Label-based Human Antibody Array 1, USA) which allows the simultaneous detection of the expression levels of 507 human proteins in plasma, figure 3.1. Target proteins include inflammatory cytokines (such as IL-1 and IL-6), chemokines, adipokine, growth factors (such as vascular endothelial growth factor, VEGF), angiogenic factors, proteases, soluble receptors, soluble adhesion molecules and other inflammatory related proteins, Appendix 1. Using this cytokine array we studied the expression profile of 507 inflammatory related cytokines in PAH patients and healthy controls.

3.2 Specific aims

3.2.1 Determine the differential expression of 507 inflammatory related cytokines in the peripheral blood of patients with PAH and normal controls using a commercially available cytokine array.

3.2.2 Correlate cytokine profile with clinical outcome

3.2.3 Correlate cytokine profile with lymphocyte profiles
The first step is to biotinylate the primary amine of the proteins in plasma. The biotin-labelled sample is added onto a glass chip (array support) and incubated at room temperature. A fluorescent dye-conjugated streptavidin is used to visualise the signals.
3.3 Materials and methods

3.3.1 Subjects

Cytokine arrays (Raybio, USA) were performed on plasma samples from 14 PAH patients (9 IPAH, 5 CTD-APAH) and 15 healthy controls by the author. Plasma samples were prepared at the time of recruitment for all participants: approximately 10ml of blood (collected in EDTA tubes and stored on ice) were spun down at 3000rpm for 10 minutes at 4°C. Plasma was separated and a subsequent spin of 3000rpm for 10 minutes was performed to remove any residual platelet debris. 400μl aliquots of plasma were placed in Greiner tubes for each participant and stored at -80°C until cytokine analysis and other investigations were performed.

3.3.2 Cytokine array technique

3.3.2.1 Prior preparation

**Cytokine kit components**

Upon receipt, the antibody array kit was stored at -20°C. The kit contained the following components: Item A (dialysis tube); Item B (labeling reagent); Item C (Internal control); Item D (stop solution); Item E (RayBio® Biotin label-based human antibody array 1 Glass Chip with Frame); Item F (blocking buffer); Item G (20X wash buffer I); Item H (20X wash buffer II); Item I (fluorescent dye-conjugated streptavidin); Item J (adhesive film) and Item K (serum buffer).

**1X PBS solution**

Three litres of 1X PBS solution was prepared by dissolving 0.6 g KCl, 24 g NaCl, 0.6 g KH₂PO₄ and 3.45 g Na₂HPO₄ into 2500 ml distilled water. The pH was
adjusted with 1M NaOH and the final volume adjusted to 3000 ml with distilled water before autoclaving (121 °C for 20 min).

3.3.2.2. Dialysis of plasma samples

The participant plasma samples which had been stored at –80 °C in Greiner tubes were placed on ice and allowed to defrost slowly. Each plasma sample was treated as follows: the plasma was diluted 5 fold with sterile 1X PBS (pH 8.0) (20μl of plasma, 80μl of 1X PBS), placed into an Eppendorff dialyzier tube (Item A) and dialyzed by placing in a beaker of 500ml 1X PBS at 4 °C for 3 hours (h). After 3 h, the 1X PBS solution was removed and replaced with 500ml fresh 1X PBS solution and left to dialyze for a further 3 h at 4 °C.

3.3.2.3. Biotin Labelling of Sample

The internal control tube (Item C) was briefly spun down before use. 100μl 1X PBS (pH 8.0) was added into the internal control tube and mixed by pipetting up and down to dissolve the powder containing the internal control. Into a sterile Eppendorf tube, the following reagents were added: 40μl of prepared internal control; 35μl dialyzed plasma sample and 155μl Serum Buffer (Item K). If the plasma total volume had been changed after the dialysis step, the quantity of dialyzed plasma and serum buffer added to the Eppendorf tube was adjusted to keep the same plasma concentration for each of the participant samples.

The 1X labeling reagent was prepared by briefly spinning down labeling reagent tube (Item B) then adding 100μl 1X PBS (pH 8.0) into the tube and gently pipetting up and down to dissolve the labeling reagent powder. 22 μl of labelling reagent solution was added into each plasma sample and mixed well (by pipetting).
This reaction solution was then incubated at room temperature with gentle shaking for 30 minutes (make and model of shaker). Over the course of this 30 min shaking incubation, the Eppendorf tubes were gently tapped every 5 minutes to ensure mixing of the reaction solution.

A 3µl aliquot of stop solution (Item D) was added into the above reaction solution in the Eppendorf tubes. The reaction solution was immediately transferred into Eppendorff tubes and dialyzed by placing into a beaker of 500ml 1X PBS (pH 8.0) at 4°C for 3 h, after which the 1X PBS (pH 8.0) was exchanged for fresh 1X PBS (500ml) and dialyzed for a further 3 h at 4°C. The samples were then centrifuged at 10000g (make and model of centrifuge) for 5 min at 4°C. Samples were then stored at -20°C until the microarray assay was carried out.

3.3.2.3 Preparation, blocking and incubation of antibody array

The antibody array glass chip (Item E) was left to air dry for 1 h within a tipbox lid and covered loosely with tin foil in a laminar flow hood.

Blocking buffer (item F) (400µl), was added slowly into each well (glass chip with frame, Item E) to ensure there were no air bubbles. This was incubated at room temperature for 30 min and then the blocking buffer was decanted from each well. The dialysed/labelled plasma was diluted 1:40 with blocking buffer (item F) (10µl plasma sample and 400µl of blocking buffer). A 400µl aliquot of this sample was carefully added into the well and incubated for 2 h at room temperature.

1X wash buffer I (ITEM G) was prepared by adding 2mls of 20X wash buffer (item G) to 38 ml of distilled water.
Samples were decanted from each well and the wells washed 3 times with 800µl of 1X wash buffer I at room temperature with gentle shaking on a rotary shaker per wash. The glass chip with frame was placed into a box with 1X wash buffer I, ensuring the whole glass slide and frame was covered with 1X wash buffer I, washing was repeated twice at room temperature with gentle shaking for 10 min/wash on a rotary shaker.

1X wash buffer II was prepared by adding 2ml of 20X wash buffer II (item H) to 38ml of distilled water. 1X wash buffer I was decanted from each well and the glass chip with frame was placed into a box and covered with 1X wash buffer II, then washed two times gentle shaking at room temperature for 5 min.

The fluorescent dye conjugated streptavidin (item I) was spun down and then and then 50µl of blocking buffer was added to this tube pipetted up and down to make a streptavidin concentrate solution. A (1X) streptavidin solution was prepared by adding 10µl of the streptavidin concentrate into an Eppendorf tube with 1ml of blocking buffer (item F) and mixed gently.

The 1X wash buffer II was decanted from the wells and 400µl of (1X) fluorescent dye conjugated streptavidin solution was added to each subarray well. The individual wells were covered with adhesive film to prevent evaporation and then the glass slide and frame covered with aluminium foil. This was incubated at room temperature for 2 h and then overnight at 4°C with gentle shaking.

The streptavidin solution was decanted from each well and the fluorescently labeled slide was disassembled out of the incubation frame and chamber by pushing the support clips outward (from the slide side) and removing the slide from the gasket, being careful not to touch the surface side of the array slide).
The glass slide was placed into a 50ml centrifuge tube with 40ml of 1X wash buffer I with gentle shaking on a rotary shaker for 10 min at room temperature. The buffer was removed and washing was repeated 2 more times for a total of 3 washes. The glass slide was then placed into a 50ml centrifuge tube containing 40ml of 1X wash buffer II and washed with gently shaking on a rotary shaker for 5 min at room temperature. The buffer was removed and washing was repeated one more time. Finally, the glass chip was washed with 40ml of distilled water for 5 minutes by gently shaking of a rotary shaker for 5 min at room temperature.

3.3.2.4 Fluorescence Detection/Slide Analysis

The glass chip was dried by placing it in a 50ml centrifuge tube and centrifuging at 1000rpm for 3 minutes. When dry, the glass slide was placed in an Axon GenePix scanner which visualised the signals on the cytokine array. Data was then analysed using the RayBio® Analysis Tool (a program specifically designed for the analysis of RayBio® Biotin Label-based Antibody Arrays). The positive, negative, blank and internal controls were used in all antibody microarray assays. By subtracting the background staining and normalising to the positive controls relative protein concentrations were obtained.

3.3.3 Statistics

All p-values reported were two-sided; values p<0.05 were considered statistically significant. SPSS 19.0 software package (SPSS Inc. USA) was used for statistical analyses while graphs were prepared using Prism 6 (GraphPad, USA).
3.4 Results

A cytokine array was performed for all 14 PAH patients and 15 healthy controls. The cytokine arrays were semi-quantitative and it was not possible to determine individual protein concentrations. Instead the fluorescence intensity of each individual antibody was established. Signal intensity comparisons could only be performed with the same antibody/antigen system (between cytokine arrays) and not between different antibodies within the same array since the fluorescence intensity detected for each individual antibody depends on its relative affinity. The cytokine arrays were normalised to allow direct comparison using the positive control fluorescent intensities (after subtracting background) using the RayBio® Analysis Tool.

The images captured by the Axon GenePix laser scanner allow a visual comparison to be made between the fluorescence patterns of PAH patients and their matched controls, demonstrated in Figure 3.2. A biotinylated protein and internal control produce positive signals which are used to identify the orientation of the cytokine array. The location of these controls, and all other antibodies, can be found on a map of the array (Appendix 2) and interpreted using the corresponding numbered antibody list (Appendix 1).

3.4.1 Cytokine Profiles of PAH Patients and Healthy Controls

The fluorescence intensity of antibodies in 14 PAH patients and 15 normal control arrays were compared. Twenty five antibodies showed (≥ 3 x) fold higher median fluorescence intensity in the PAH group compared with the normal control group.
Figure 3.2 Images captured using an Axon GenePix laser scanner showing the RayBio® Biotin-label-based human antibody array probed with plasma from (a) PAH patient and (b) the matched healthy control.
and reached statistical significance. One antibody was identified where the median fluorescent intensity was higher in the control group than in the PAH group, but this did not reach statistical significance. Table 3.1 summarises a list of these antibodies, median values, fold expression and p-values.

Graphs showing the individual immunofluorescence values for PAH and normal controls for each of the twenty five antibodies listed can be found in Appendix 3. Graphs were reviewed and eight antibodies chosen which appeared to have meaningful differences in immunofluorescence values between PAH and control values with potential roles in PAH pathogenesis, figure 3.3 and figure 3.4.

3.4.2 Cytokine profile of PAH patients according to clinical outcome

3.4.2.1 PAH cytokine profiles according to 1 year transplant free survival.

The fluorescence intensity of 507 antibodies recorded at baseline were compared between PAH patients who were deceased (N=3) and alive (N=11) at 1 year follow up. Matrix metalloproteinase (MMP)-10 was the only antibody found to have a statistically significant difference in median fluorescence intensity between deceased and alive PAH groups. Twelve antibodies demonstrated a (≥ 3 x) fold difference in median fluorescence intensity between PAH groups, but none reached statistical significance. Five antibodies demonstrated a (≥ 2x) fold difference in median fluorescence intensity in the PAH deceased group compared to PAH alive group, but again none reached statistical significance. Table 3.2 summarises the median values and p-values for all eighteen antibodies.
TABLE 3.1: Summarises the list of antibodies with a ≥ 3 fold difference in the median value between PAH patients and normal controls which reaches statistical significance.

<table>
<thead>
<tr>
<th>Antibody list</th>
<th>Median PAH patients</th>
<th>Median Normal controls</th>
<th>Fold expression</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activin RIA / ALK-2</td>
<td>569.08</td>
<td>92.61</td>
<td>6.14</td>
<td>0.046</td>
</tr>
<tr>
<td>Activin RIB / ALK-4</td>
<td>247.82</td>
<td>80.34</td>
<td>3.08</td>
<td>0.006</td>
</tr>
<tr>
<td>Angiopoietin-4</td>
<td>354.27</td>
<td>105.88</td>
<td>3.35</td>
<td>0.02</td>
</tr>
<tr>
<td>BTC</td>
<td>316.17</td>
<td>93.86</td>
<td>3.37</td>
<td>0.033</td>
</tr>
<tr>
<td>CCR4</td>
<td>373.92</td>
<td>123.78</td>
<td>3.02</td>
<td>0.033</td>
</tr>
<tr>
<td>Chordin-Like 2</td>
<td>434.63</td>
<td>130.89</td>
<td>3.32</td>
<td>0.002</td>
</tr>
<tr>
<td>Cryptic</td>
<td>265.78</td>
<td>78.23</td>
<td>3.40</td>
<td>0.033</td>
</tr>
<tr>
<td>EGF</td>
<td>331.04</td>
<td>72.59</td>
<td>4.56</td>
<td>0.016</td>
</tr>
<tr>
<td>Eotaxin-3 / CCL26</td>
<td>364.51</td>
<td>80.93</td>
<td>4.50</td>
<td>0.029</td>
</tr>
<tr>
<td>ErbB3</td>
<td>1493.67</td>
<td>391.45</td>
<td>3.82</td>
<td>0.023</td>
</tr>
<tr>
<td>GFR alpha-3</td>
<td>322.07</td>
<td>104.98</td>
<td>3.07</td>
<td>0.045</td>
</tr>
<tr>
<td>IL-2 R beta /CD122</td>
<td>493.49</td>
<td>74.74</td>
<td>6.60</td>
<td>0.029</td>
</tr>
<tr>
<td>IL-9</td>
<td>371.41</td>
<td>78.76</td>
<td>4.72</td>
<td>0.029</td>
</tr>
<tr>
<td>IL-17E</td>
<td>254.98</td>
<td>84.62</td>
<td>3.01</td>
<td>0.029</td>
</tr>
<tr>
<td>IL-22 BP</td>
<td>363.23</td>
<td>95.35</td>
<td>3.81</td>
<td>0.046</td>
</tr>
<tr>
<td>MIP-3 alpha</td>
<td>754.24</td>
<td>174.25</td>
<td>4.33</td>
<td>0.018</td>
</tr>
<tr>
<td>MMP-9</td>
<td>116.86</td>
<td>24.91</td>
<td>4.69</td>
<td>0.041</td>
</tr>
<tr>
<td>MMP-19</td>
<td>487.34</td>
<td>98.49</td>
<td>4.95</td>
<td>0.02</td>
</tr>
<tr>
<td>MSP alpha Chain</td>
<td>1150.18</td>
<td>119.75</td>
<td>9.61</td>
<td>0.029</td>
</tr>
<tr>
<td>NRG1-beta1 / HRG1-beta1</td>
<td>336.45</td>
<td>98.62</td>
<td>3.41</td>
<td>0.033</td>
</tr>
<tr>
<td>PD-ECGF</td>
<td>362.33</td>
<td>60.89</td>
<td>5.95</td>
<td>0.046</td>
</tr>
<tr>
<td>SDF-1 / CXCL12</td>
<td>553.52</td>
<td>129.63</td>
<td>4.27</td>
<td>0.041</td>
</tr>
<tr>
<td>Thymopoietin</td>
<td>146.45</td>
<td>40.75</td>
<td>3.59</td>
<td>0.018</td>
</tr>
<tr>
<td>TRAIL R1 / DR4 / TNFRSF10A</td>
<td>239.48</td>
<td>55.08</td>
<td>4.35</td>
<td>0.012</td>
</tr>
<tr>
<td>Vasorin</td>
<td>276.06</td>
<td>54.45</td>
<td>5.07</td>
<td>0.014</td>
</tr>
</tbody>
</table>
Figure 3.3: Graphs showing the median and individual fluorescent intensities between PAH patients and normal controls.

- ● IPAH ▲ CTD-APAH ■ Normal controls
Figure 3.4: Graphs showing the median and individual fluorescent intensities between PAH patients and normal controls.
TABLE 3.2 Summarises the list of antibodies with a ≥ 3 fold difference in the median value between deceased and alive PAH patients at 1 year follow up.

<table>
<thead>
<tr>
<th>Antibody list</th>
<th>Median dead PAH patients</th>
<th>Median alive PAH patients</th>
<th>Fold expression</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR4</td>
<td>171.88</td>
<td>411.42</td>
<td>2.4 *</td>
<td>0.073</td>
</tr>
<tr>
<td>CD27-TNFR SF7</td>
<td>33.34</td>
<td>95.79</td>
<td>2.9 *</td>
<td>0.073</td>
</tr>
<tr>
<td>CD30 ligand TNFSF8</td>
<td>683.96</td>
<td>2862.67</td>
<td>4.2</td>
<td>0.243</td>
</tr>
<tr>
<td>DAN</td>
<td>68.28</td>
<td>248.67</td>
<td>3.6</td>
<td>0.073</td>
</tr>
<tr>
<td>DKK-1</td>
<td>172.68</td>
<td>379.13</td>
<td>2.2 *</td>
<td>0.073</td>
</tr>
<tr>
<td>ERB B4</td>
<td>75.42</td>
<td>252.09</td>
<td>3.3</td>
<td>0.243</td>
</tr>
<tr>
<td>Glut1</td>
<td>155.61</td>
<td>51.87</td>
<td>3.0</td>
<td>0.312</td>
</tr>
<tr>
<td>IL17 B</td>
<td>1164.69</td>
<td>4657.76</td>
<td>4.0</td>
<td>0.312</td>
</tr>
<tr>
<td>IL21</td>
<td>27.97</td>
<td>65.43</td>
<td>2.3 *</td>
<td>0.052</td>
</tr>
<tr>
<td>IL23</td>
<td>1715.09</td>
<td>868.15</td>
<td>2.0 *</td>
<td>0.052</td>
</tr>
<tr>
<td>LFA 1 alpha</td>
<td>1161.51</td>
<td>140.24</td>
<td>8.3</td>
<td>0.186</td>
</tr>
<tr>
<td>Luciferase</td>
<td>3162.69</td>
<td>905.45</td>
<td>3.5</td>
<td>0.392</td>
</tr>
<tr>
<td>Lymphotoxin-beta</td>
<td>704.62</td>
<td>215.61</td>
<td>3.3</td>
<td>0.186</td>
</tr>
<tr>
<td>MDC</td>
<td>4393.75</td>
<td>958.22</td>
<td>4.6</td>
<td>0.392</td>
</tr>
<tr>
<td>MMP-10</td>
<td>264</td>
<td>145</td>
<td>1.8</td>
<td>0.032**</td>
</tr>
<tr>
<td>Neuropilin-2</td>
<td>974.78</td>
<td>2988.90</td>
<td>3.1</td>
<td>0.586</td>
</tr>
<tr>
<td>Osteoactivin</td>
<td>2000.29</td>
<td>617.56</td>
<td>3.2</td>
<td>0.139</td>
</tr>
<tr>
<td>VEGF D</td>
<td>131.90</td>
<td>505.00</td>
<td>3.8</td>
<td>0.243</td>
</tr>
</tbody>
</table>

*median > 2 fold change, p-value nearing statistical significance
** the only antibody with a statistically significant difference (p=0.032)
Graphs showing the median and individual fluorescent intensities for six antibodies (with p-values < 0.075), highlighting the association with lymphocyte profile depletion are illustrated in figure 3.5.

Figure 3.6 shows four images captured using the Axon genepix laser scanner highlighting the differential fluorescence intensity of the anti-CC chemokine receptor (CCR)-4 at position 61 (Appendix 2) according to whether PAH patients were deceased or alive at 1 year follow up.

3.4.2.2 PAH cytokine profiles according to 3 year transplant free survival

The fluorescence intensity of 507 antibodies recorded at baseline was compared between PAH patients who were deceased (N=8) and alive (N=6) at 3 year follow up. Ten antibodies showed a (≥ 3 x) fold difference and four antibodies a (≥ 2 x) fold difference in baseline median fluorescent intensity between deceased and alive PAH patients, Table 3.3. The only antibodies which had a statistically significant difference in immunofluorescence values between these PAH groups were Granzyme A, IL-1 F7 FIL1 zeta (also named human IL-37) and monocyte chemotactic protein (MCP)-3, figure 3.7; other graphs have been included to illustrate the association between immunofluorescence levels and lymphocyte depletion but these did not reach statistical significance.
Figure 3.5: Graphs showing the median and individual fluorescent intensities recorded at recruitment according to transplant free survival at 1 year follow up.

- **IPAH** (black=normal lymphocyte count; red=NK and CD8+ depletion; blue=NK cell depletion/CD8+ lower end normal; purple=CD8+depletion)

- **CTD-APA H** (black=normal lymphocyte count; red=NK and CD8+ depletion; green=CD4+depletion)

- **Normal controls**
Figure 3.6 Cytokine array images demonstrating the higher fluorescence intensity of the antibody CCR4 (position 61 Appendix 2) in two PAH patients (a,b) who were alive compared with the lower fluorescence intensity of CCR4 in two PAH patients (c,d) who were deceased at 1 year follow up.
TABLE 3.3: Summarises the list of antibodies with a ≥ 3 fold difference in the median value between deceased and alive PAH patients at 3 year follow up

<table>
<thead>
<tr>
<th>Antibody List</th>
<th>Median dead PAH patients</th>
<th>Median Alive PAH patients</th>
<th>Fold expression</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP-4</td>
<td>331.5</td>
<td>834.0</td>
<td>2.5 *</td>
<td>0.085</td>
</tr>
<tr>
<td>CCR-9</td>
<td>3160.2</td>
<td>1580.1</td>
<td>2.0*</td>
<td>0.08</td>
</tr>
<tr>
<td>Cerebrus 1</td>
<td>68.3</td>
<td>556.3</td>
<td>8.1</td>
<td>0.223</td>
</tr>
<tr>
<td>CLC</td>
<td>1203.0</td>
<td>4137.1</td>
<td>3.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Glut-1</td>
<td>144.0</td>
<td>27.9</td>
<td>5.2</td>
<td>0.465</td>
</tr>
<tr>
<td>Granzyme A</td>
<td>463.0</td>
<td>178.0</td>
<td>2.6*</td>
<td>0.029</td>
</tr>
<tr>
<td>IL-1 F7/F1L/Zeta</td>
<td>434.2</td>
<td>109.7</td>
<td>4.0</td>
<td>0.029</td>
</tr>
<tr>
<td>IL-23</td>
<td>1303</td>
<td>627</td>
<td>2.1*</td>
<td>0.08</td>
</tr>
<tr>
<td>LFA 1 alpha</td>
<td>745.9</td>
<td>75.1</td>
<td>9.9</td>
<td>0.186</td>
</tr>
<tr>
<td>MCP-3</td>
<td>274.9</td>
<td>62.8</td>
<td>4.4</td>
<td>0.0426</td>
</tr>
<tr>
<td>TL1A/TNFSF15</td>
<td>24.2</td>
<td>7.4</td>
<td>3.3</td>
<td>0.08</td>
</tr>
<tr>
<td>TNFR II/TNFRSF1B</td>
<td>1404.2</td>
<td>368.7</td>
<td>3.8</td>
<td>0.167</td>
</tr>
<tr>
<td>TRAIL R2/DR5/TNFRSF10B</td>
<td>381.0</td>
<td>1510.0</td>
<td>4.0</td>
<td>0.123</td>
</tr>
<tr>
<td>VEGF D</td>
<td>139.0</td>
<td>648.4</td>
<td>4.7</td>
<td>0.291</td>
</tr>
</tbody>
</table>

* median fold expression > 2 fold change
Figure 3.7 Graphs showing the median and individual fluorescent intensities recorded at recruitment according to transplant free survival at 3 year follow up

- **IPAH** (black=normal lymphocyte count; red=NK and CD8+ depletion; blue=NK cell depletion/CD8+ lower end normal; purple=CD8+depletion)
- **CTD-APAHI** (black=normal lymphocyte count; red=NK and CD8+ depletion; green=CD4+depletion)
- **Normal controls**
3.4.3 PAH cytokine profile correlated with lymphocyte counts

The cytokine profile of PAH patients with no lymphocyte depletion (N=8) was compared with that of PAH patients with NK cell depletion (N=4). Three of the four patients with NK cell depletion also had CD8+ depletion whereas one had a CD8+ count of 210 cells/μL (normal range ≥ 200 cells/μL). Two patients were excluded from this analysis: one patient with CD4+ cell depletion (but a normal NK and CD8+ cell count) and one patient with a CD8+ count of 190 cells/μL but a normal NK cell count.

PAH patients with NK and CD8+ cell depletion had elevated IL-23 levels (p=0.008) and decreased levels of IL-21 (p=0.05) compared to PAH patients with normal lymphocyte counts, figure 3.8. Despite the lack of statistical significance the graph for BMP-4 is included given its role in T-lymphopoiesis and PAH pathogenesis\(^5^8\) and that for Chemokine (C-X-C) motif ligand 14 (CXCL 14) given its role in NK cell migration and inhibition of angiogenesis.\(^5^9\)
Figure 3.8 Graphs illustrating the immunofluorescence profiles of PAH patients according to whether they have NK cell depletion

• IPAH (black=normal lymphocyte count; red=NK and CD8+ depletion; blue=NK cell depletion/CD8+ count lower end normal)

▲ CTD-APAHP (black=normal lymphocyte count; red=NK and CD8+ depletion)

■ Normal controls
3.5 Discussion

3.5.1 Protein immunofluorescence profile of PAH patients and normal controls.

This study demonstrates that PAH patients have an altered protein immunofluorescence profile compared to normal controls. Twenty five antibodies showed a three fold difference in median immunofluorescence levels between PAH patients and normal controls which reached statistical significance, Table 3.1.

The small number of patients in the study together with the semi-quantitative technique (without normal reference ranges for individual antibodies), however, led to a wide range of immunofluorescence values particularly for PAH patients. It is therefore difficult to know whether statistically significant findings are real and a true representation of the “relative protein concentration” in plasma and if they translate into clinical importance.

Activin A Receptor type 1 (ACVR1), chordin-like 2, Epithelial growth factor (EGF), Eotaxin 3/chemokine (C-C motif) ligand 26 (CCL26), IL2-R beta/CD122, macrophage inflammatory protein-3-alpha (MIP-3-α), Matrix metalloproteinase (MMP-19) and MSP- α-chain appear to have meaningful differences in immunofluorescence values between PAH patients and normal controls. Of these proteins EGF has been reported to be a potential biomarker in paediatric PAH.

ACVR1 and chordin-like 2 are involved in the transforming growth factor beta superfamily of signaling proteins (interacting with BMP’s and the BMPR-II receptor). ACVR1 levels are down-regulated in monocataline models of PAH resulting in impairment of BMP signaling, however, their role in human PAH is unknown.
This study did not identify a difference in immunofluorescence values for cytokines previously reported to be elevated in IPAH patients (macrophage inflammatory protein-1-alpha, monocyte chemo attractant protein-1, P-selectin, Tumour Necrosis Factor alpha (TNF-α), IL-1 and IL-6).\textsuperscript{18,28} This may be due to the fact that the large cytokine array used was semi-quantitative, that our IPAH patients and CTD-APAHI were pooled or were at a different stage of disease to those in other studies.

This study gives a preliminary snapshot view of the expression profile of a large number of proteins and identifies which proteins deserve further in-depth review. An enzyme-linked immunosorbent assay (ELISA) needs to be performed for each antibody of interest, to confirm that immunofluorescence values obtained correlate with actual protein concentration in plasma.

3.5.2 The protein immunofluorescence profile associated with an increased risk of death 1 year follow up.

This study demonstrates an altered protein immunofluorescence profile in the deceased PAH patients (N=3) compared to the alive PAH patients (N=11). Deceased PAH patients had higher median levels of MMP-10 (p=0.03) and IL-23 (p=0.052) and reduced levels of IL-21 (p=0.052) when compared with alive PAH patients.

Soon et al using a quantitative cytokine assay demonstrated that patients with higher levels of IL-2, IL-4, IL-8, IL-10 and IL-12p70 had worse outcomes at 1 and 5 years than patients with lower levels\textsuperscript{31} but the underlying mechanism is unclear.
These cytokines however, were not associated with an increased risk of death in our study.

MMP-10 has an important role in vascular remodeling and has been implicated in lung tumorigenesis and tumour progression. \(^{63}\) MMP-10 is up-regulated on microarray analysis in experimental models of PAH but its role in human PAH is unclear. \(^{64}\)

IL-23 expression is increased in human tumours; increasing angiogenesis and reducing CD8+ T-cell infiltration into the cancerous growth thereby providing a protective cancer promoting environment for early malignancies. \(^{65}\) Elevated peripheral IL-23 levels are seen in a large number of auto-immune conditions, such as psoriasis and systemic sclerosis. \(^{66}\) Patients with systemic sclerosis are protected from developing PAH if they possess a single nucleotide polymorphism of the IL-23 receptor. \(^{66}\)

The IL-21 receptor is expressed on NK cells and cytotoxic T cells and IL-21 is known to have a key role in regulating NK cells and cytotoxic T cells and enhances their ability to kill tumour cells and inhibit viral replication. \(^{67,68}\)

This is the first time that elevated immunofluorescence levels of IL-23 and MMP-10 and reduced levels of IL-21 have been shown to be associated with an increased risk of death in PAH patients. Establishing the actual protein concentration of these antibodies in stored plasma will confirm these findings which may lead to the identification of novel adverse prognostic markers in PAH.
3.5.3 The protein immunofluorescence profile associated with an increased risk of death at 3 year follow up.

This study identified that deceased PAH patients (N=8) at 3 year follow up expressed an altered immunofluorescence profile to PAH patients who were alive at this time (N=6). Deceased PAH patients had elevated Granzyme A, IL-1F7 FIL1 zeta levels and MCP-3 levels compared to alive PAH patients, which reached statistical significance, figure 3.8. Granzyme A is an enzyme that is stored within cytotoxic T lymphocytes and elevated levels (associated with CD8+ depletion) suggest this may be due to apoptosis of these cells but its role in PAH is unknown. MCP-1,-2 and-3 are chemotactic for human T-lymphocytes. Although MCP-1 is known to play an important role in vascular remodeling in PAH the role of MCP-3 is unknown.

This is the first time that the immunofluorescence levels of these proteins has been reported in PAH patients and shown to be associated with an adverse outcome but again accurate quantification of protein levels is required before any firm conclusions can be made.

3.5.4 PAH cytokine profile associated with NK cell depletion

This study demonstrates that PAH patients with NK and CD8+ cell depletion have elevated IL-23 and reduced IL-21 immunofluorescence values compared to patients with normal lymphocyte counts. Mutations in the IL-23 receptor protect patients with systemic sclerosis from developing PAH (as elevated IL-23 levels are associated with increased angiogenesis). IL-21 has a key role in regulating NK cells and
cytotoxic T cells and enhances their ability to kill tumour cells and inhibit viral replication. 67,68

It will be interesting to confirm that the actual concentrations of IL-23 and IL-21 are elevated in the plasma of PAH patients with NK cell depletion (and premature death) since this could be a potential new adverse prognostic marker and a target for IL-23 and IL-21 directed therapy which may reverse the lymphocyte depletion and dire outcome.

**Study limitations**

Whilst the cytokine array technique provides a large quantity of preliminary data it has some limitations: it is expensive, time consuming and most importantly does not give readily quantifiable results. These limitations are compounded by the small number of participants in our study (due to the rarity of the disease).

Future work will focus on confirming that immunofluorescence levels correlate with actual plasma protein concentrations. This may lead to a number of potential protein markers which can be validated in other studies.
CHAPTER 4

Brain Natriuretic Peptide, Cardiac Troponin I and Endothelin-1 and their role as prognostic markers in Pulmonary arterial Hypertension
4.1 Introduction

A non-invasive bio-marker which identifies patients at the earliest stage of disease and gives an accurate estimation of risk and clinical outcome is highly desirable. Although a number of markers have been explored in PAH patients the majority only become positive when there is evidence of advanced disease. In this chapter the utility of plasma BNP, cTnI and ET-1 as prognostic markers of PAH are explored.

4.1.1 BNP

BNP is one of several members of the natiuretic peptide family of hormones which have important roles in cardiovascular homeostasis. BNP is released from both right and left ventricular myocardial cells in response to increased myocardial pressure and/or volume overload as occurs with the failing heart; once released it acts on the kidney causing natiuresis and diuresis. The synthesis and secretion of BNP is regulated at gene level with no hormone being stored in the myocyte. Healthy individuals have a plasma BNP concentration of around 3.5pg/ml, whereas in patients with congestive heart failure values can be increased by 200-300 fold.

The precursor (pre), pre-pro BNP is cleaved during translation to form the pro hormone pro BNP which in turn releases c-terminal BNP (the focus of this study) and the N terminal fragment NT-proBNP. C-terminal BNP and NT-proBNP have both been used as biomarkers in PAH. Although NT-proBNP has advantages over BNP (less susceptible to degradation and thought to be more sensitive and specific)
NT-proBNP assays are not readily available in clinical laboratories and difficult to introduce into routine clinical practice.\textsuperscript{72,73}

**BNP and PAH**

A large number of studies have identified elevated plasma BNP levels in patients with PAH, although the numbers of patients investigated are small.\textsuperscript{22,74,75} More recently, in the REVEAL study of over 2000 patients, plasma BNP levels were found to be elevated in all PAH subgroups compared with normal controls and on subgroup analysis patients with CTD-APAH (N=641) had a higher mean BNP level than patients with IPAH (N=1251).\textsuperscript{9-11}

BNP levels have been shown to correlate with: echocardiographic measurements such as right ventricular systolic pressure (RVSP); haemodynamic parameters such as mean pulmonary arterial pressure (mPAP) and pulmonary vascular resistance; exercise capacity tests such as the 6 minute walk test (6MWT) and with the NYHA functional class.\textsuperscript{9,10,22,74,75} Elevated plasma BNP levels are not usually seen until the mean PAP pressure is high enough to cause right ventricular strain.\textsuperscript{76} Therefore a normal BNP level does not exclude the presence of early PAH and has limited diagnostic value as a screening tool for excluding PAH in low risk populations. In high risk groups such as those with systemic sclerosis (where the prevalence of PAH is estimated to be between 12-35%) BNP is a good preliminary screening test. If BNP is elevated, further investigations are required to prove that PAH is present (echocardiogram +/- RHC).\textsuperscript{72}

The prognostic capability of BNP was first demonstrated by Nagaya et al who showed that patients with baseline median BNP level ≥ 150pg/ml had a worst outcome than those with a recording < 150pg/ml, and if BNP levels were persistently
raised (≥ 180pg/ml) at 3 month follow-up there was an increased risk of death.  

The REVEAL study confirmed that BNP levels ≥ 180pg/ml were associated with an increased risk of death at 1 year, and that BNP levels < 50pg/ml were associated with increased survival at 1 year.  

BNP is useful in monitoring the response to therapy. In the SERAPH (Sildenafil versus Endothelin Receptor antagonist in PAH) study, plasma BNP levels were elevated in all patients from both groups at baseline. BNP levels decreased in the group of patients receiving sildenafil which correlated with a reduction in right ventricular mass size.  

This current study aims to confirm the prognostic ability of BNP in IPAH and CTD-APA patients and use it as the “gold-standard” biomarker for comparison throughout this study.

4.1.2 Cardiac troponin

Cardiac troponin T (cTnT) and cardiac troponin I (cTnI) are regulatory proteins that control the calcium mediated interaction between actin and myosin filaments of cardiac muscle. Disruption of the cardiac myocyte membrane causes release of cTnT and cTnI and the quantities released (which equates to the extent of cardiac muscle ischaemia/necrosis) can be detected in serum using quantitative troponin assays (monoclonal antibodies to epitopes on cTnI and cTnT). These antibodies are highly specific for cardiac troponin and have no cross-reactivity with skeletal muscle troponin.
The detection of cardiac troponins in peripheral blood (in conjunction with symptoms and signs of ischaemia) are diagnostic of an acute myocardial infarction. Elevated cardiac troponins are also seen in other conditions including chronic renal impairment, left ventricular dysfunction and in patients in the critical care setting when there is no other evidence of an acute myocardial infarction.

Rapid advances in immunoassay technologies have resulted in the development of highly sensitive assays. Between 1995 and 2007 the limit of detection fell from 0.5ng/ml for some cTnT assays to 0.006ng/ml for the cTnI-Ultra a 100 fold improvement in assay sensitivity. Detectable cTnI levels (below the consensus defined diagnostic range) were commonly observed in stable patients undergoing elective cardiac evaluation using a highly sensitive cTnI assay. Figure 4.0 is a Kaplan Meier curve illustrating the risk attributed to detectable cTnI (below the usual diagnostic level) and demonstrates the usefulness of high sensitivity assays (despite their reduced specificity) in identifying patients who would benefit from risk reduction measures.

Figure 4.0: Kaplan-Meier analysis for 3 year major adverse clinical events according to definite (cTnI 0.009-0.029ng/ml), probable (cTnI 0.001-0.008ng/ml) and no (cTnI<0.001ng/ml).
**Cardiac troponin and PAH**

Torbicki et al found elevated cTnT in 8 out of 56 patients with stable PH (51 PAH and 5 CTEPH). Serial cTnT analyses revealed persistent elevation of cTnT in 4 out of the 8 patients with elevated cTnT (all deceased at follow up) whereas 4 out of 8 patients with undetectable cTnT on repeat analysis were all alive at follow up. This study suggests that cTnT is not a sensitive marker of PH but if persistently elevated on serial analysis is of prognostic significance.

Highly sensitive cTnI assays had not been reported in PAH patients at the commencement of this study we therefore set out to establish whether a higher sensitivity cTnI assay would improve the prognostic ability of cardiac troponins in PAH.

4.1.3 Endothelin-1 (ET-1)

The endothelin (ET) system is composed of three endothelin genes (ET-1, ET-2 and ET-3 genes), pre-pro ET peptides, peptidases, three 21 amino acid peptide ligand isoforms (ET-1, ET-2 and ET-3) and two G-protein coupled receptors. ET-1 is mainly produced by endothelial cells in the pulmonary circulation, ET-2 plays only a minor role if any in the lung and ET-3 is produced by multiple cell types including endothelial cells but its role in the lung is unknown.

The ET-1 gene is translated to prepro-ET-1 which is then cleaved to form the biologically inactive big ET-1. ET converting enzymes further cleave this to form functional ET-1. There are two ET receptor isoforms, type A (ETA), located predominantly on vascular smooth muscle cells, and type B (ETB), predominantly
expressed on vascular endothelial cells but also on arterial smooth muscle. Activation of ETA and ETB receptors on pulmonary vascular smooth muscle cells induces pulmonary vascular vasoconstriction and smooth muscle cell proliferation. Conversely, activation of endothelial ETB receptors results in the release of vasodilator and anti-proliferative agents.\textsuperscript{73,81,82}

ET-1 is not stored in endothelial cells but specific stimuli (such as hypoxia) instigate its synthesis and secretion which occurs over minutes. The plasma half life of ET-1 is about 4-7 minutes and as a result vascular cells can rapidly adjust ET-1 levels.\textsuperscript{81}

**ET-1 and PAH**

Activation of the endothelial system has been demonstrated in both plasma and lung tissue of patients with pulmonary hypertension,\textsuperscript{82-86} however, it is unclear whether elevated plasma ET-1 levels are a cause or consequence of PAH. In patients with IPAH (N=16) Rubens et al demonstrated significantly elevated plasma ET-1 and big ET-1 (which has a longer half-life and less tissue extraction than ET-1) when compared to normal controls; plasma levels of ET-1 (and big ET-1) showed positive correlation with pulmonary vascular resistance and mean PAP and a negative correlation with cardiac output, cardiac index and the 6MWT.\textsuperscript{85} There are no data on the use of ET-1 in PAH screening. The prognostic ability of ET-1 is unclear, although, plasma ET-1: ET-3 ratios were reported to predict transplant free survival in a study of 33 PAH patients.\textsuperscript{82} ET-1 has also been suggested to have a role in monitoring therapy since the ratio of big ET-1 in the radial artery to the pulmonary artery decreased after treatment with inhaled iloprost.\textsuperscript{88}
4.2 Specific Aims

4.2.1 Investigate the utility of BNP as a prognostic marker in IPAH and CTD-APAH patients.

4.2.2 Investigate the utility of cTnI as a prognostic marker in IPAH and CTD-APAH patients.

4.2.3 Investigate the role of ET-1 as a prognostic marker in IPAH and CTD-APAH patients.

4.3 Materials and methods

A peripheral venous blood sample (approximately 40ml) was collected from each participant at recruitment; plasma BNP, cTnI and ET-1 analysis was performed by Christchurch Hospital laboratories using standard protocols. In addition, BNP levels were repeated at follow up visits as this is the standard of care at Christchurch PAH clinic.

4.3.1 Brain Natriuretic Peptide assay

The ARCHITECT BNP assay (Abbott Diagnostics, Chicago, Illinois, USA) is a two-step immunoassay for the quantitative determination of human BNP in human EDTA plasma which uses chemiluminescent microparticle immunoassay (CMIA) technology. In the first step, sample and anti-BNP coated paramagnetic microparticles are combined. BNP present in the sample binds to the anti-BNP coated microparticles. After washing, anti-BNP acridinium-labeled conjugate is added to create a reaction mixture in the second step. Following another wash cycle, pre-trigger and trigger solutions are added to the reaction mixture. The resulting
chemiluminescent reaction is measured as relative light units (RLUs). A direct relationship exists between the amount of BNP in the sample and the RLUs detected by the ARCHITECT i System optics. Within run coefficient of variation at 30pmol/L is 3.5%.

4.3.2 Cardiac Troponin I assay

The ARCHITECT STAT Troponin-I assay (Abbott Diagnostics, Chicago, USA), a two-step immunoassay was used to determine the presence of cTnI in human plasma using CMIA technology. In the first step, sample, assay diluent and anti-troponin I antibody coated paramagnetic microparticles were combined. Troponin-I present in the sample binds to the anti-troponin-I coated microparticles. After incubation and wash, anti-troponin-I acridinium-labeled conjugate was added in the second step. Following another incubation and wash, pre-trigger and trigger solutions are then added to the reaction mixture. The resulting chemiluminescent reaction is measured RLU’s. A direct relationship exists between the amount of troponin-I in the sample and the RLUs detected by the ARCHITECT i* System optics. The concentration of troponin-I is read relative to a standard curve established with calibrators of known troponin I concentrations. The limit of detection is 0.01ng/ml, 99th percentile is 0.028 ng/ml and coefficient of variation was <10% at a concentration of 0.032 ng/ml.

4.3.3 Endothelin-1 Assay

Five ml of blood was collected into an EDTA tube, separated and the plasma frozen at –80 °C. Two ml of plasma was extracted on C18 sep-pak cartridges (Waters, USA) pre washed with 5mL methanol and 5mL of 0.1 % trifluoroacetic acid (TFA).
Endothelin was eluted with 2mL of 80% Isopropanol in 0.1 % TFA, dried under an air stream at 37 °C, and reconstituted with 0.5mL of 0.1M phosphate buffer (pH 7.4) containing 0.05M NaCl, 0.1% BSA, 0.1% Triton x-100 and 0.01% NaN₃ (assay buffer).

Endothelin was assayed by adding 50uL of standard or extract to micronic tubes and 50uL of Endothelin antiserum (cat. number RAS6901, Bachem USA), which had the following crossreactivity as supplied by the manufacturer:- Endothelin-1 (Human, Porcine) 100%; Endothelin-2 (Human) 7%; Endothelin-3 (Rat, Human) 7%; Big Endothelin (Porcine) 35%; Big Endothelin (Human) 17%; Big Endothelin 22-38 (Human) 0%; Alpha-ANP (Human) 0%. The assay was incubated at 4°C for 22-24 hours, after which 50uL of ^125^I labelled Endothelin (2,000-2,500 cpm) was added and incubated at 4°C for 22-24 hours. Bound and free Endothelin were separated using a solid phase second antibody method in which 0.5mL of 5% Sac-Cel (IDS – UK) in assay buffer was added, vortexed, and incubated at room temperature for 30 minutes and centrifuged at 2,000g for 15 minutes at 20 °C. After decanting the liquid, the pellet was then counted in a gamma counter.

Endothelin was iodinated by a Lactoperoxidase method (1) where 7.5ug Endothelin-1 and 25uL 0.4M Na Acetate buffer were reacted with 18MBq of Na^{125}I (Amersham – UK), 20ug Lactoperoxidase and 100ng H₂O₂ for 10 minutes. Another 100 ng H₂O₂ was then added and incubated for an additional 10 minutes, after which the mixture was separated on a 10cm Brownlee Aquapore RP300 7micron HPLC column using a gradient of 20-100% acetonitrile in 49mM phosphate buffer pH 2.9 over 40 minutes.

Using the above method, recoveries of Endothelin-1 from human plasma were 96% at 10 pmol/L and extracts of human plasma diluted in parallel with the
standard curve. The assay had a mean detection limit of Endothelin in plasma of 0.65pmol/L over 34 assays. Within assay coefficient of variation was 12.8% over 27 assays at 1.8-3.0pmol/L and the between assay coefficient of variation at 2.1pmol/L was 15.9 % over 16 assays. The Reference Range for the assay measured in 200 normal subjects selected at random from the Christchurch electoral roll is: 0.9-2.3 pmol/L.
4.4 Results

Brain Natriuretic peptide

BNP levels <100 pg/ml are considered to be within the normal reference range at Christchurch Hospital.

4.4.1 BNP levels of PAH patients and normal controls at recruitment

PAH patients had a higher median BNP level than normal controls: 321 pg/ml compared to 24 pg/ml (p<0.0001), figure 4.1a.

4.4.2 Transplant free survival at 1 year follow up according to baseline BNP levels

PAH patients deceased at one year had a higher median BNP level at baseline than PAH patients alive at one year: 146.33 pg/ml compared to 66.82 pg/ml but this did not reach statistical significance, figure 4.1b.

Figure 4.1 (a) Graph showing the baseline plasma BNP levels for PAH patients and healthy controls and (b) the transplant free survival at 1 yr follow up according to baseline plasma BNP levels.

● IPAH ▲ CTD-APA □ Normal controls * Active transplant list
4.4.3 Transplant free survival according to baseline BNP levels and aetiology.

IPAH patients alive at one year had a higher median baseline BNP level than CTD-APAH patients alive at one year: 321 compared with 62 pg/ml (p= 0.07), figure 4.2a. Two CTD-APAH patients and eight IPAH patients had BNP levels >100 pg/ml: both CTD patients were deceased at 1 year follow up whereas only one IPAH patient was deceased, figure 4.2a. This may suggest that BNP levels above the normal reference range are more predictive of adverse outcome at 12 months in CTD-APAH than in IPAH patients. One IPAH patient and three CTD-APAH patients had BNP levels <100 pg/ml: all these patients were alive at 12 months follow up, figure 4.2a.

All three CTD-APAH patients with BNP levels <100 pg/ml at recruitment were deceased at 3 year follow up whereas the IPAH patient with a BNP level <100 pg/ml was alive at 3 year follow up. This suggests that BNP is not a sensitive marker of long term prognosis in CTD-APAH patients.

Figure 4.2: Graphs showing transplant free survival according to aetiology and baseline BNP level at (a) 1 year and (b) 3 year follow up.

● IPAH ▲ CTD-APAH
4.4.4 Transplant free survival according to whether plasma BNP levels <100pg/ml

Figure 4.3 demonstrates that although baseline BNP levels <100pg/ml are able to predict transplant free survival at 1 year follow up their ability to predict survival at 3 year follow up is poor. The cut-off >180pg/ml used in the REVEAL study was also used for comparison but survival outcomes were almost identical at 1 and 3 year follow up. Whereas, BNP levels <100pg/ml and <50pg/ml always predicted survival at 1 year follow up, at three year follow up even a BNP level <50pg/ml at recruitment did not always predict survival at 3 year follow up.

![Graph a](image1)

![Graph b](image2)

**Figure 4.3**: Kaplan Meier curves showing the transplant free survival according to whether BNP levels <100pg/ml at (a) 1 year follow up and (b) 3 year follow up
4.4.5 BNP levels correlated with haemodynamic status

The correlation of BNP levels with haemodynamic parameters such as PAP was not performed given that the time from diagnostic RHC to baseline BNP measurements varied from 0-22 months.

4.4.6 BNP levels correlated with functional status

There was no statistically significant association between baseline BNP levels and functional assessments (NHYA III v NHYA I & II; 6MWD) recorded at recruitment, figure 4.4.

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**Figure 4.4:** Graphs showing the association between BNP and (a) NYHA functional classification and (b) 6 minute walk distance.

- ● IPAH  ▲ CTD-APAH  ■ Normal controls
4.4.7 Serial BNP analysis

Repeat BNP analysis was performed at routine follow up appointments (3-6 months post recruitment) or if patients were admitted requiring escalation of therapy. Figure 4.5 illustrates the ability of serial BNP to predict transplant free survival at 1 year.

Figure 4.5: Serial BNP analysis and their ability to predict transplant free survival at 1yr follow up.
Cardiac Troponin I

A cTnI detection level of 0.01ng/ml was used in this study. Values greater or equal to 0.03ng/ml are considered diagnostic of a myocardial infarction in the appropriate clinical context.

4.4.7 Cardiac Troponin I levels in PAH patients and healthy controls

PAH patients had a greater median cTnI level compared to normal controls: 0.02ng/ml compared to 0.01ng/ml (P=0.008), figure 4.6.

Figure 4.6: Graph showing the distribution of cTnI in peripheral blood of patients with PAH and normal controls

● IPAH ▲ CTD-APAH ■ Normal controls

Undetectable cTnI <0.01ng/ml was entered as 0.005 to enable comparison between detectable 0.01ng/ml and undetectable readings.
4.4.8 Transplant free survival according to cTnI levels at recruitment

PAH patients deceased at one year had a greater median cTnI than PAH patients alive at one year but this did not reach statistical significance: 0.03ng/ml compared with 0.02ng/ml, figure 4.7. An undetectable cTnI was noted in one of the patients deceased at 1 year follow-up suggesting that a positive cTnI at recruitment has poor sensitivity as a marker of survival at one year follow-up. However, sensitivity may increase later in the disease process: as repeated cTnI measurements in this patient revealed elevation to 0.08ng/ml in the weeks preceding death (7 months post recruitment). Of the eleven patients alive at one year follow-up: one patient had cTnI <0.01; seven patients had cTnI levels between 0.01 and 0.02 (detectable but considered within the normal range); two patients had cTnI levels of 0.03ng/ml (cTnI ≥0.03ng/ml is diagnostic of an MI in the correct clinical context) and one patient an elevated cTnI of 0.07ng/ml (which incidentally became undetectable at follow-up a year later after being established on PAH therapy).

Figure 4.7: Transplant free survival at 1 year according to baseline cTnI levels
4.4.9 Transplant free survival according to baseline cTnI levels

Figure 4.8 Compares the transplant free survival according to whether cTnI levels at recruitment were undetectable <0.01ng/ml, detectable (≥ 0.01 and < 0.03) or raised ≥ 0.03ng/ml. This demonstrates that the cTnI highly sensitive assay used in this study was an insensitive marker of prognosis in PAH when the detection limit was set at 0.01ng/ml.

![Kaplan Meier curves comparing the transplant free survival at 3 year follow up according to whether cTnI levels at recruitment were undetectable <0.01ng/ml, detectable (≥ 0.01 and < 0.03) or raised ≥ 0.03ng/ml.](image)

**Figure 4.8:** Kaplan Meier curves comparing the transplant free survival at 3 year follow up according to whether cTnI levels at recruitment were undetectable <0.01ng/ml, detectable (≥ 0.01 and < 0.03) or raised ≥ 0.03ng/ml.
Endothelin-1 (ET-1)

The Reference Range for the assay measured in 200 normal subjects selected at random from the Christchurch electoral roll is: 0.9-2.3 pmol/L.

4.4.10 ET-1 levels in PAH patients and normal controls at recruitment.

PAH patients had a greater median ET-1 level compared to normal controls: 3.94 pmol/L compared to 2.94 pmol/L which reached statistical significance (p=0.04), figure 4.9. Although the normal range set by the laboratory was 0.9-2.3 pmol/L this was established using historical controls and had not been repeated despite a change in the assay reagents. The normal controls in this study had ET-1 levels higher than the historical normal reference range quoted in the method section.

Figure 4.9: Graph showing the distribution of plasma ET-1 levels in the peripheral blood of PAH patients and normal controls at recruitment.
4.4.11 Transplant free survival according to ET-1 levels recorded at baseline.

PAH patients deceased at one year had a greater median ET-1 than PAH patients alive at one year, but this did not reach statistical significance: 4.7 pmol/L compared with 3.0 pmol/L, figure 4.10 (a). PAH patients deceased at three year follow had a greater median ET-1 level than alive PAH patients, but this did not reach statistical significance; 4.8 pmol/L compared with 3.9 pmol/L, figure 4.10 (b). However, IPAH patients deceased at three year follow up had a greater median ET-1 level than IPAH patients alive at 3 years which reached statistically significance; 6.1 pmol/L compared to 3.4 pmol/L (p=0.02), figure 4.10 (c).

**Figure 4.10:** Graph showing the distribution of baseline ET-1 levels according to:

(a) transplant free survival at 12 months; (b) transplant free survival at 36 months and (c) transplant free survival at 36 months and IPAH aetiology

● IPAH ▲ CTD-APA  ■ Normal controls
4.4.12 Survival curves according to ET-1 levels

The ET-1 cut-off was established by using the highest ET-1 level recorded for normal controls in this study. Figure 4.11 demonstrates the survival according to whether ET-1 levels were above or below 4.37pmol/L.

![Survival Graph]

**Figure 4.11:** Kaplan Meier graph demonstrating the transplant free survival according to whether ET-1 ≥ 4.37 pmol/L
4.5 Discussion

4.5.1 BNP

This study demonstrates that a BNP level < 100pg/ml was always predictive of survival at 12 months for patients with both CTD-APAH and IPAH. A BNP level ≥ 100pg/ml was always predictive of an adverse outcome in CTD-APAH at 12 months whereas it was not as discriminatory in patients with IPAH. Baseline BNP levels were unable to predict outcome at 3 year follow up; one patient with a BNP level < 50pg/ml was deceased at 3 year follow up and three patients with BNP levels > 300 pg/ml were alive at 3 year follow up.

In the REVEAL study\textsuperscript{9} lower than average BNP levels < 50pg/ml were predictive of 1 year survival whereas REVEAL and Nagaya et al found that BNP levels ≥ 180pg/ml were predictive of an adverse outcome. \textsuperscript{9,22} In this study we chose to adopt values above and below the normal reference range at Christchurch Hospital but these results compared favorably (with no significant difference in outcome) when the cut offs used by REVEAL and Nagaya et al were used in data analysis. \textsuperscript{9,22}

Baseline BNP levels showed no statistically significant association with functional status such as NYHA class or 6MWD. We did not assess the association of BNP with haemodynamic variables since the median time from RHC study was 22 months. Nagaya et al showed a positive correlation between BNP levels and increasing NYHA class, mean PAP, PVR and negative correlation with cardiac output. \textsuperscript{22} This may reflect the larger number of purely IPAH patients in that study who were recruited following diagnostic RHC in comparison to our smaller number of pooled IPAH and CTD-APAH patients with a median time to diagnostic RHC of 8 months.
This study demonstrates that baseline BNP levels < 100pg/ml always predicts survival at 1 year whereas BNP levels ≥ 100pg/ml can predict adverse outcome in CTD-APAHA but is not as discriminatory in IPAH patients. Baseline BNP measurement were unable to predict survival at 3 year follow up. Serial BNP measurements appear to enhance the prognostic ability but require validation in larger studies.

4.5.2 CTnI

This study demonstrates that the detection limit of 0.01ng/ml in the cTnI assay used is not sensitive enough to allow cTnI to be used as a prognostic marker in PAH. However, when serial cTnI measurements were made in this study increasing levels predicted adverse outcome and decreasing levels predicted survival. This result together with that reported by Torbicki et al\textsuperscript{33} suggests that serial cardiac troponin measurement are more helpful at predicting outcome than one off recordings in PAH patients.

In 2010, Heresi et al using a highly sensitive cTnI assay identical to the one used in this study (except they used a lower detection limit of 0.001ng/ml) demonstrated that cTnI is a good prognostic marker in PAH patients.\textsuperscript{89} Undetectable cTnI was defined as <0.001ng/ml, detectable cTnI level was defined as ≥ 0.001 and, cTnI level>0.03ng/ml was diagnostic for MI). CTnI was detected in 25% of PAH patients in contrast to only 6.5% of PAH patients having detectable cTnT using a classical cTnT assay. In patients with detectable cTnI 36 month transplant free survival was 44% compared to 85% in patients with undetectable cTnI.\textsuperscript{89} (4) Detectable cTnI was associated with a 4.7 fold increased risk of death related to right ventricular failure or transplant (p=0.001).\textsuperscript{89} Their study shows that by using a highly
sensitive cTnl assay with a lower detection limit that there is a higher chance that PAH patients at adverse risk will be identified. Serial cTnl measurements may enhance this further.

4.5.3 Endothelin-1

This study demonstrates that PAH patients have higher median ET-1 levels than matched normal controls. There was no statistically significant difference in ET-1 levels between deceased PAH patients and alive PAH patients at 1 and 3 year follow up. However, when the ET-1 levels of IPAH patients (CTD-APAH patients excluded) were analysed according to transplant free survival at 3 years there was a statistically significant difference between deceased and alive patient ET-1 levels. This is the first time that ET-1 is shown to be a useful prognostic marker in IPAH patients.

Montani et al found that ET-1 levels were elevated in 33 PAH patients (from all aetiological sub-groups) and demonstrated correlation between ET-1 levels and haemodynamic parameters but did not find an association between ET-1 levels and the ability to predict survival.\textsuperscript{82} In addition ET-3 levels were found to be low in PAH patients, correlated with haemodynamic parameters but not with clinical outcome measures (death or transplantation).\textsuperscript{82} However, they demonstrated that the ET-1: ET-3 ratio appeared to be a good prognostic marker in their cohort of patients.\textsuperscript{82}

Further research is required with larger patient numbers (from all aetiological groups) to confirm the utility of ET-1 and ET-3 as prognostic markers in PAH and to
confirm our findings that elevated ET-1 levels in IPAH patients are good prognostic markers of death at 3 year follow up.
CHAPTER 5

Summary, Conclusion and Recommendations
5.1 Summary

5.1.1 Lymphocytes and PAH

This study shows that NK cell depletion and CD8+ T-lymphocyte depletion occurs in IPAH and CTD-APAH patients suggesting a common immune-pathophysiology. PAH patients with a high CD4:CD8 ratio, CD8+ lymphocyte depletion and NK cell depletion at recruitment appear to have an increased risk of death at 1 and 3 years; the first time that the potential clinical importance of lymphocyte depletion in PAH patients has been reported.

Combined depletion of NK and CD8+ cell depletion was associated with death within 10 months of analysis. Depleted NK and CD8+ cell counts were also independently associated with adverse outcome. However, it is unclear whether CD8+ and NK cell depletion in PAH are a consequence of or a predisposing factor in this disease.

The protein immunofluorescence profile (elevated IL-23 and reduced IL-21 levels) identified in PAH patients with deficiency of NK cells and cytotoxic T- cells offers an insight as to potential mechanistic pathways involved in the adverse outcome associated with lymphocyte depletion in PAH patients.

Although high CD4:CD8 ratios and depleted NK and CD8+ cell counts are associated with elevated BNP levels these immune biomarkers appear superior to BNP as a prognostic marker and could aid our clinical decision making, particularly with the timing of active listing for lung transplantation and optimisation of pulmonary vasodilator therapies.
5.1.2 Inflammatory related proteins and PAH

Using a cytokine array that simultaneously analysed the immunofluorescence profile of 507 inflammatory related proteins, this study identified: a difference in protein immunofluorescence between PAH patients and normal controls; a difference in protein immunofluorescence between deceased PAH patients and alive PAH patients at 1 and 3 year follow up and elevated IL-23 and reduced IL-21 immunofluorescence levels in PAH patients with NK and CD8+ T-lymphocyte depletion.

Differentially expressed proteins should now undergo further analysis (using individual ELISAs) to ensure that the immunofluorescence profiles are an accurate reflection of protein levels in plasma.

If confirmed, the identified protein profiles can be analysed in larger PAH cohorts and may lead to new inflammatory protein markers of PAH some of which would offer prognostic information. In addition, the protein immunofluorescence profile associated with NK and CD8+ cell depletion (and poor clinical outcome) may help to unravel the pathogenesis of PAH and could potentially offer new IL-23 and IL-21 directed therapy.

5.1.3 BNP, cTnl and ET-1 and PAH

This study confirmed the utility of baseline BNP measurements as an adverse prognostic marker in PAH patients at 1 year follow up but highlighted that its ability to predict adverse outcome at 3 year follow up was poor. A baseline BNP level above the normal reference range in CTD-APAH patients always predicted adverse outcome
whereas similar reading in IPAH patients were not as discriminatory. Although, baseline BNP levels below the normal reference range always predicted survival at 1 year follow up this was not true for predicting survival at 3 year follow up. Serial BNP measurements appear to be better than one off measurements but require validation with larger patient numbers.

This study did not find that the cTnI high sensitivity assay (at a limit of detection of 0.01ng/ml) was any better than standard cTnT assays at identifying PAH patients at adverse risk. However, serial cTnI measurements did improve the ability to assess risk. The cTnI higher sensitivity assay with a lower detection limit of 0.001ng/ml used by Heresi et al (increased sensitivity but lower specificity) was much better at identifying patients at risk of adverse outcome. Using this cTnI high sensitivity assay at the lower detection limit together with serial analysis would undoubtedly offer improved prognostic information to the clinician.

This study confirmed that median endothelin-1 levels were elevated in IPAH and CTD-APAH patients compared to normal controls similar to other studies. It also demonstrated that ET-1 levels in deceased IPAH patients were higher than in IPAH patients who were alive at 3 year follow up which reached statistical significance, (p=0.02). This is the first time that ET-1 levels have been shown to predict adverse outcome in IPAH patients.

Study limitations

There are a large number of limitations in our study. The most significant being the small number of patients available for recruitment (due to the rarity of the disease). This however is a common problem with single centre studies in PAH patients and we did manage to include all available patients from our regional
centre. Another limitation was the recruitment of patients with both IPAH and CTD-APAH whereas the majority of other studies in PAH focuses on one or other and seldom pool aetiologies. The benefit of including both aetiologies in our study is that both groups have almost identical pathologies and are thought to have a common immunopathophysiology. It was interesting that NK and CD8+ cell depletion was found in both aetiological groups, further evidence for a common immune pathophysiology.

Patients were recruited at various stages during the disease process. This was largely due to the fact that only six patients underwent diagnostic RHC during the two year recruitment period. But again this is a common finding in a number of single centre PAH studies.

Patients were receiving different drug regimens at the time of blood sampling due to their various stages of disease (some only being recruited on the day of diagnosis). Although one patient receiving immunosuppressive therapy had evidence of NK and CD8+ depletion another patient receiving similar therapy did not.

In retrospect it would have been useful to monitor serial lymphocyte counts. One patient had repeat lymphocyte analysis performed as part of routine rheumatological follow up and the extent of NK and CD8+ cell depletion did mirror her progressive decline prior to death.

Finally, there was a lack of functional assays. It would have been interesting to assess the expression levels of inhibitory cell surface receptors on NK and CD8+ cells to establish whether these were responsible for the observed pattern of depletion.
Nevertheless, the findings in this study suggest important avenues for further research and warrant further assessment in larger multicentre studies in PAH patients from all aetiological sub-groups.

5.2 Conclusion

Depletion of NK cells and cytotoxic CD8+ T-cells in the peripheral blood of IPAH and CTD-APA patients appears to have a significant association with decreased survival and the favoured prognostic marker BNP. The mechanism responsible for the adverse outcome associated with NK and CD8+ depletion in PAH is unclear. However, the protein immunofluorescence profile identified in PAH patients NK and CD8+ depletion offers an insight as to potential mechanistic pathways and will be the focus of future studies.

Further research is required in larger PAH cohorts (from all aetiological sub-groups) to confirm the preliminary findings from this study. This would allow us to refine the phenotype of NK and CD8+ cells at different stages of disease and explore the potential of therapy which could reverse the lymphocyte deficiencies and the fatal outcome.
5.3 Recommendations

5.3.1 Lymphocytes and PAH

   a. Large prospective multicentre study investigating the lymphocyte profile of PAH patients at diagnostic RHC, and repeated as they progress through the various stages of PAH (on therapy).

   b. Analysis of receptors on the cell surface of NK and CD8+ cells as patients progress through the stages of PAH to identify mechanism for lymphocyte ‘exhaustion’ in PAH.

   c. Bone morphogenetic protein receptor 2 mutation analysis and IL-23 receptor analysis and correlation with lymphocyte depletion.

   d. Serology of PAH patients for viruses associated with lymphocyte depletion

5.3.2 Inflammatory related cytokines and PAH

Perform specific ELISA on each protein identified as being differentially expressed in PAH patient’s v normal controls to confirm that protein immunofluorescence reflects actual protein concentrations.

5.3.3 cTnI and ET-1

   a. Perform serial measurement of cTnI using high sensitivity cTnI assay (lower detection limit of 0.001ng/ml) and correlate with functional status, BNP and survival.

   b. Perform serial measurements of ET-1 & Endothelin-3 from all aetiological sub-groups and correlate with functional status, BNP and survival.
References and bibliography


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Appendices
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Note: The table contains a list of proteins and their corresponding codes, which are likely used in a specific context or experiment. The codes may refer to specific antibodies or other biological markers. The table is part of an appendix or continuation of a larger list, as indicated by the header "Appendix 1 continued."
Appendix 2

RayBio® Biotin Label-based Human Antibody Array 1

|------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|

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Appendix 3

Graphs showing the median and individual fluorescent intensities in PAH patients and normal controls.
Appendix 3 cont...

Graphs showing the median and individual fluorescent intensities in PAH patients and normal controls.
Appendix 3 continued

Figure 3.3: Graphs showing the median and individual fluorescent intensities between PAH patients and normal controls.

● IPAH ▲ CTD-APAH ■ Normal controls
Figure 3.4: Graphs showing the median and individual fluorescent intensities between PAH patients and normal controls.