INVESTIGATION OF POTENTIAL BIOMARKERS FOR LYMPHANGIOLEIOMYOMATOSIS (LAM) IN BLOOD

A thesis submitted in partial fulfilment of the degree of Master of Science (MSc) by Research

by

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CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Ayesha Javed declare that this thesis is submitted in fulfilment of the requirements for the award of Master of Science by Research, in the School of Life Sciences and Faculty of Science at the University of Technology Sydney. This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. This document has not been submitted for qualifications at any other academic institution. This research was supported by Australian Government Research Training Scheme and the LAM Australia Research Alliance.

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ABSTRACT

LAM is a rare multisystem disease primarily involving lungs. There is an average lag time of 4 years from the onset of symptoms till the diagnosis is made. Current methods of diagnosis are invasive and associated with a lot of limitations. Clinically, interstitial lung disease (ILD) resembles LAM and there are no methods available that are less invasive, and yield quicker results, in order to differentiate the two. The aims of this study are to identify potential biomarkers for LAM diagnosis and also determine its clinical severity just by using a simple blood test.

This pilot study was conducted to investigate and validate the differential expression of 10 proteins in the blood of patients suffering from lymphangioleiomyomatosis (LAM) as compared to interstitial lung disease (ILD) (idiopathic pulmonary fibrosis) and normal healthy subjects in order to determine if any of the proteins could serve as a potential biomarker for the early diagnosis of LAM. The 10 proteins selected for study were lipopolysaccharide binding protein (LBP), Fetuin-B, apolipoprotein A1 (APO A1), apolipoprotein A4 (APO A4), heparin cofactor 2, phospholipid transfer protein (PLTP), plasma serine protease inhibitor 5 (SERPIN A5 gene product), N-acetylmuramoyl-L-alanine amidase (PGLYRP2), sex hormone binding globulin (SHBG) and Talin 1. These proteins were selected on the basis of a proteomics study (LC/MS) which showed altered expression levels (either increased or decreased) of these proteins in sera from LAM patients, as compared to normal controls.

Validation of differential expression of the selected protein targets was carried out using either ELISA or western analysis using sera from LAM patients (n=10), ILD patients (n=10) and normal age matched individuals (n=10). Blood samples were obtained from and a blood biobank

established. The expression of each of the 10 candidate proteins was validated in all three sample groups and data obtained was analysed to establish the statistical significance of any detected protein level alterations.

The validation experiments carried out showed that Fetuin-B was significantly decreased in LAM patient sera as compared to ILD patients ($p=0.023$). Heparin cofactor 2 was significantly decreased in LAM patients as compared to normal healthy individuals ($p=0.030$). PGLYRP2 was significantly reduced in LAM as compared to ILD patients ($p=0.00047$) and normal healthy human subjects ($p=0.0061$). Further, clinical severity of disease (ILD and LAM) was also correlated with the protein level differences observed. Correlation analysis showed that, in LAM, levels of heparin cofactor 2, PGLYRP2 and Fetuin-B decrease with increasing severity of the disease. In ILD, levels of APO A4 were shown to be decreased with increasing disease severity in ILD, while plasma serine protease inhibitor levels were increased with the increasing disease severity.

This study data identified some novel potential biomarkers for diagnosing LAM by differentiating it from other similar diseases and normal individuals. These include Fetuin-B, Heparin cofactor 2 and PGLYRP2. Additionally, some potential protein biomarkers of severity were also identified for LAM and ILD. These potential biomarkers may, in future be used for diagnostic purposes in LAM and also for determining stage/severity of LAM and ILD.

CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Overview of Lymphangioleiomyomatosis

Lymphangioleiomyomatosis (LAM) is an extremely rare disease of the lungs which mostly affects females in their reproductive years [1, 2]. This condition is characterised by the formation of cysts in the lungs that increase in number over the time. The approximate age of patients at the time of diagnosis is 35 years [3]. There are two forms of this disease, namely sporadic LAM (S-LAM) and tuberous sclerosis complex LAM (TSC-LAM) which both are thought to be due to mutations in the TSC gene. [4]. Almost 1 in 400,000 female adults are affected by LAM and 30-40% of women with TSC have TSC-LAM [5, 6]. There are currently less than 1000 women in the USA that have been confirmed to have S-LAM [7]. Tuberous sclerosis is a syndrome that is caused by mutations in the TSC gene and, in addition to having some clinical features in common with LAM, has a number of unique clinical features involving neurological impairment and the formation of tumours throughout the body [8].

1.1.1 Histological features of LAM

The typical histological characteristics of LAM include the presence of 'lookalike' smooth muscle cells known as LAM cells. These cells tend to proliferate abnormally, leading to the formation of cysts inside the lungs. This excessive proliferation causes blockage of lymphatic vessels resulting in lymphatic fluid (chyle) accumulation, as well as obstruction of air passages, which ultimately results in respiratory failure in patients [9]. LAM cell behaviour resembles that of malignant cells, including excessive, uncontrolled and abnormal growth and survival capacity, invasion and infiltration capacity and ability to metastasise to local and distant sites primarily through the lymphatic channels. Surprisingly, histological findings in LAM cells are not consistent with those of other malignant cells. Malignant cells show some typical histological features such as excessive mitosis, nuclear cytoplasmic asymmetry and changed nuclear cytoplasmic size ratio, but these features have not yet been described in LAM cells [3].

Diagnosis of LAM is chiefly based on surgical biopsy and radiology. High resolution computed tomography (HRCT) scanning alone is not a reliable method for the diagnosis of LAM. This became evident after examination of a lung that had been explanted from a LAM patient who had undergone lung transplantation surgery. This patient was later found out to be suffering from another cystic respiratory condition in which the clinical picture resembled that of LAM [10, 11]. Therefore, the mainstay of LAM diagnosis remains as tissue biopsy with corresponding histological findings. A further study has revealed that some other parameters may also be used to identify LAM without the use of interventional procedures such as tissue biopsy. This may be done through determining the presence or absence of lung cysts together with the occurrence of angiomyolipoma, associated TSC and/or chyle collections [12].

Biomarkers in the blood could also provide a diagnostic means for LAM, as well as provide a means for monitoring prognosis and progression. There are currently a number of biomarkers already identified for LAM, and, among these, vascular endothelial growth factor D (VEGF-D) is considered to be the most useful [13]. The levels of this product have been found to be raised in the blood of patients suffering from LAM as compared to healthy adults and patients with other cystic lung diseases [14]. Although found to be elevated in a large number of LAM patients, it has been shown to be an unreliable means of accurately diagnosing LAM, and has therefore been deemed to have questionable clinical utility. The prime reason for this is that it has not been shown to be elevated in all LAM patients and this is further explained in the following sections [15]. Despite this, a number of studies have suggested that VEGF-D levels of greater than 800 pg/ml, along with the characteristic cyst formation in the lungs, points towards a diagnosis of LAM [16]. However, VEGF-D levels of less than 800 pg/ml do not totally rule out the possibility of LAM [17].

1.1.2 Morbidity associated with LAM

Ten years post diagnosis, it is expected that possibly more than half of all LAM patients will experience shortness of breath on mild exertion, and an even greater number of patients will suffer shortness of breath with light exercise. Of these, approximately 20% will become dependent on oxygen supplementation and 10% will reach the terminal stages of the illness and death [18].

1.1.3 LAM is often misdiagnosed

Over all it is believed that the diagnosis of LAM is delayed by approximately 4 years as a result of misdiagnosis due to the close resemblance of the symptoms of LAM with those of other respiratory diseases, such as chronic obstructive pulmonary disease (COPD) and asthma. The symptoms are explained in detail in subsequent sections of this chapter. The main symptom that is almost always the presenting feature of these conditions is progressive shortness of breath.

1.2 Prevalence of LAM

Due to the milder symptoms of TSC-LAM, compared to S-LAM, as well as occasional differences in the clinical picture seen in TSC-LAM (such as cognitive impairment, seizures or renal impairment), TSC-LAM remains under-diagnosed [8]. Despite this, it is known that TSC-LAM is 5-10 fold more prevalent than S-LAM [7, 19]. Both TSC and S-LAM occur mainly in females with only 4 males having a confirmed diagnosis of LAM by biopsy [20-22]. Three of these cases were proven to be LAM, based on histological findings, whereas in the last case, the particular male was diagnosed with LAM in the presence of pre-existing Klinefelter syndrome (i.e. possessing an additional X chromosome and therefore having an XXY chromosomal makeup). This patient had been taking exogenous sex hormones for many years, suggesting a probable role for sex hormones (androgens as well as estrogen) in the development of LAM in this particular male [23].

Populations suffering from TSC that are screened for LAM show that only a third of TSC-LAM patients have lung features consistent with those found in LAM [24-26]. Sporadic LAM was diagnosed for the first time in 1937 [27] and currently there are almost 10,000 known cases of S-LAM across the world [28] .

1.3 Differential Diagnosis of LAM

There are several diseases that mimic LAM on clinical presentation, however these diseases also possess some features that can distinguish them from LAM. In Langerhans cell histiocytosis (LCH), there is the presence of irregular cysts that are mainly distributed in the middle and upper lung areas, unlike LAM. In Emphysema, also unlike LAM, there is lack of a definite boundary

wall in the cysts and most patients generally have a long smoking history [29]. Lymphangiomatosis is another condition very closely resembling LAM and, although very rare, shows features similar to LAM including lymphadenopathy, lymphangiomas and smooth muscle cell infiltration of thoraco-abdominal lymphatics [30, 31]. Other diseases that resemble LAM in many ways are Birt-Hogg-Dube´ (BHD) syndrome, which consists of leiomyosarcomas of lower grade (grading represents malignant potential of any tumour cells, with a higher grade indicating a greater potential for malignancy and a lower grade indicating lower malignant potential) [32], lymphocytic interstitial pneumonitis, follicular bronchiolitis [33], light chain deposition disease, amyloidosis [11] and hypersensitivity pneumonitis [34]. Furthermore, in chronic cigarette smokers, multiple cysts can be found present in a diffuse pattern which, when examined through radiography of the chest, mimic LAM to a great degree [35].

1.4 The genetic basis of LAM

S-LAM, as well as TSC-LAM, have a genetic basis and are thought to be caused by mutations of the TSC gene [4]. Tuberous sclerosis is an autosomal dominant disorder and the commonality between TSC and LAM is an important aspect that needs to be understood in order to gain advanced knowledge of LAM pathogenesis and thus target therapies [36]. There is evidence of germline mutations in both the TSC-1 and TSC-2 genes in women with TS and LAM. TSC-1 and TSC-2 are large genes consisting of 23 and 41exons [37] respectively, with the majority of mutations occurring in TSC-2, leading to severe clinical manifestations that are also common among TS patients [38]. Noteworthy is the fact that a study looking for activating and inactivating mutations and genetic abnormalities in angiomyolipoma confirmed the presence of mutations only in TSC-2, and not mutations in associated genes such as TSC-1. These findings hint towards a role for TSC mutations in tumorigenesis [39].

The protein encoded by the TSC-2 gene is tuberin [40], while hamartin is the protein encoded by the TSC-l gene. These two proteins are found in the cytoplasm and form a complex to function together in a heterodimer that participates in signalling pathways involved in controlling proliferation and cellular growth [41]. Rheb/mTOR/P70S6K is the pathway through which both these genes participate to regulate the cell cycle. Rheb GTPase inactivates Rheb whereas tuberin activates Rheb GTPase. When tuberin is altered, it leads to hyperactivity of Rheb which

stimulates the mTOR pathway and ultimately leads to stimulation of cellular growth and proliferation [42]. This is explained further in a later section along with the actions of various drugs that targe different sites in the pathway.

Tuberin maintains Rheb, a Ras homolog, in an inactive state [43, 44]. The triad comprising TSC-1/TSC-2/Rheb acts as an important cellular signalling complex that senses inputs from growth factors [45]. There is inactivation of the wild-type (wt) TSC-2 allele in LAM cells of women with TSC-LAM characterised by loss of heterozygosity (LOH) at chromosome 16pl3 [46]. LOH at the TSC-2 locus has been identified in cells from chyle, urine, blood and bronchoalveolar lavage fluid in up to 90% of patients with sporadic LAM [47, 48]. Some studies have also revealed the presence of TSC-2 mutations in LAM cells cultured in the laboratory [49]. Although the para-aortic lymph nodes and angiomyolipomas from S-LAM patients have shown evidence of TSC-2 loss of heterozygosity [50], women with sporadic LAM did not have TSC-1 mutations by definition [51]. In another study aimed at identifying TSC-2 mutations in LAM cells, fixed lung tissue was studied histologically and then examined using both laser capture microdissection (LCM) and next generation sequencing (NGS) technology. Histologically, characteristics of LAM lesions, including LAM nodules containing cells positive for HMB45, smooth muscle actin and the formation of multiple cysts, were observed. LCM was also used to examine cells from the LAM nodules. Extracted DNA was amplified and NGS analysis detected 9 different pathogenic variations in the TSC-2 sequence in eight cases of sporadic LAM. Additionally, of all the samples tested using NGS techniques, two were shown to lack mutations in either TSC-1 or TSC-2. Interestingly, these cases without mutation in TSC-1 and TSC-2 also showed a lack of expression of phospho-S6 kinase and thus normal TSC-1/TSC-2 function, as well as mTOR regulation. This finding suggests that there was no activation of mTORC1, which is known to be the target of rapamycin complex 1. Thus the indication is that there are potential alternate causative mechanisms contributing to the development of LAM, acting independently of, and in addition to, the known mechanisms [52].

Angiomyolipoma occurring in LAM behaves similarly to a benign tumour and grows within the renal parenchyma. Although the definition of LAM as a neoplastic disease is not clear, it is included in the family of perivascular epithelioid cell tumours (PEComa), due to the similar molecular characteristics, and therefore points to its neoplastic nature [53]. Activation of the

mTOR pathway provides an unusual survival advantage to the angiomyolipoma and other PEComas [47].

By definition, "a neoplasm is an abnormal mass of tissue, the growth of which exceeds, and is uncoordinated with, that of the normal tissues, and persists in the same excessive manner after cessation of the stimulus which evoked the change (which is autonomous, purposeless and preys on the host)" [54].

1.5 Oestrogen and Progesterone Receptor Expression in LAM cells

Immunohistochemically, progesterone and oestrogen receptors can be detected in almost all LAM cells, pointing towards its predominance in the female gender [55], although the number of progesterone receptors has been shown to be significantly higher in comparison to the number of oestrogen receptors in almost 80% of biopsies tested. This is unlike other female predominant neoplasms in which there is higher expression of oestrogen receptors. Thus LAM cells appear to have a higher PR/ER expression ratio [56].

1.6 Molecular Pathogenesis of the PEComas and LAM

It has been suggested that LAM belongs to the group of tumours called “perivascular epithelioid cell tumours” (PEComas) [57-59]. The World Health Organisation (WHO) has classified PEComas as a family of proliferative lesions consisting of mesenchymal cells that have similar histological and immunohistochemical perivascular epithelioid cells [60]. This is a heterogeneous group of neoplasms sharing the presence of PECs as a common feature. A PEC is a mesenchymal cell with a distinctive immunophenotypic profile and also expresses some markers including HMSA-1 (a protein, a melanocytic marker), HMB-45(a monoclonal antibody having putative melanoma cell specificity) and, less frequently, Desmin [57, 61, 62].

The morphological appearance of PECs is epitheloid and having a clear cytoplasm. The nucleus is located in the cell centre with an inconspicuous nucleolus [57-59]. Some ultrastructurally distinctive features are also seen in these perivascular epitheloid cells and these include the presence of bundles of microfilaments that have many mitochondria, membrane-bound dense granules and electron dense condensation [63, 64]. Mostly there is visibility of mild cytological

atypia in the perivascular region [57-59]. Therefore it can be assumed that these cells could be derived from mesenchymal stem cells that are genetically modified and having their origin in the perivascular region [65].

The distinctive group of neoplasms referred to as the PEComas have similar molecular alterations irrespective of the location in which they are found. Their common basis emerges from the fact that they have inactivation and loss of either the TSC-1 or TSC-2 genes [19, 24-26, 66]. PEComas are a group of benign tumours, and, in order to become malignant, they most likely require a number of genetic modifications [42].

Similarly, LAM cells show no evidence of atypia (i.e. there is no abnormal cell structure) or any visible features of proliferative behaviour. However there is progressive invasion and infiltration of lung parenchyma by LAM cells, confirming its metastatic behaviour, which is further revealed by the recurrence of LAM cells in the lungs post transplantation [54]. It has been suggested that this is due to the migration of LAM progenitor cells to newly transplanted lungs [67]. Allografts of the new lung tissue have revealed the presence of cells with similar gene alterations to those that were present in the explanted lung LAM cells, as well as the occurrence of LAM cells in the lymph node of the same patient. This supports the theory that recurrence is patient derived [54].

The genetic abnormalities that are common in LAM and PEComas are alterations of particular tumour suppressor genes, providing a growth advantage to cells and leading to the formation of clones of cells that are dependent on epidermal growth factor (EGF) [68]. There exist a variety of malignant neoplasms with phenotypic and genotypic abnormalities characteristic of PEComas [69-71] .

1.7 Clinical Features of LAM

Patients with LAM typically present with dyspnoea (shortness of breath) on exertion, that becomes progressive, pneumothorax, or accumulation of chyle in the pleural space [72]. On physical examination, there may be findings of wheezing, clubbing (drumstick like deformity of the finger tips) and crackles (rattling and clicking sounds produced in the base of the lungs) [73]. Less often, there is incidental discovery of a retroperitoneal abdominal mass resembling lymphoma or ovarian cancer but is confirmed as LAM on biopsy [74].When screened, asymptomatic patients with TSC often get diagnosed with LAM earlier [20-22].

1.7.1 Angiomyolipoma

Symptomatic angiomyolipoma (a type of benign tumour of the kidney) is highly prevalent in both TSC-LAM and S-LAM patients and poses equal risk of bleeding. Angiomyolipoma can present at any time during the course of the illness and therefore these tumours need to be monitored continuously using MRI [75]. Interestingly, patients with renal angiomyolipomas rarely present with flank pain, hematuria (presence of blood in urine), hydronephrosis (swelling of the kidney due to accumulation of urine in the renal calyses and pelvis) or renal failure as might be expected. S-LAM patients tend to have multiple tumours that are larger and more prone to heamorrhage. Angiomyolipomas are benign tumours of the kidney that are composed of smooth muscle cells, fat cells and blood vessels [76, 77]. There may be co-existence of pulmonary LAM and bilateral renal angiomyolipomas, however this is associated with more devastating outcomes in terms of mortality and morbidity [78].

Additionally, while more than half of the S-LAM patients have angiomyolipomas, nearly all, or most TSC-LAM patients have these tumours [8-10][61, 79, 80]. The most common of these angiomyolipomas is that found in kidney, known as renal angiomyolipoma and is benign in nature [10].

1.7.2 Other clinical signs of LAM: pneumothorax, chylothorax and haemoptysis

Pneumothorax: Pneumothorax is the entry of air within the pleural space causing lung collapse. The occurrence rate of pneumothorax is almost 60-70% in LAM patients with a recurrence rate of 70%. This is the highest rate among all pulmonary diseases [81, 82].

Chylothorax: The occurrence of chyle collections in the pleural space may be unilateral or bilateral and is found in approximately one third of LAM patients [83].

Haemoptysis: A recent study has also documented long-standing haemoptysis as a presenting feature in patients with underlying LAM. In addition, other features including cough and dyspnoea were also observed at a later stage [84].

1.7.3 Lymphedema in LAM

One of the rare complications of LAM is lymphedema, which does not correspond with the severity of the disease in terms of pulmonary cystic involvement. Axial lymphatics are involved in the development of this lymphedema. In a group of 228 patients, all confirmed cases of LAM, only 3.8% (8 patients) showed the presence of lymphedema as an additional feature and only 2.2% (5 patients) of these patients showed lymphedema as the prominent or presenting feature. All these patients presented with enlarged lymph nodes (lymphangioleiomyomas) either in the pelvic cavity or retroperitoneum, but the exact incidence is yet to be determined [85]. Lymphedema develops when lymph from normal capillary filtration does not drain properly, resulting in accumulation of protein rich fluid in the interstitium [86]. Lymphedema can occur either due to (1) an inherent defect or abnormality in the lymphatic channels, (2) secondary to pressure exerted by an enlarged tumour in the region, (3) as a post-surgical complication following lymph node removal or (4) due to the formation of scar tissue following radiation exposure. It has remarkable psychological consequences on patients because of the lack of an established therapy for lymphedema [87]. However it would be difficult to diagnose the presence of underlying LAM based on identification of lymphedema alone [85]. Thus, it is clear that the symptoms of LAM are not confined to the lungs alone.

1.8 Air travel and pregnancy for LAM patients

Patients with LAM travelling by air mostly do not experience adverse symptoms. In the 10-20% of patients that do experience symptoms during flights, the symptoms generally experienced include anxiety, chest pain, cyanosis, shortness of breath, haemoptysis and evidence of pneumothorax, however none of the incidents have resulted in hospitalisation [88]. Those patients who have experienced pneumothorax are advised against travelling by air or to take necessary precautions as available. Women with LAM have been shown to have exacerbation of their symptoms, especially pneumothorax, during pregnancy and are advised to avoid air travel if possible [88]. Advice regarding pregnancy is generally given on a case by case basis and is dependent on the severity of exacerbations and the stage of disease advancement.

1.9 Screening and follow-up of LAM patients

The LAM Foundation Pleural Disease Alliance recommends that all women suffering from TSC, and who are 18 years of age or above, should be screened for LAM at least once a year by pulmonary HRCT [89]. Measurement of serum VEGF-D has utility in terms of screening and aiding early diagnosis of LAM in women with TSC or pulmonary cysts. Routinely, for follow-up of LAM patients, the recommendation is to undergo pulmonary function tests once or twice a year and pulmonary HRCT screening after 1-3 years [90].

1.10 Mortality rate associated with LAM

The mortality rate associated with LAM is approximately 10-20% after 10 years from the time of onset of symptoms but can be variable [5, 18]. It has been recently reported that those who present with pneumothorax at a younger age have better prognosis with an 89% survival rate at 10 years compared with those patients who present with dyspnea at a later age. This could possibly be due to the fact that dyspnea arises after at least 1/3 of the lung is affected and the disease has progressed, whereas episodes of pneumothorax at a younger age draws attention towards early treatment and better outcomes in terms of improving quality of life and thus prolonging survival [91].

1.11 The clinical course of LAM

Following the appearance of initial symptoms, almost 50% of patients experience dyspnea while walking [18]. The presentation and the clinical course vary to a great degree making it an unpredictable clinical condition with some patients developing respiratory failure many years earlier than others. This makes it almost impossible to plan an early targeted therapy [6]. There exists an association, or relation, between cyst size and occurrence of pneumothorax, whereby cysts that are larger than 0.5 cm tend to rupture more often than those of a size that is less than 0.5 cm [92].

1.12 Treatment and clinical management of LAM

First line treatment of LAM includes general management of patients. General management improves patient quality of life. This depends on a number of factors including socioeconomic status, presence or absence of complications, stage of progression of the disease and its severity. All patients should be evaluated and recommended for assessment of disease severity. For this purpose, all patients should be examined clinically along with recording their medical history and conducting a series of baseline tests that include pulse oximetry, pulmonary function tests, arterial blood gas measures, VEGF-D level measurement, electrocardiogram, ultrasonic cardiogram, a 6 minute walking test (6MWT), a St George respiratory questionnaire (SGRQ), pulmonary HRCT of the chest and CT/MRI of the abdomen and pelvis. The HRCT scan of the chest can be repeated every 12 months for disease assessment as elaborated below.

1.12.1 General management of LAM

General measures that need to be taken as part of management are vaccination for influenza and pneumococcus, appropriate counselling if pregnancy is to be planned, as it can worsen the symptoms, and similarly precaution against air travel in case of an existing pneumothorax or having had one within the past month. Supplemental oxygen therapy for those with hypoxemia is advised. Patients should be encouraged to participate in pulmonary rehabilitation programs [93]. Patients with lymphedema gained benefits after changing to diets containing a limited quantity of fat, in combination with physiotherapy. The reasons for the health benefits are not clearly known. Gonadotropin-releasing hormone analogues were also given to some patients. All the therapies, when combined, gave effective control of lymphedema [85].

1.12.2 Surgical/ invasive therapy

In the case of development of lung chylous pleural effusion, the LAM Foundation has recommended pleurodesis of the same side of the lung after the development of pneumothorax. Most patients respond well to pleurodesis, however, for some patients, thoracocentesis is preferred because it is less invasive. A small number of patients with chylothorax are simply kept under observation. Those patients with angiomyolipomas less than 4 cm in size are usually observed using ultrasonography or CT scan, however if the tumour size exceeds 4 cm then interventional therapies are considered. These include embolisation, enucleation, radioablation, or electrocautery, which are all nephron sparing techniques, or partial nephrectomy. Most patients with recurring angiomyolipomas will have undergone a prior resection [94, 95]. There appears to be an increased chance of recurrence of pneumothorax in patients who have undergone treatment comprising conservative procedures including aspiration and chest tube drainage as compared to those who have undergone permanent procedures such as chemical or surgical pleurodesis. Recurrence rate after conservative and permanent procedures is about 66% and 27-32% respectively [94]. Chemical pleurodesis is the treatment of choice in the event of a first pneumothorax. Finally, patients with severely diminished lung function are recommended to undergo lung transplantation [86]. Lung transplantation is the ultimate solution for those suffering from end-stage disease. Both single or double lung transplants have the same outcomes in terms of quality of life and 1, 3 and 5 year survival rates [96].

1.12.3 Pharmacological therapy for LAM

Ongoing research has been fruitful in terms of identifying potential molecular targets of drugs that can be used to treat LAM. Potential target drugs include lymphangiogenesis inhibitors (e.g. anti VEGF-D antibody), metalloproteinase inhibitors (e.g. doxycyclin), selective estrogen antagonists (e.g. fespemifene), angiogenesis inhibitors (e.g. bevacizumab), tyrosine kinase inhibitors and Rheb inhibitors (e.g. farnesyltransferase inhibitors). The classes of drugs mentioned below have already been approved, or are under trial for other conditions [95, 86].

Sirolimus and everolimus are two known immunosuppressant drugs that work by inhibiting mTORC1 [97]. When treated with sirolimus, both S-LAM and TSC-LAM patients were observed to show regression of renal angiomyolipomas, whereby the tumours were reduced to half the

original size following therapy. However, the tumours grew back to their original size after discontinuation of the drug treatment. These findings suggest that this drug is cytostatic, only reduces the size of the cells but not causing cell death or apoptosis. Additionally, VEGF-D levels were shown to decline during sirolimus therapy but tended to rise again after discontinuation of therapy [87]. Patients with LAM respond to treatment with Sirolimus in terms of slowed decline in lung function and slowed progression towards cyst formation. Sirolimus is well tolerated by most patients [88] and, even at low doses, has proven to be efficacious in decreasing chyle fluid collections and improving lung function [89]. Treatment with this drug also leads to a decline in the levels of LAM cells present in the blood and passed on in the urine. This was more pronounced in postmenopausal women as compared with younger age groups probably indicating a role for sex hormones in this function [90].

While LAM patient response to Sirolimus is good, there are, however, side effects associated with sirolimus therapy. The most common side effects include hypercholesterolemia, diarrhoea, hypertension, proteinuria, peripheral oedema, upper respiratory tract infections, leucopoenia, thrombocytopenia, delayed wound healing, acne, headaches and stomatitis. It is therefore recommended that patients be kept under observation during treatment [98]. In this scenario, routine laboratory tests need to be performed throughout the therapy, including urine analysis, blood chemistry and cell count, blood levels of sirolimus and urinary protein creatinine ratio. Lung function tests are also to be performed at 6 monthly intervals. Guidelines are not yet available with respect to optimal starting dose and continuation of lifelong therapy as little is known about post-therapy resistance development in LAM patients. Current dosage recommendations are based on sirolimus efficacy in the prevention of lung graft rejection [99]. In patients with reversible airflow limitation, therapy with bronchodilators improves lung function. It has also been seen that LAM patients tend to have abnormal bone density and therefore also respond well to therapy with bisphosphonates [91].

In mouse models, simvastatin, a HMG-CoA reductase inhibitor, inhibits RHoA activity, together with inhibition of tumour growth and cell proliferation and promotes apoptosis (Figure 1.1). When it is administered in combination with sirolimus it had a preventative effect on the recurrence of tumours in mice [100].

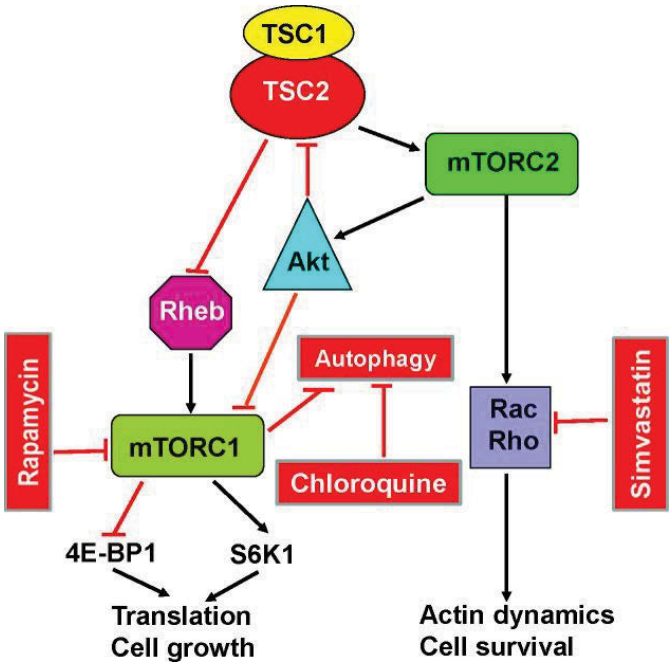

Figure 1.1. Schematic diagram of the mTOR pathway showing the different sites of action of various drugs used to treat LAM. The main players in the mTOR pathway are shown with the upstream and downstream activators indicated by the arrows. Key: Protein kinase B is also called Akt, Rheb is for Ras homologue enriched in brain, TSC1 is for tuberous sclerosis complex 1, TSC 2 is for tuberous sclerosis complex 2, Rho is for Rho Protein [101]).

1.13 Diagnostic criteria for LAM

LAM is usually diagnosed using pulmonary and/or abdominal HRCT scan or lung biopsy [102]. Patient history and HRCT may or may not be taken into account for diagnosis if biopsy has been taken from lymphangioleiomyomas or lymph nodes as that alone will provide sufficient evidence for a definitive diagnosis. LAM cell morphology is characteristic and provides a reliable means to make a pathological diagnosis, especially when HMB-45 and smooth muscle actin immunoreactivity are also taken into consideration. However, there is currently a trend towards replacing the combination of biopsy and patient history for the use of HRCT alone for the diagnosis of LAM and there exist a number of conditions that very closely resemble HRCT

findings of lungs in LAM [12]. Characteristic findings on HRCT are the presence of multiple diffuse very thin walled cysts and a large collection of chyle [103].

In 2010, the first official diagnostic criteria for LAM were established and termed the "European Respiratory Society Guidelines for diagnosis of LAM". According to the criteria, a patient would have a definitive diagnosis of LAM if he/she has a characteristic HRCT along with lung biopsy findings. Otherwise, a characteristic HRCT finding in combination with any of the following findings, including thoracic or abdominal chlye accumulation, presence of lymphangioleiomyoma or involved lymph node, presence of TSC and angiomyolipoma (kidney) would be diagnostic for LAM. A patient would be classified as having probable LAM if he/she has HRCT findings compatible with LAM and a favourable clinical history or has renal angiomyolipoma or abdominal/thoracic chylous effusion in addition to characteristic HRCT findings. Lastly, a characteristic/ compatible HRCT would be suggestive of possible LAM in the absence of other compatible findings [12].

Apart from the diagnostic modalities discussed above, there exist potential biomarkers that could indicate LAM far more easily. Currently the only marker for this purpose is VEGF-D but this is neither diagnostic nor prognostic. Its levels are elevated in some LAM patients and its elevation in serum is related to the extent of lymphatic involvement. All these factors hint towards the requirement for more sensitive and specific biomarkers that could simplify and aid in early definitive diagnosis [103, 104].

1.13.1 Pathologic diagnosis

The presence of smooth muscle cells occurring in multifocal nodules, immature in appearance and showing excessive proliferation, together with cysts, represents a characteristic pathological lesion. There is also the presence of epithelioid cells, termed LAM cells, that are mostly found in the perivascular region (Figure 1.2) [105-107]. In early disease, pathological findings are not prominent and in later stages, occur in variable degrees with non-specific sensitivity. However, if such immature smooth-muscle like cells are found together with epithelioid cells and cysts, this is considered sufficient for a diagnosis of LAM. Routine staining with haematoxylin and eosin is used for this purpose, in addition to supportive HRCT and suggestive history.

Transbronchial biopsy specimens can also be used to perform HMB45, smooth muscle actin and desmin immunohistochemistry, which serves as an important adjunct to diagnosis. Even in the absence of positive HMB45 staining, pathological findings alone provide sufficient evidence for making a diagnosis of LAM [63, 108]. However, positive history and radiology would raise the level of confidence in the LAM diagnosis. 50% of patients can also be detected positive for oestrogen/progesterone receptors using immunohistochemistry [109, 110].

Overall, it is recommended that a pathologist experienced in LAM diagnosis should examine biopsy specimens from suspected cases with presence of cysts, perivascular epithelioid cells and smooth muscle like cells. Immunohistochemistry for HMB45 and oestrogen/progesterone receptors is only required in the cases of doubtful pathological findings but can only be considered an adjunct to diagnosis [12].

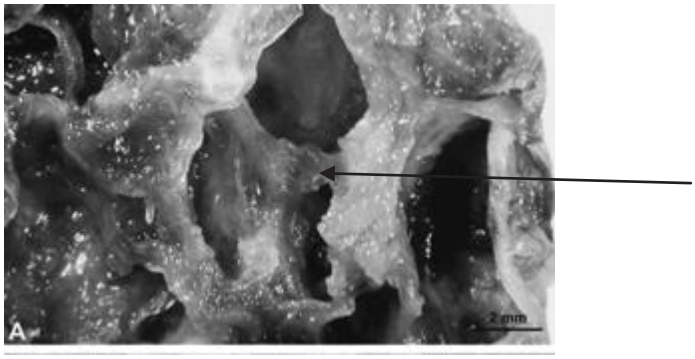

Figure 1.2. Lung section of a LAM patient. Lung section showing diffusely distributed thin walled cysts characteristic of LAM. These cysts are usually thin walled structures with 2-4 mm wall thickness with variable sizes ranging from 2-5 mm in diameter for the smaller cysts and as large as 30 mm in diameter for the larger cysts. This specimen was obtained during surgery for lung transplantation. (Figure taken from [111]).

1.14. Biomarkers: an introduction

A biomarker is a biological molecule that is representative of a variation or disturbance in a normal physiological process. It can also be described as a molecule that can be of some clinical utility. In other words, a biomarker is a molecule that is specific, as well as sensitive, for a particular disease condition and is reflective of the ongoing underlying processes. Biomarkers may represent the stage that the disease process has reached and, in the case of therapeutic intervention, may also prove to be an indicator of the effect or efficacy of the drug or treatment being used. These features make biomarkers a very important discovery from a clinical point of view. There is a deficiency of biomarkers in most fields of clinical science and where they do exist they are mostly not specific. For example, alpha fetoprotein is considered a biomarker for liver disease, but although elevated, it is not specific for non-neoplastic liver diseases like alcoholic liver disease, acute viral hepatitis or drug induced hepatitis [128]. Despite being a non-specific marker for non-neoplastic liver disease, when serum levels are raised, it is almost always indicative of a hepatocellular carcinoma [129]. Another example is C reactive protein which is an indicator of inflammation in general but not specific for any type or causative agent of inflammation [130].

There are various proteomic strategies that can be adopted to discover protein biomarkers [131]. Proteomics are studies conducted to determine the protein content of an entire organism, cell or a system, whether structural or functional [132]. Determination of protein content or modification can be carried out using different techniques including mass spectrometry, image analysis, amino acid sequencing, gel electrophoresis and others. These can be used to detect and quantify the protein content in a given sample. These techniques provide avenues for the discovery of new biomarkers for diagnostic, prognostic and therapeutic assessment purposes [133].

1.14.1 Development of non-invasive diagnostic and prognostic biomarkers for LAM

Biomarkers present in circulating blood are a very promising, minimally invasive tool to aid in the diagnosis of a disease, monitor its progression and outcomes and provide prognostic information. VEGF-D has been found to be elevated in LAM but, as previously mentioned, it is neither diagnostic nor prognostic [14]. It has been shown, however, that VEGF-D levels can be useful in differentiating LAM from some other diseases such as Birt Hogg Dube disease,

pulmonary Langerhans cell histiocytosis and emphysema [14]. One reason for the unreliability of this biomarker is that the levels of this marker are not always elevated in all LAM patients [103, 104]. Another study has shown that serum VEGF-D levels appear to be elevated in those patients who have significant development of cysts in the lung, as seen on semi quantitative CT, therefore confirming an association of VEGF-D levels with the extent of cystic lung involvement [134]. Other potential indicative biomarkers include metalloproteinases (MMPs) as altered levels have been seen in a number of respiratory conditions, including LAM, but there is a limitation in terms of specificity for LAM [135].

Increased levels of membrane bound angiotensin converting enzyme (ACE) inhibitor have also been observed in LAM patients [22], together with elevated serum levels of ACE. Evaluating the clinical utility of serum ACE, VEGF-D and total MMP using the European respiratory society (ERS) guidelines, and comparing the accuracy of diagnosis, has shown that VEGF-D levels could differentiate LAM patients with substantial lymphatic involvement only from LAM patients with other predominant features not including lymphatic involvement, but was not indicative of renal angiomyolipoma. Thus VEGF-D appears to be indicative of lymphatic involvement only and not LAM as a whole, or even lung function, and can reduce the need for biopsy by 10%. Total MMP-2 has shown some relation with lung function and disease activity. Together the biomarkers and clinical guidelines can reduce the requirement for invasive procedures like biopsy in order to improve the clinical care of patients [15]. The levels of MMP have been found to be elevated in the blood of LAM patients as compared to normal matched individuals. In this case MMP-9 levels are increased and not MMP-2 levels as confirmed using ELISA and gelatine zymography [135]. One study showed that there is a correlation between pulmonary cysts on HRCT and other factors such as air trapping and airway obstruction. It also confirmed that there is an insignificant relationship between serum VEGF-D levels and pulmonary cyst formation and no association between pulmonary cyst development and serum MMP-2 and MMP-9 levels [136].

1.14.2 Previous work related to this study

To date, a proteomics study has been conducted by our research group comparing the serum protein content of LAM patients with that of healthy, age and gender-matched, adults. Isobaric tags were used to identify proteins and to determine their absolute and relative quantities in the

serum of the two study groups. This study detected 14 proteins showing altered expression (either increased or decreased levels) in the serum of LAM patients compared with that of healthy individuals (Table 1.1) [137]. Of the 14 differentially expressed proteins identified, four have been validated (as part of another research project) using ELISA and histological methods (Table 1.2). These 4 validated proteins were fibronectin, Von Willebrand factor, kallikarein III and plasminogen. Validation of the remaining 10 proteins is described in this thesis. Identification of altered serum protein levels that could act as potential biomarkers for diagnosing LAM and predicting its clinical outcome over time is a way forward to significantly reduce the time required to make correct diagnoses and avoid delayed management and invasive diagnostic procedures. Additionally, the proteins identified could also identify altered cellular signalling pathways that may, in future, become the target of therapy [137].

Serum analysis provides a convenient source for identifying a dysregulated pathway, mechanism or disease state. A dynamic network of growth factors, fluids and integrins are utilised in the extracellular matrix (ECM) to allow tissue and cell organisation. A significant imbalance in these proteins can impact assembly and disassembly of the ECM [138]. ECM disorganisation is known to play a role in many respiratory diseases such as asthma [139] and also in some other diseases including cancer. Another ECM glycoprotein, fibronectin, is known to be essential for healthy maintenance of the ECM that surrounds cells and tissues. Reductions in serum fibronectin levels and increased deposition in the lung in LAM patients shows an imbalance in its levels (Table 1.2). Tables 1.1 and 1.2 summarise the altered protein level changes in LAM versus healthy adults.

Table 1.1: Differentially expressed proteins identified in LAM patient serum, relative to normal individuals, to be validated as part of this project.

Proteins Significantly Increased in LAM Patient Serum (vs Serum of Healthy Controls)	Protein Molecular Weight (kDa)*	Proteins Significantly Decreased in LAM Patient Serum (vs Serum of Healthy Controls)	Protein Molecular Weight (kDa)*
Apolipoprotein A1	30.78	Heparin cofactor 2	57
Plasma serine protease inhibitor	45.68	Sex hormone binding globulin	43.78
Talin 1	269.77	Lipopolysaccharide binding protein	53.38
N-acetylmuramoyl-L-alanine amidase	35	Fetuin-B	42
Apolipoprotein A4	45.4		
Phospholipid transfer protein (PLTP)	54.74		

*Molecular weight information has been taken from the Uniprot database of proteins (https://www.uniprot.org/).

Table 1.2: Differentially expressed proteins identified in serum samples of LAM patients as compared to normal individuals and validated by ELISA and immunohistochemistry as part of another related research project.

Validated Differentially Expressed Proteins in LAM	Trend in Expression level in LAM vs Normal	Protein Molecular Weight (kDa)*
Fibronectin	Decreased in LAM	262.6
von Willebrand factor	Decreased in LAM	309
Plasminogen	Decreased in LAM	90.6
Kallikrein III	Increased in LAM	71.37

*Molecular weight information has been taken from the Uniprot database of proteins (https://www.uniprot.org/).

(A) Validated differentially expressed proteins

1.14.2.1 Von Willebrand factor (validated)

Von Willebrand factor, which is known for its role in haemostasis, has recently been found to be a marker for acute respiratory distress syndrome and COPD [140]. Von Willebrand factor levels are decreased in children and adults with hereditary and idiopathic pulmonary arterial hypertension and its levels are inversely correlated with the severity of disease [141].

1.14.2.2 Kallikrein 111 (validated)

Kallikrein 111, also known as prostate specific antigen (PSA), is a glycoprotein enzyme belonging to the protease family. It is secreted into semen by epithelial cells of the prostate gland and liquefies the ejaculate to allow for free motility of the sperm [142].

1.14.2.3 Plasminogen (validated)

Plasminogen is converted to its active form, plasmin, by proteolytic cleavage. This protein is a serine protease normally present in the serum where it functions to break down fibrinogen in blood clots [143].

1.14.2.4 Fibronectin (validated)

Animal studies as well as those conducted on patients with epilepsy have shown that levels of fibronectin are increased in brain tissue. Serum and cerebrospinal fluid levels of fibronectin are also elevated in patients with epilepsy, thus pointing towards its significance as a biomarker in these patients [144].

(B) Differentially expressed proteins yet to be validated (as part of this project)

A brief overview of the detected differentially expressed proteins, their function and current knowledge of their value as diagnostic biomarkers for other diseases is outlined. The described following potential biomarkers have not yet been validated by any other technique and are the focus of this study. This research project involves the validation of the following proteins as potential serum-based diagnostic biomarkers for LAM. A brief description of the role(s) of each protein in general, in the body and as potential biomarkers elsewhere is provided.

1.14.2.5 Apolipoprotein A1 (APO A1) and apolipoprotein A4 (APO A4)

Apolipoprotein A4 is a protein, which when in plasma, can be found in either high density lipoprotein (HDL) particles or chylomicrons and can also be freely circulating [145]. It is synthesised in the intestine and then secreted into the lymph. In blood, it plays a role in the absorption and secretion of lipids in the diet. [146]. Apolipoprotein levels have been found to be elevated in patients with prostatic disease. It has been shown to be increased even when the levels of prostate specific antigen were normal. These observations point towards its probable importance as a biomarker for detecting prostatic cancer [147]. Apolipoprotein A4 synthesis and secretion is increased in the intestine when there is an increase in fat absorption, however its precise role in lipid metabolism is not clear. Apolipoprotein A1 levels are associated with risk of atherosclerosis, cardiovascular disease and thrombosis [148].

1.14.2.6 Talin-1

This protein, found in humans [149], is a cytoskeletal protein that activates integrins and is very large in size. It is expressed in the kidney, spleen, liver, stomach, lungs and vascular smooth muscle [150]. Integrins are cell surface receptors that can pass signals across cell membranes in either direction (i.e. into or out of cells) [151]. Talin-1 is upregulated at the mRNA and protein levels in patients with nasopharyngeal carcinoma [152]. Talin-1 promotes adhesion and cancer cell invasion as well as migration [153]. These characteristics indicate that there could be some association with the malignant behaviour of LAM cells and Talin-1 could act as a potential biomarker for LAM.

1.14.2.7 Phospholipid transfer protein (PLTP)

Plasma phospholipid transfer protein (PLTP) helps in the transfer of cholesterol and phospholipids from triglyceride abundant lipoprotein into HDL [154]. In patients with diabetes or those with suspected coronary artery disease, elevation of plasma PLTP corresponds with increased risk of mortality from all causes [155]. Recently it has been found to play a role in inflammation and modulates the proinflammatory effects of $CD4^+$ T cells [156]. It also aids in the provision of phospholipids from circulating lipoprotein to aid pulmonary surfactant release. This is shown by increased production in lung following hypoxia in type II alveolar cells [157].

1.14.2.8 Heparin cofactor 2

Heparin cofactor 2 is a protease inhibitor which inhibits the serine protease thrombin, which has an important role in the pro-coagulation pathway [158]. A study has shown accumulation and elevated levels in rabbit lung tumours [159]. Heparin cofactor 2 increases metastasis in non-small-cell lung carcinoma. Levels of this protein in tissues and plasma are a good means of predicting treatment course in patients with non-small-cell lung cancer (NSCLC) [160].

1.14.2.9 Fetuin-B

Fetuin-B is a protease inhibitor belonging to the cystatin protein super family. It has been shown to play a role in tumorigenesis, angiogenesis and even clotting [161]. One study has used proteomics techniques to show altered levels of fetuin-B in the serum of mice with lymphoma, thereby revealing an association between altered fetuin-B levels and tumour development [162].

1.14.2.10 Sex hormone binding globulin (SHBG)

The role of this glycoprotein is to bind with the sex hormones oestrogen and progesterone [163]. SHBG has high affinity for its receptors found on prostatic cell membranes and it provides supplemental means to steroids that are bound to it to enter cells [164]. Serum levels of SHBG, together with testosterone levels, when low, have a good predictive value for metabolic syndrome and type II diabetes in middle aged men [165]. A breast cancer study conducted to determine the association of SHBG with oestradiol levels showed an inverse relationship, with higher levels of bioavailable oestradiol being associated with lower levels of SHBG [166].

1.14.2.11 Lipopolysaccharide binding protein (LBP)

LBP is a trace plasma protein that binds to the lipid A moiety of bacterial lipopolysaccharide (LPS). It has an amino acid sequence similar to that of cholesterol ester transporting plasma protein and LPS binding protein found in granulocytes and permeability increasing protein [167] and plays a pivotal role in initiating an immune response which occurs due to the fact that, when bound to LBP, bacterial LPS is presented to cell surface receptors [168].

1.14.2.12 N-acetylmuramoyl-L-alanine amidase

This human protein, known as PGRPs-L, is a peptidoglycan recognition protein with a role that is not yet known in humans [169]. PGRP-L is expressed in the liver and, to a lesser extent, in other organs such as intestine, thymus, pancreas, heart and bone marrow [170]. PGRPs are found in fruit flies and other organisms where their function is to recognise bacteria through their peptidoglycan walls [171]. PGRPs also act as enzymes and play a role in the biosynthesis of bacterial peptidoglycan [172].

1.14.2.13 Plasma serine protease inhibitor (gene name: SERPIN A5)

The plasma serine protease inhibitor is synthesised in the liver in humans [173] and then released into the blood where it is found in the form of a single chain glycoprotein [174]. The protein is an inhibitor of protein C when present extracellularly [175]. Plasma protein C is a vitamin K dependent serine protease that plays a role in blood coagulation [176]. A recent study has highlighted LAM signature in serum exosomes which could be potential biomarkers for LAM and may also help in understanding pathogenesis of LAM [177]. Another study has identified Vitamin D binding protein as potential biomarker of severity of LAM [178] which is also a very interesting and relevant discovery.

The expression of the abovementioned proteins has been shown to be altered in LAM patients compared to normal individuals and hence shows their importance as potential biomarkers in LAM. The outlined information has provided some current knowledge of these proteins and their potential role(s) as biomarkers in some respiratory and other conditions. Functions of these proteins extending outside the respiratory domain, is beyond the scope of this study.

1.15 The necessity for biomarkers for LAM and background to the current project

1.15.1 Limitations of current diagnostic procedures for LAM

Current techniques used to establish a diagnosis of LAM have limitations and currently the only definitive mode of diagnosis is tissue biopsy, which is an extremely invasive procedure. It requires a trained surgeon and has morbidity associated with post-operative management due to

increased risk of infections, respiratory failure or even death. Approximately 1% of patients experience post-operative bleeding for which 17.8 % required blood transfusion. The risk of total lung collapse and respiratory failure is almost 15%, of which about 6% subsequently require chest intubation to expand the lung. The rate of death post biopsy is about 4.3%. Tissue biopsy takes more time to plan, perform and reach a diagnosis as compared to less invasive techniques such as radiology. It also requires longer post-operative management. HRCT exposes patients to higher levels of radiation in comparison to X-ray, which is very harmful for humans and therefore is not a suitable means for repeated evaluation for measuring progression or monitoring prognosis when a patient is under treatment. High radiation exposure has been known to be a risk factor for the development of cancer and genetic abnormalities in the offspring of such individuals. Also, a number of other conditions resemble LAM on HRCT and therefore this technique cannot be relied upon solely in the absence of other features required for diagnosis.

1.15.2 Developing new serum-based diagnostic biomarkers for LAM

The forerunner proteomics biomarker study described earlier utilised two small and different cohorts of patients (LAM patients and matched normal individuals). The proteomic findings are interesting and now require validation of the altered protein expression using an alternative means, such as ELISA and/or western blotting, in a LAM cohort compared to normal cohort, to determine if the changes detected by the proteomics study are indeed detected in patient cohorts. Additionally, the previous study only compared protein expression levels in LAM and normal subjects and did not include any other diseases clinically resembling LAM. It is very easy to clinically differentiate LAM patients from normal individuals, rendering this comparison of limited clinical utility unless such molecular markers are identified that can differentiate LAM in early stages before the manifestation of irreversible clinical features The patients selected for this study have been accurately diagnosed using established diagnostic criteria so that there is no doubt of the authenticity of the diagnosis of LAM in the subjects.

1.16 Hypotheses and aims of the project

1.16.1 Hypotheses

1. There will be increased serum apolipoprotein A (1 and 4), plasma serine protease inhibitor (SERPIN A5), talin-1, N-acetylmuramoyl-L-alanine amidase, apolipoprotein A4 and phospholipid transfer protein (PLTP) levels in LAM patients when compared to other respiratory diseases.
2. There will be decreased serum heparin cofactor 2, sex hormone binding globulin, lipopolysaccharide binding protein and fetuin-B protein levels in LAM patients compared to the levels of these proteins in normal individuals and patients with other respiratory conditions.
3. These differentially expressed proteins will be potential diagnostic and/or prognostic biomarkers for LAM.

1.16.2 Aims of the project

The overall aim of this project is to determine the potential of any one, or more, of the detected differentially expressed proteins as serum-based diagnostic or prognostic biomarkers for LAM. This will be accomplished by completion of the following specific aims:

1. To validate the altered expression of the potential biomarker proteins in LAM through ELISA or western blotting experiments, using serum/plasma specimens from LAM patients, normal controls and ILD patients, to ascertain their utility as diagnostic biomarkers.
2. To determine the biomarker specificity for LAM as compared to interstitial lung diseases (ILD) and ultimately develop a diagnostic algorithm.
3. To determine if any correlations exist between biomarkers levels and severity of disease in LAM and ILD.

1.16.3 Significance of the project

The discovery of a sensitive and specific biomarker could improve the quality of life of many patients who have to wait a long time in order to get a definitive diagnosis and also need to

undergo highly invasive diagnostic procedures that carry their own harmful implications. Determining the stability of the identified biomarkers over time could further authenticate the utility of this as a diagnostic and prognostic modality. There is a possibility that other pulmonary disease may be ruled out or even diagnosed using these findings.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 General Materials and Reagents

General materials, reagents and buffers were purchased from various providers which are provided in the following sections as appropriate.

2.1.2 Materials for ELISA analysis

The 96 well microplates used for ELISA assays, phosphate buffered saline (PBS), wash buffer, 0.05% (v/v)Tween 20 in PBS, reagent diluent, 1% (w/v) bovine serum albumin in PBS, substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine) and stop solution were all purchased from R&D Systems Australia. Specific antibodies were purchased from various sources as listed in section 2.1.2.1.

2.1.2.1 Antibodies for ELISA analysis

Choice of antibodies for ELISA was determined by availability, from commercial sources, for specific detection and quantification of the proteins of interest. Available antibodies for ELISA were the following and all antibody information is presented in Table 2.1.

For detection of human lipopolysaccharide binding protein (LBP), the LBP Duoset ELISA development system (manufactured by R&D Systems Australia) was used. It contained human LBP capture antibody, human LBP detection antibody, human LBP standard and streptavidin-HRP (horseradish peroxidase).

For detection of human Fetuin-B, the Human Fetuin-B Duoset ELISA development system (manufactured by R&D Systems Australia) was used. It contained human Fetuin-B capture antibody, human Fetuin-B detection antibody, human Fetuin-B standard and streptavidin-HRP.

2.1.3 Materials for Western Analysis

Sodium dodecyl sulphate (SDS) was purchased from Merck (Australia). Tris glycine electrophoresis buffer (10X), containing Tris base, glycine and SDS and 2X SDS loading buffer were also purchased from Merck. Transfer buffer (25 mM Tris, glycine, 10% methanol), wash solution (PBS with 0.05% Tween 20), blocking solution (5% skimmed milk/ 5% BSA in washing buffer) were purchased from Thermo Fisher Scientific. Acrylamide/bis-acrylamide, TEMED and

ammonium persulfate (APS) were provided by Sigma Chemicals. Immobilon membranes, sandwich and blotting paper were purchased from Merck Millipore (Australia).

2.1.3.1 Antibodies for Western Analysis

The antibodies used in the western detection and quantification of the target proteins were based on commercial availability and proven specificity from literature sources. The antisera were raised against either the whole, or part of, each of the target protein(s) as outlined below.

Anti-heparin cofactor 2 antibody was purchased from Abcam. This antibody is one that had been raised against the full-length native heparin cofactor 2 protein (purified) from human plasma.

The anti-Talin 1 antibody was purchased from Abcam. This antibody had been raised against a recombinant fragment corresponding to amino acid residues 780-850 of Talin 1. A second anti-Talin 1 antibody was also used in this work and was purchased from Merck Millipore (Australia).

Anti-phospholipid transfer protein (PLTP) antibody was purchased from Abcam. This antibody had been raised to a synthetic peptide corresponding to specific N-terminal amino acid residues in human PLTP. An additional anti PLTP antibody also used in this project was purchased from Abcam. This antibody had been raised against a recombinant full length human PLTP (corresponding to amino acids 1-422).

The plasma serine protease inhibitor antibody was purchased from Sapphire Biosciences Pty Ltd. This antibody was raised against the full-length recombinant human protein (derived from the SERPIN A5 gene).

The anti-APO A4 antibody used was purchased from Abcam. This antibody was raised against a synthetic peptide corresponding to a segment of the human APO A4 N-terminus (amino acid residues 49-79).

Anti sex hormone binding globulin antibody was purchased from Abcam. This antibody was raised against a synthetic peptide derived from the N-terminus of the protein (amino acid residues 50-150).

Anti-apolipoprotein A1 (APO A1) antibody was purchased from Abcam. This antibody was raised against a synthetic peptide corresponding to residues near the N terminus of human apolipoprotein A1.

Anti N-acetylmuramoyl-L-alanine amidase (PGRLY2) antibody was purchased from My BioSource. This antibody was raised against peptidoglycan recognition protein purified from human protein.

Secondary antibodies were also used and these included both anti-mouse and anti-rabbit antibodies. These were purchased from R&D Systems Australia.

2.1.4 Protein standards for electrophoresis

The protein standards used for polyacrylamide gel electrophoresis of proteins were the dual color Precision Plus protein standards, which were purchased from Bio-Rad Laboratories, and the spectra multicolored high range protein ladder from Thermo Fisher Scientific. The Precision Plus protein standard contains the following molecular weight bands, in increasing order of molecular weight: 10 kDa, 15 kDa, 20 kDa, 25 kDa, 37 kDa, 50 kDa, 75 kDa, 100 kDa, 150 kDa and 250 kDa. The spectra multicolored high range protein ladder contained the following molecular weight bands: 40 kDa, 50 kDa, 70 kDa, 100 kDa, 130 kDa, 180 kDa, 250 kDa and 300 kDa.

2.2 Methods

2.2.1 Blood biobank establishment and sample collection

Blood sample collection was carried out according to the ethics protocol and was continued in order to expand the biobank intended for use in this study. This biobank contained 14 blood samples from LAM patients. Blood samples were regularly collected from patients attending the Interstitial Lung Disease (ILD) Clinic at Royal Prince Alfred Hospital (Sydney, Australia) over a period of 1.5 years.

Upon collection, samples were processed and stored at the Woolcock Institute of Research, Sydney, Australia. Demographic and diagnostic details of the patients were collected and all

patient details were de-identified. The biobank database was password protected with restricted access. Additionally, patient and clinical details, including severity of clinical features and pharmacological treatments were also recorded.

2.2.2 Study Populations

Approval for this study was granted from the University of Technology Human Research Ethics Committee. The inclusion criteria were that patients needed to have a confirmed diagnosis of LAM and/or ILD (a common form of interstitial lung disease, henceforth referred to as ILD in this thesis) based on the standard diagnostic guidelines for LAM and ILD, as outlined by the European Respiratory Society. Factors such as age, gender, concomitant diseases, severity/stage of disease, medical treatment given and lifestyle factors were not included as criteria for inclusion or exclusion.

Before collecting samples, subjects were informed of all possible adverse effects associated with the venepuncture procedure. All subjects provided written informed consent according to the human ethics guidelines. Patients that were booked into the ILD clinic at Royal Prince Alfred Hospital, at the particular date, were enrolled into the study. After obtaining written informed consent from the patients, blood samples of the individuals recruited for the study were collected and clinical features recorded to estimate the severity of the disease simultaneously. These blood samples were processed and stored in a specialised biobank established for the purpose of this study (Figure 2.1).

2.2.3 Study cohorts and sample processing

To prove the presence of altered protein expression in LAM patients, as compared to those suffering from interstitial lung diseases (ILD) and normal subjects, blood sample collection from these three subject groups was initiated with appropriate institutional human ethics approval. Collected samples were classified into 3 study groups: (i) samples from healthy individuals (n=10), (ii) samples from LAM patients (n=10) and (iii) a pool of samples from patients with ILD (n=10), which represents a group of diseases within which idiopathic pulmonary fibrosis (IPF) belongs (Figure 2.3).

Serum and plasma samples were collected in vacutainers by venepuncture. Samples were inverted three times and then allowed to stand for 40 minutes to clot at room temperature. Samples were then centrifuged at 4000 rpm for 10 minutes. The supernatant (plasma/serum) was collected and the serum and plasma samples were prepared and aliquoted (200 uL) using sterile pipette tips. Approximately 10 serum samples and 10 plasma samples were prepared from each blood sample and stored at -80^{o}C. The storage details and location of each aliquot was recorded in the biobank database.

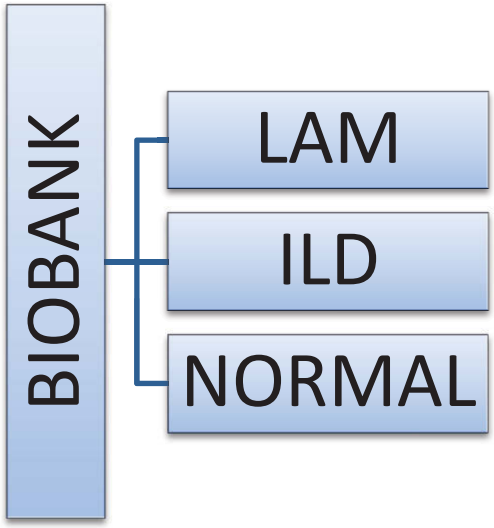

Figure 2.1: Collection and biobanking of samples from the 3 study groups used in this study. The biobank contained serum and plasma samples from normal individuals (n=10), LAM patients (n=14) and ILD patients (n=198).

2.2.4 Study Design and Plan

After obtaining consent and enrolment of the subjects, blood samples were collected, processed and stored at -80^{o}C. Antibodies for western blotting and ELISA were sourced from various locations to be able to detect and quantify the novel candidate proteins of interest (Table 2.1).

The methodological plan is summarised in figures 2.1 and 2.2. After selection of antibodies, respective protocols for western blotting and ELISA were established and optimised prior to any analysis of experimental; samples. Each candidate protein was quantified in the sample cohorts to validate any differential expression between the sample groups (normal vs LAM vs ILD). Clinical features of the patients were to be reviewed within three months of obtaining the samples where feasible. Correlation of clinical features of the subjects with any detected and validated differential protein expression was made where the data was available and allowed such correlations to be made (Figure 2.3).

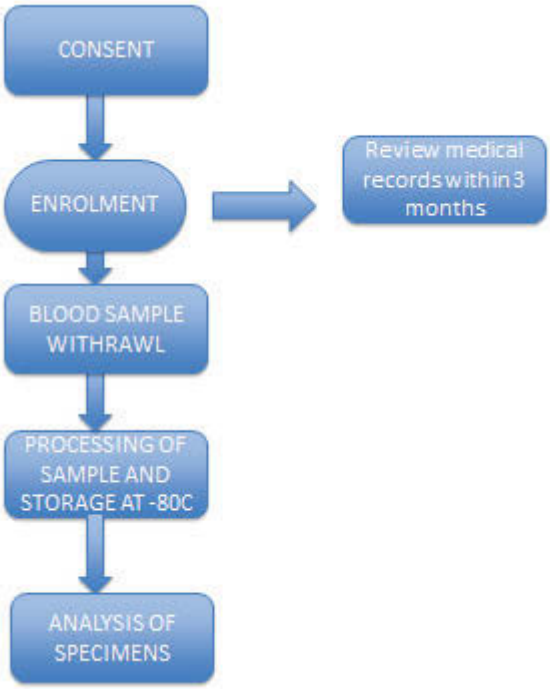

Figure 2.2: Methodological plan for the project. After obtaining informed consent, subjects were enrolled into the study and blood samples collected. These samples were then processed and stored at -80^{o}C to be analysed at a later date.

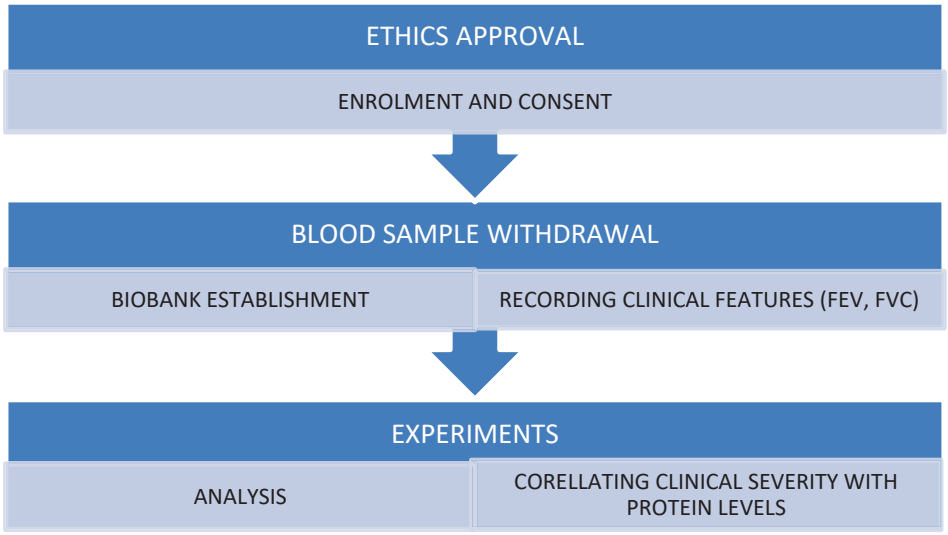

Figure 2.3 Overall project plan. Clinical features recorded at the time of blood sample collection would later be correlated with any differential protein expression, where the data allowed. Key: FEV, forced expiratory volume; FVC, forced vital capacity.

Table 2.1: List of some of the shortlisted ELISA kits available, at the time of commencement of this project, for the quantification of the proteins of interest.

PRODUCT	COMPANY	SAMPLE REQUIREMENT	DETECTION RANGE	REACTIVITY	PRECISION
Human Plasma Serine Protease Inhibitor (product of SERPIN A5 gene)	CUSABIO	Serum / plasma (50-100 uL volume)	0.16-10 ng/mL	human	No information available
Apolipoprotein A4	LifeSpan Bio Sciences	Serum/plasma (100 uL/well)	7.8-500 pg/mL	mouse	Intra-Assay CV <10% Inter-Assay CV <12%
N-acetylmuramoyl-L-alanine amidase (PGLYRP2)	LifeSpan Bio Sciences	Serum/Plasma (100 uL volume)	0.16-10 ng/mL	human	Intra-Assay CV <10% Inter-Assay CV <12%
Talin 1	LifeSpan BioSciences	Serum/plasma (volume not specified)	0.16-10 ng/mL	human	Intra-Assay CV <10% Inter-Assay CV <12%
Serine peptidase inhibitor (heparin cofactor 2)	My Bio Source	Serum/plasma (volume not specified)	1.25-80 pg/mL	human	Intra-Assay CV <8% Inter-Assay CV <10%
Sex hormone binding globulin	R&D	Serum/plasma (volume not specified)	0.06-4 nmol/L	human	No information available
Lipopolysaccharide binding protein	Abnova	Serum/plasma (100 uL volume)	1.5-50 ng/mL	human	Intra-Assay CV <3.5% Inter-Assay CV <5%
Fetuin-B	Biovendor	Serum/plasma (10 uL/well)	0.019 ng/mL	human	Intra-Assay CV <3.7% Inter-Assay CV <5.2%
Phospholipid transfer protein (PLTP)	Bio-compare	Serum/plasma (100 uL volume)	78.125-5000 pg/mL	mouse	No information available
Apolipoprotein A1	Cell Biolabs	Serum/plasma (100 uL volume)	1 ng/mL	human	No information available

2.2.5 ELISA Protocols

The following sections describe the establishment and optimisiation of the ELISA protocols and their use for the quantitative validation of differential expression of the candidate proteins.

2.2.5.1 Preparation of ELISA reagents

All the reagents were prepared according to the manufacturers' instructions and as per protocol. The prepared reagents were aliquoted and stored at -80ºC until required. The steps involved in preparation are detailed in subsequent sections.

Reconstitution of ELISA reagents

All plasticware was sterilised at 121^{o}C for 20 minutes in an autoclave and glassware heated at 180^{o}C for 2 hours before use. PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH_2PO_4) was prepared and filtered before use.

Streptavidin-HRP was diluted to working concentrations, as specified by the manufacturer, using reagent diluents (1% BSA in PBS). Human LBP capture antibody (240 ug/ml) was subjected to serial dilutions, after initial reconstitution with 0.5 ml of PBS, until the desired dilution of 2.0 ug/ml was obtained. Human LBP detection antibody (4.5 ug/ml was reconstituted using 1.0 ml of 1% BSA in PBS (reagent diluents) and then further diluted to a working concentration of 75 ng/ml. 6 aliquots each of both antibodies were prepared with 83 ul of capture antibody solution and 167 ul of detection antibody solution in each aliquot to be used immediately. Human LBP standard (1150 ng/ml) was first reconstituted with 0.5 ml of 1% BSA in PBS. Then 2-fold serial dilutions were performed with reagent diluents using a 7 point standard curve as depicted in figure 2.4.

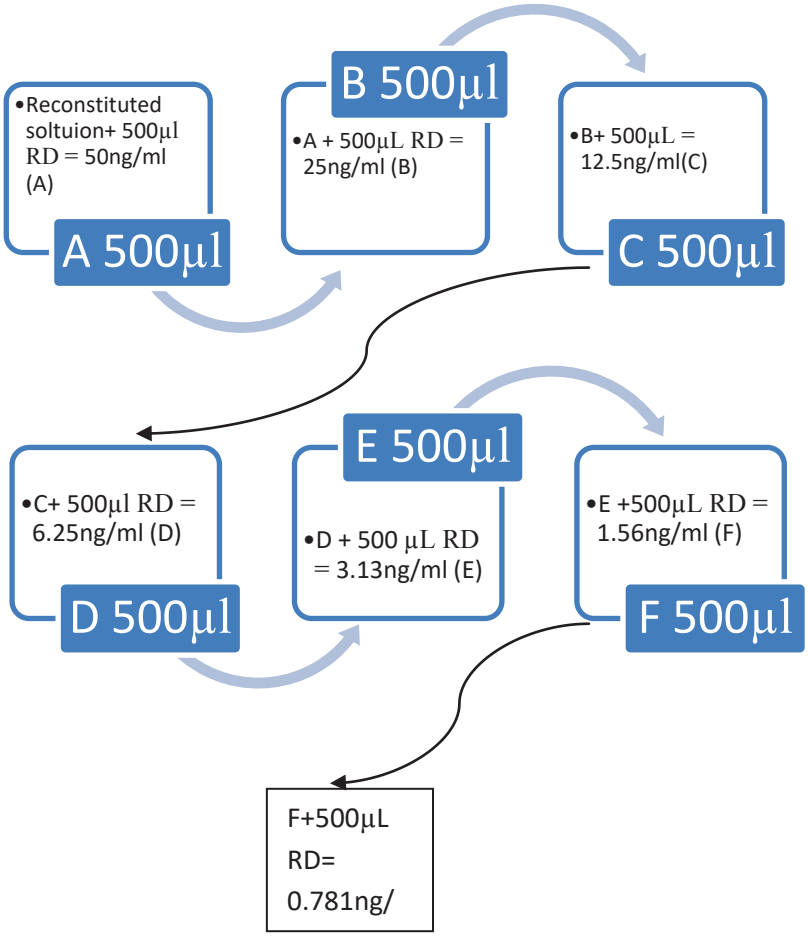

Figure 2.4: Preparation and dilution of the LBP standard for ELISA analysis. Serial dilutions of the standard were made until the required concentration was obtained, RD stands for reagent diluent which is 1% BSA in PBS. Ultimately the final concentration required (0.781 ng/ml) is obtained. A, B, C, D, E, F is the diluted solutions shown in a series.

The Fetuin-B capture antibody (720 ul/ml) was reconstituted using 1 ml of PBS. 17 aliquots were prepared (with 56 ul in each) and stored at -80^{o}C. In order to make the working concentration, it was further diluted with PBS to a concentration of 4 ug/ ml. Fetuin-B detection antibody (360 ug/ml) was diluted with 1 ml of PBS. 17 aliquots, each containing 56 ul of solution were stored at -80oC. Immediately before use, it was brought to a working concentration of 2 ug/ml. Fetuin-B standard was reconstituted with 0.5 ml of reagent diluent and brought to its working concentration immediately before usage.

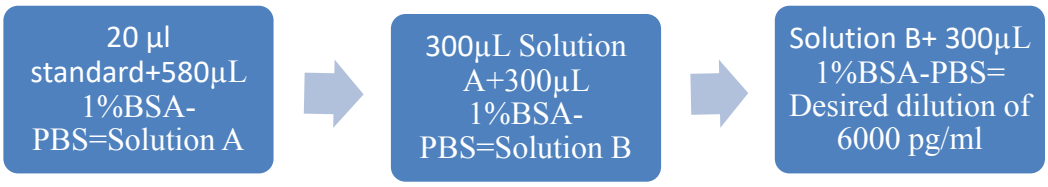

Figure 2.5: Schematic of the protocol for preparing the 2-fold serial dilutions required to obtain the final concentration of standard for Fetuin-B. A and B are the diluted solutions.

2.2.5.2 ELISA protocol optimisation

The protein targets that were analysed using ELISA were Fetuin-B and LBP. In order to validate the protocol for Fetuin-B detection and quantification, the following steps were performed in the order described below.

Preparation of ELISA plates

After diluting the capture antibody to the working concentration (56 ul of 720 ug/ml concentration was added into 10 ml of PBS), immediately before performing the experiment, all well of a 96 well microplate were coated with 100 ul of capture antibody using multichannel pipettes. This microplate was sealed and incubated overnight at 4ºC.

On the following day, each well was aspirated and washed three times with 400 ul of wash buffer (0.05% Tween 20 in PBS). The microplate was inverted and blotted against paper towels after each wash. The wells were then blocked by the addition of 300 ul per well of reagent diluents. Plates were then sealed and incubated at room temperature for one hour, after which they were washed again three times as before. Following this, 100 ul per well of standards and samples were added to the plates in duplicate. Standards were diluted in reagent diluent (180 ng/ml into 0.5 ml of 1% BSA in PBS). Serial dilutions (2 fold) were performed for standards (Figure 2.2).

2.2.5.3 Protocol for sample testing using ELISA

For sample testing, samples were diluted 20 times (by adding 5 ul of sample into 95 ul of reagent diluent) before placing in wells. Plates were then sealed and incubated at room temperature for 2

hours. Plates were then washed three times using wash buffer according to the protocol. 100 ul of diluted detection antibody (56 ul of a 360 ug/ml solution was added to 10 ml 1% BSA in PBS) was added to each well of the microplate and plates were covered, sealed and incubated for 2 hours at room temperature. After this, plates were again washed three times. Streptavidin was brought to a working concentration of 1:200 by adding 100 ml into 20 ml of 1% BSA in PBS to make solution A, and then adding 50 ul of solution A to 10 ml of 1 % BSA. 100 ul of streptavidin was added per well after washing. The plates were then covered and left to incubate at room temperature for 20 minutes, away from direct sun light. Plates were again washed three times with wash buffer and 100 ul of substrate solution, tetramethlybenzidine (TMB), was added per well. The plates were then left to incubate for 20 minutes at room temperature, avoiding light. In the final step, 50 ul/well of stop solution ($2NH_2SO_4$) was added and plates were gently tapped to ensure thorough mixing. The optical density of each well was immediately determined at 540 and 570 nm using a microplate reader.

Estimation of LBP was carried out using the described procedure but with some differences in the sample concentrations. All the experiments included quantitative standards and all samples were analysed in duplicate.

For optimisation of Fetuin-B, normal samples were initially tested at a 1/20 dilution, but to determine the best working concentration, samples were tested using 1/50, 1/100, 1/200 and 1/1000 dilution. Sample dilutions were prepared as shown in Table 2.2.

Table 2.2. Serial dilutions carried out in order to obtain the various serum concentrations for ELISA. The listed concentrations were used to identify the most optimal serum concentration.

Serum Volume	**Reagent Diluent Volume (µl)**	**Dilution Obtained**
10 µl serum	70	1/8 dilution
50µl of 1 in 8 dilution	450	1/50 dilution
250µl of 1 in 50 dilution	250	1/100 dilution
200µl of 1 in 100 dilution	200	1/200 dilution
100µl of 1 in 200 dilution	400	1/1000 dilution

It was determined that the 1/1000 sample dilution worked the best for the samples and this dilution was used to perform the ELISA experiments as shown in results section. For the experiments, human serum samples were used from each disease group (LAM and ILD) and from normal subjects.

For human LBP, ELISA optimisation showed that the 1/30 dilution gave the best outcome compared to all the other dilutions.

2.2.5.4 ELISA method and optimisation

ELISA was employed to validate differential expression of Fetuin-B and LBP. The ELISA methodology was optimised for antibody concentrations prior to conducting the quantitative analysis on patient samples. Optimisation is an essential step as it determines the actual conditions and concentrations of reagents that work best to give optimal and reproducible results. This step is described in the later sections in the thesis.

Plate wells were coated with equal volume of capture antibody, after diluting it to the working concentration, and incubated overnight. Desired sample dilutions were prepared after identification of most optimal serum dilution following initial optimisation experiments. Blocking agents were then put in wells. Equal volumes of standard and samples were put in each well with various washing steps in between. After that detection antibody was put in each well in equal volume using calibrated pipettes. Then streptavidin was added to each well and after washing followed by substrate solution TMB. Again, there were washing steps in between. In the end stop solution was added to each well.

ELISA quantification was carried out with the standards provided with the ELISA antibody kit(s). Absorbance detection for each well was carried out using a microplate reader and proteins were quantified against the standards provided.

2.2.6 Western blot protocols for optimisation and validation

All remaining protein candidates were validated using western analysis after establishing and optimizing the protocols as outlined in the following sections.

2.2.6.1 Preparation of the stacking and analysis/separating gels

The stacking and separating gels were prepared manually and allowed to set for at least 2 hours prior to usage. 20 ml of polyacrylamide gel solution was prepared using 5 ml each of 4X SDS/Tris and Acrylamide/Bis (40% Bio-Rad) and 9.78 ml H2O. To this was added 20µl TEMED and 200 µl 10% APS. After cleaning with alcohol and assembling the equipment, the gel solution was poured into gel frame to a level approximately 1.5 cm from the top of the plate. 500µl of butanol was added on top this layer to remove any water and flatten the gel. This gel was allowed to set for 30 minutes, after which the butanol was removed. The 4% polyacrylamide stacking gel was then prepared using 2.5 ml 4X SDS-Tris, 1 ml acrylamide/Bis and 6.5 ml H2O, to which was added 10 µl TEMED and 100 µl 10%APS. The stacking gel was poured into the gel frame and the gel combs were inserted. The stacking gel was allowed to set for at least 30 minutes before removing the combs.

2.2.6.2 Polyacrylamide gel electrophoresis of proteins

5X SDS electrophoresis running buffer was prepared using 0.125 M Tris-Base (15.1g), 0 .96 M Glycine (72.0 g), 0.5% w/v SDS (5g), H2O water was added to make 1 liter. This solution was diluted 5 times (to 1X concentration) before use. 2X SDS loading buffer was prepared using 400 mM Tris-HCl (3.125 g), 5% SDS (2.5 g), 20% Glycerol (10 ml), 0.006% Bromophenol blue (0.003 g) and H2O to make 50 ml total volume. Required dilutions of samples were prepared in the loading buffer and dye added to each sample. These samples tubes were then heated at 95ºC for 5 minutes to denature proteins and 10 µL of each sample were loaded on to the gel. Samples were electrophoresed at either 125 Volts (V) for 1.5 hours or at 80 V for the first 30 minutes and then at 120 V for 1 hour.

2.2.6.3 Transfer of proteins to membranes for western analysis

10X Tris/glycine transfer buffer was prepared using 250 mM Tris-Base (30.2 g), 1920 mM Glycine, (144 g) and H2O was added to make up 1 liter. 100 ml of this buffer was taken and 100 ml of methanol added to it along with 750 ml of water. This buffer was poured into the buffer tank till the desired level. Immobilon PVDF membranes were activated by immersing in 100% methanol. The gel holding cassettes for transfer were prepared by placing wet foam pads on two sides with two wet blotting papers in between. These were wetted using transfer buffer. The

activated membrane was placed on one of the wet blotting papers and gel placed on top of it. More blotting paper and a foam sheet are placed on to the gel to complete the sandwich. It was immediately immersed in transfer buffer and placed in the buffer tank in which a cooling unit had been placed in order to prevent overheating. The transfer process was allowed to occur at 25 V, 400 mA for 2 hours (for 2 tanks) and at 200 mA for 1 hour (for 1 tank).

2.2.6.4 Post-transfer blocking of PVDF membranes

Blocking solution was prepared using 5% dry skim milk in T-TBS (5 g/100 ml). After the completion of the transfer process, PVDF membranes were carefully removed from the gel cassettes and blocked immediately by placing in 10 ml of blocking solution per membrane for 1 hour at room temperature with gentle agitation.

2.2.6.5 Washing of membranes and addition of primary antibody

Membranes were washed once with T-TBS and placed in the dishes containing 10 ml of 2% BSA-TBS-T (0.2 g/10 ml) each. Now the membrane was exposed to a specific concentration of primary antibody. For optimization purposes, this experiment was repeated using 6 different concentrations of primary antibody in order to obtain the best working concentration. After addition of primary antibody, the dishes were covered and placed on a shaker overnight at 4ºC. Variable antibody concentrations were achieved by specific dilutions (Table 2.3).

Table 2.3 Preparation of the various primary antibody dilutions used in the optimisation of the western blotting experiments for each protein.

Primary antibody dilution	Dilution achieved
20 µl primary antibody (PA) +10 ml 2% BSA-TBS-T	1:500
10 µl PA +10 ml 2% BSA-TBS-T	1:1000
6.6 µl PA+10 ml 2% BSA-TBS-T	1:1500
5 µl PA+10 ml 2% BSA-TBS-T	1:2000
4 µl PA+10 ml 2% BSA-TBS-T	1:2500
3.3 µl PA+10 ml 2% BSA-TBS-T	1:3000

2.2.6.6 Membrane washing and addition of secondary antibody

The membranes were then washed 5 times, for 3 minutes each wash, with T-TBS. The membranes were then exposed to secondary antibody at a dilution of 1:2000. Secondary antibody type used, whether anti mouse or anti rabbit, was according to the protein type being analysed and was varied with different proteins used (Table 2.4). Membranes were kept immersed in 5 µl secondary antibody in 10 ml of 2% BSA-TBS-T for 1 hour. After 1 hour the membranes were washed with T-TBS for 3 minutes. The washing step was repeated 5 times with 3 minutes duration each time. After washing, the membranes were kept in TBS-T. Membranes (blots) were now ready to be visualized. The secondary antibodies used for particular proteins are summarised in Table 2.4.

Table 2.4: Secondary antibodies used for each primary antibody being validated. PLTP represents phospholipid transfer protein, SERINA5 represents plasma serine protease inhibitor, SHBG represents sex hormone binding globulin and APO stands for Apolipoprotein.

Protein to be analysed	Specific secondary antibody used
Anti PLTP	Anti mouse antibody
plasma serine protease inhibitor	Anti mouse antibody
SHBG	Anti rabbit antibody
APO A1, APO A4	Anti rabbit antibody
Heparin cofactor 2	Anti mouse antibody
Talin 1	Anti mouse antibody
N-acetylmuramoyle-L-alanine amidase	Anti mouse antibody

2.2.6.7 Chemiluminescent detection of proteins

An enhanced chemiluminescent system (ECL) was used for the chemiluminescent detection of the proteins according to the manufacturer's instructions. The chemiluminescent reagent system was prepared by mixing both the luminal reagent and oxidizing reagent in equal volumes. It was then immediately poured onto the membrane and incubated for 1 minute. 0.125 ml of ECL

reagent mixture was used per cm^2 of membrane. Excess chemiluminescent reagent was removed with blotting paper and the membrane was placed on a propylene sheet protector on the Kodak imaging station. The exposure time was optimized and repeated for 3-5 minutes for all the proteins and images were taken and analysed using Care Stream Molecular Imaging software.

2.2.6.8 Western Blot optimisation and patient sample analysis

Western analysis was used to validate the differential expression of SHBG, PLTP, plasma serine protease inhibitor, Talin 1, Heparin cofactor 2, N-acetylmuramoyl-L-alanine amidase, APO A1 and APO A4. The choice of western analysis for these proteins was used due to non-availability of reliable ELISA kits for the target proteins. Once selected, experiments were carried out to establish optimal protocols for the quantification of each of the abovementioned proteins in the 3 sample cohorts.

After preparation of polyacrylamide gels, diluted serum, from each of the samples to be analysed, was loaded in each well in equal volumes using calibrated pipettes. Prior to loading, the serum was stained and incubated at 95^oC for 5-10 minutes. A separate protein standard for comparison was not required as the same volume of serum was added into each well in all cases. Serum dilutions of 1/50, 1/100, 1/500 and 1/100 were used depending on the protein being analysed/quantitated. After electrophoresis, proteins were transferred onto immobilion membranes which had been activated by dipping in 100% methanol. The membranes were then exposed to different primary antibody concentrations of 1/3500, 1/3000, 1/2500, 1/2000, 1/1500, 1/1000, 1/500 to determine the most optimal primary antibody concentration for the detection of each protein of interest. Membranes were then blocked with blocking agents and exposed to secondary antibody, followed by a series of washing steps. Finally, the membranes were visualised using chemiluminescence detection and visualisation of the protein bands using a Kodak camera station. Bands corresponding to the detection of the correct molecular weight protein(s) (as listed in Table 1.1) were determined by comparing with the standard protein ladder present on the gel and the membrane.

From the chemiluminescent images, the intensity of each band was determined using densitometric analysis with the Care Stream Molecular Imaging software. Band intensity determination was standardised by the selection of a defined window of dimensions 0.5 cm x

0.75 cm (i.e. 0.5 cm^2). Band intensity was measured and given arbitrary units of pixels/0.5cm^2. The intensity of the band for a particular protein was then compared across the 3 different sample groups for which equal volumes of serum had been loaded. In all cases, samples were analysis was repeated 3 times in order to ensure the data obtained was consistent.

2.2.7 Data handling and data analysis

Whether estimated by ELISA or western analysis, the expression level of each protein target was compared across the three-sample cohort (normal, LAM and ILD). In each case, the results for each protein are presented as the means ± SD for each sample cohort. Two tailed students' T-Test was used to determine if the protein levels were significantly different between two sample cohorts, with a $p<0.05$ indicating significance. Correlations between severity of ILD and LAM with differential protein expression were determined using Pearson's correlation. All statistical analyses were conducted using GraphPad Prism (version 7.04).

CHAPTER 3

RESULTS 1

VALIDATION OF POTENTIAL SERUM BIOMARKERS FOR LAM DIAGNOSIS

3.1 Introduction

As outlined in the background and aims (chapter 1), the identified marker proteins were to be validated for their potential as biomarkers for LAM using either ELISA or western blot techniques with appropriate antibodies. Routine collection of new blood samples was from the Royal Prince Alfred Hospital (RPA) Interstitial Lung Diseases (ILD) Clinic in Sydney, Australia. Collected samples were processed and aliquots stored at -80^{o}C for use in this project. Western blotting and ELISA techniques for the detection and quantification of target proteins were established and optimised. Optimisation of experimental conditions for the analysis and validation of the 10 protein targets (both ELISA and western as relevant) were performed as part of this project.

The following sections describe the results obtained after protein validation. The markers selected for validation, and the method(s) to used are summarised in table 3.1

Table 3.1 Summary of the target proteins to be validated for differential expression and the techniques employed for validation of each candidate protein.

Protein to be validated for differential expression	**Validation technique employed***
Fetuin-B	ELISA
LBP	ELISA
plasma serine protease inhibitor	Western Blotting
PLTP	Western Blotting
SHBG	Western Blotting
APO A4	Western Blotting
APO A1	Western Blotting
Heparin cofactor 2	Western Blotting
PGLYRP2	Western Blotting
Talin 1	Western Blotting

*All analyses were conducting on serum, or diluted serum, samples collected from individuals from within the three study populations (normal, LAM and ILD)

3.2 Selection of differentially expressed proteins and validation of differential expression using ELISA and western analysis

In order to identify potential blood-based biomarkers for a disease, protein quantification from blood samples derived from a disease cohort is compared with that of a control cohort. One of the emerging techniques to perform such quantifications is mass spectrometric based proteomics. This technique can assesses quantitative differences in proteins in different sets of samples, as well as identify the proteins showing altered abundance. In previous work in our laboratory, mass spectrometry based proteomics identified the differential expression of 14 proteins in LAM patient serum. Among various techniques available for protein quantification, enzyme-linked immunosorbent assay (ELISA) and western blot analysis are the most common techniques used to validate protein expression levels post proteomic analysis. In order to investigate the detected potential protein biomarkers in LAM, the differential expression of these proteins need to be validated using ELISA in the first instance. ELISA was selected as the preferred technique of analysis and western blot analysis was used in the cases where reliable ELISA analysis kits were unavailable for the particular protein.

ELISA can be used to detect the presence, and or quantitate the amount of a protein in a sample using a specific antibody for the target protein. In ELISA an unknown sample, containing the protein of interest, is affixed to a surface, and then a specific antibody is added over the surface so that it can bind to the protein (or vice versa). The antibody used is linked to an enzyme, and in the final step a substrate specific for that enzyme is added and the reaction generates a detectable signal, which is then measured. Western blot analysis is a technique that detects proteins in a given sample using a specific antibody to that protein. It involves protein electrophoresis, transfer of separated proteins to a membrane and the use of a specific antibody to detect the protein of interest, usually by chemiluminescent detection.

ELISA has some advantages over western blotting in that ELISA is usually a quicker procedure, is quantitative and has greater sensitivity. ELISA is more specific for the target protein because western blot uses one specific antibody against the target whereas ELISA usually uses two specific antibodies in a sandwich format. Also, more samples (up to 40-80) can be analysed at one time with ELISA as compared to western blotting in which usually only 10-15 samples can

be analysed at one time. Western blotting also takes a significant amount of time and performance to optimize conditions for each protein before starting the analysis.

The first task was to investigate the availability of commercially available ELISA kits for the proteins of interest. There were not many options for the availability of kits for most of the proteins to be validated. Most of the kits that were found had not been used extensively and there were no previous publications in which the kits had been used, making their reliability somewhat questionable (Table 2.1). Therefore, as a start, ELISA kits from R&D (Human LBP Duoset and Human Fetuin-B Duoset, were tested. Western blot analysis was to be used for the rest of the proteins.

The number of subjects in each study group was limited to ten, as mentioned earlier, partly due to limited numbers of diagnosed LAM patients available in Australia and across the world. If these criteria were also considered this would make recruitment of sufficient LAM patients a very difficult and could not be achieved within the time frame of the study.

3.2.1 Optimisation of ELISA and Western Blot prior to validation of altered protein expression

This section is further sub divided into two separate sections: one for ELISA and one for western blotting.

3.2.1.1 Optimisation of the ELISA technique prior to validation of differential protein expression

The ELISA optimisation protocols were established for the experiments. Initially, the ELISA protocols were tested using interleukin-6 (IL-6) and interleukin-8 (IL-8) as protocols for the detection and quantitation of these proteins were already established and well known in the laboratory.

It was determined that the 1/1000 sample dilution worked the best for Fetuin-B in all the samples and this dilution was used to perform the ELISA experiments. The ‘best’ dilution/concentration was that which produced detectable and reproducible (i.e. at least 3 replicates per sample and at

least 3 repetitions of the quantitation experiments) ELISA readings. These criteria were used to determine the optimal serum dilutions for all ELISA quantitation experiments.

Using the same criteria for LBP, the best working serum concentration was a 1/30 dilution. The step wise dilutions and procedures are given in the materials and methodology section.

To conclude here, for Fetuin-B, the best dilution was 1/1000 and for LBP, the best was 1/30 and these dilutions were used for all samples that subsequently used for ELISA from this point onwards.

3.2.1.2 Optimisation of Western blotting technique prior to validation of altered protein expression

Western detection protocols were initially validated using fibulin 1 protein for which conditions for optimal detection were already known. Due to the non-availability of reliable ELISA kits for some of the target proteins, western blot analysis was used instead. Overall, western analysis was used to estimate 8/10 target proteins. Following sections describe how each protein was optimized by testing under different conditions in order to obtain most optimal conditions for the final validation experiments. The optimal conditions to detect these proteins as identified here are summarized in table 3.2.

3.2.1.2.1 Optimisation of plasma serine protease inhibitor

For plasma serine protease inhibitor levels, Serpin A5/Protein C inhibitor antibody (catalogue number NBP2-01833) from Novus Biologicals USA was chosen. This is a monoclonal human anti mouse IgG1 antibody. In order to determine the optimal conditions for western blotting, a series of experiments were performed using different concentrations of serum and with different primary antibody concentrations. In this case, serum was diluted 1/50, 1/100, 1/500 and 1/1000, and these dilutions were tested by electrophoresis and the relevant primary antibody was applied at a 1/1000 concentration. To optimise it further, all of the abovementioned serum concentrations were tested with more primary antibody concentrations of 1/2000, 1/2500 and 1/3000. With all the optimisation experiments, the secondary antibody concentration was consistently kept at the same dilution of 1/2000. Multiple optimization experiments were performed using different conditions until the most suitable conditions were found at which the protein visualisation

appeared maximal. Briefly described in the subsequent section are some of the optimisation experiment results.

The optimisation experiments were carried out using all the above mentioned serum concentrations and primary antibody concentrations. Plasma serine protease inhibitor levels were then estimated in human serum samples from all the three groups (LAM, ILD and normal). The most optimal conditions for the estimation of plasma serine protease inhibitor were determined to be the one with serum concentration of 1/500 and primary antibody concentration of 1/3000.

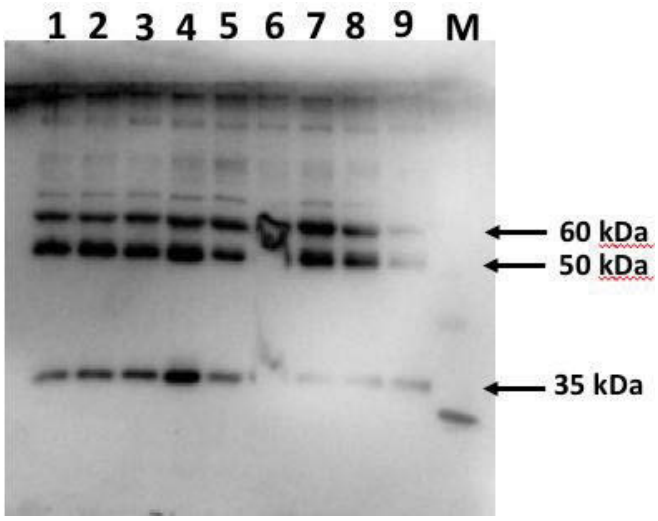

Figure 3.1: Optimisation of detection and western analysis of plasma serine protease inhibitor at 1/500 serum dilution and 1/2000 antibody dilution. Serum proteins were electrophoresed on SDS PAGE gels and transferred to a membrane for western analysis of plasma serine protease inhibitor. Equal volumes of serum (diluted 1/500) were subjected to protein electrophoresis and western analysis using an antibody for plasma serine protease inhibitor (1/2000 dilution) with expected molecular weight of 35 kDa. Key: Lanes 1-3, contain normal samples; Lanes 4-6, ILD patient samples; Lanes 7-9, LAM samples; Lane M, protein molecular weight marker.

3.2.1.2.2 Optimisation of PLTP for western blotting

Anti-PLTP antibody ab57273 (catalogue number ab57273) from Abcam was used according to the manufacturer's guidelines. It is a monoclonal anti-mouse antibody. Multiple optimisation experiments were performed as previously described (including a series of primary and secondary antibody dilutions) to find the most suitable conditions. Although the relevant band appeared at the correct place (here 55kDa), there were other concurrent multiple bands also present, most prominent at 76 kDa and 50 kDa (Figure 3.2, Figure 3.3). Despite omitting the primary antibody application step in the experiments, the additional bands were still present, albeit more faintly. The reasons for the appearance of multiple bands could therefore not be identified. An alternative PLTP antibody was then utilised to determine if the primary antibody used here might be non-specific.

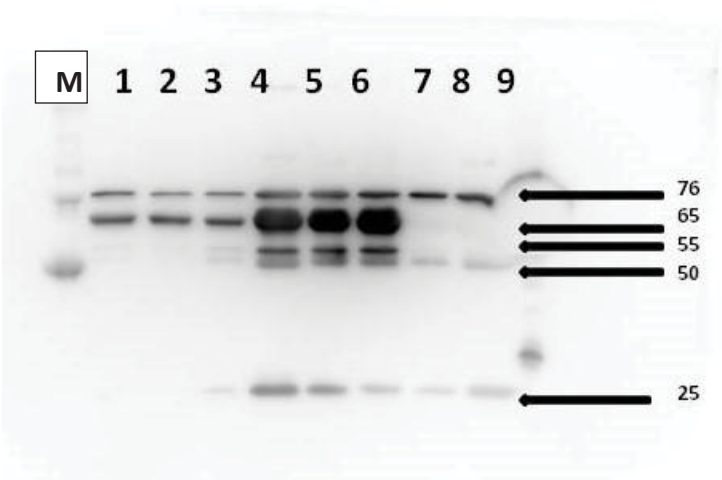

Figure 3.2: Optimisation of detection and western analysis of PLTP at 1/1000 serum dilution and 1/2000 antibody dilution. Serum proteins were electrophoresed on SDS PAGE gels and transferred to a membrane for western analysis of PLTP. Equal volumes of serum (diluted 1/1000) were subjected to protein electrophoresis and western analysis using an antibody for PLTP (1/2000 dilution) with expected molecular weight of 55-65 kDa. Key: Lane M, protein molecular weight marker, Lanes 1-3, LAM patient samples; Lanes 4-6, ILD patient samples; Lanes 7-9, normal samples.

In order to determine the optimal conditions for western blotting for this new antibody, a series of experiments were performed as before, varying serum/plasma and primary antibody concentrations. Multiple bands were seen with almost all conditions, despite changing the antibody (Figure 3.3).

During the optimisation steps multiple bands were observed on the membrane with almost all the concentrations. In an attempt to eliminate this issue, further serum dilutions were used with different primary antibody dilutions to minimize the chances of nonspecific protein binding. Additionally, various other measures were taken to eliminate the problem as discussed in the results section. Ultimately, the conditions of the western analysis were optimised to a level where the additional bands, although present, were very faint, relative to the target band, allowing quantitative analysis. Hence, PLTP concentration was estimated in human serum samples from all the three groups (LAM, ILD and normal) using the initial antibody from Abcam.

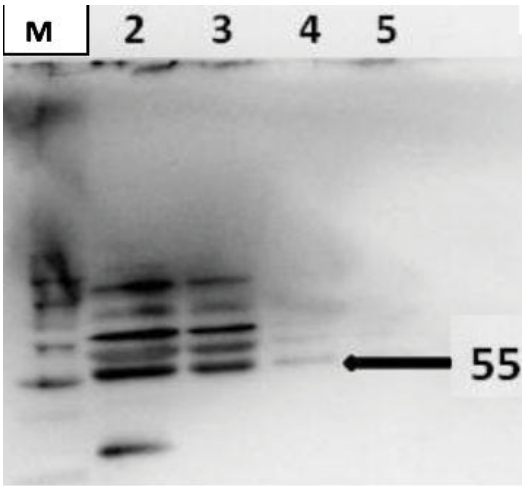

Figure 3.3: Optimisation of detection and western analysis of PLTP at varying serum dilutions with 1/2000 antibody dilution. Serum proteins were electrophoresed on SDS PAGE gels and transferred to a membrane for western analysis of PLTP. Equal volumes of serum (diluted to 1/50, 1/100, 1/500, 1/1000) were subjected to protein electrophoresis and western analysis using an antibody for PLTP (1/2000 dilution) with expected molecular weight of 55-65 kDa. Key: Lane M, protein molecular weight marker; Lane 2, normal sample (1/50 dilution); Lane 3, normal sample (1/100 dilution); Lane 4, normal sample (1/500 dilution); Lane 5, normal sample (1/1000 dilution).

3.2.1.2.3 Optimisation of SHBG for Western blotting

Following the standard optimisation protocol, the most optimal conditions estimated for the detection of SHBG were those with serum concentration at 1/100 dilution and primary antibody concentration at 1/3000 dilution (Figure 3.5).

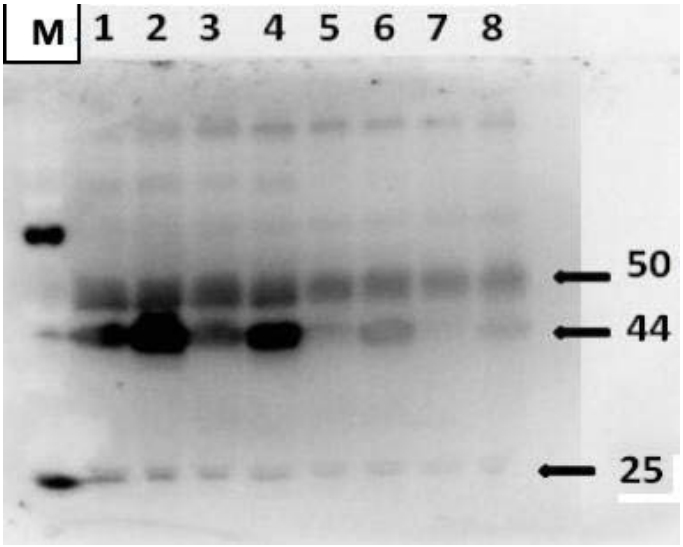

Figure 3.4: Optimisation of detection and western analysis of SHBG at varying serum dilutions. Serum proteins were electrophoresed on SDS PAGE gels and transferred to a membrane for western analysis of SHBG. Equal volumes of serum (diluted to 1/50, 1/100, 1/500, 1/1000) were subjected to protein electrophoresis and western analysis using an antibody for SHBG (1/2000 dilution) with expected molecular weight of 44 kDa. Key: Lane M, protein molecular weight marker; Lanes 1-2, normal samples (1/50 dilution); Lanes 3-4 normal samples (1/100 dilution); Lanes 5-6, normal samples (1/500 dilution); Lanes 7-8 normal samples (1/1000 dilution).

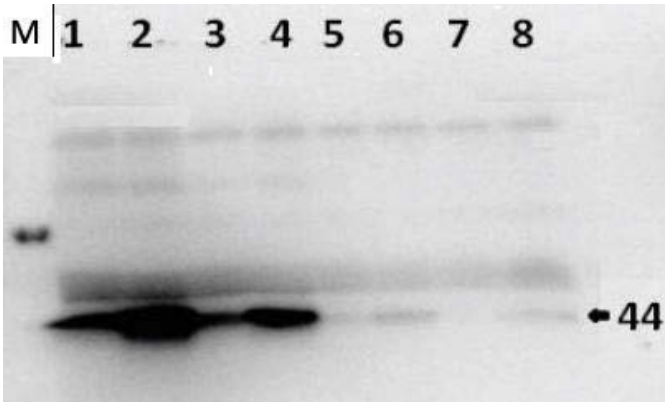

Figure 3.5: Optimisation of detection and western analysis of SHBG at varying serum dilutions with 1/3000 antibody dilution. Serum proteins were electrophoresed on SDS PAGE gels and transferred to a membrane for western analysis of SHBG. Equal volumes of serum (diluted to 1/50, 1/100, 1/500, 1/1000) were subjected to protein electrophoresis and western analysis using an antibody for SHBG (1/3000 dilution) with expected molecular weight of 44 kDa. Key: Lanes 1-2, contain Normal samples (1/50 dilution); Lanes 3-4 contain Normal samples (1/100 dilution); Lane 5-6 contain Normal samples (1/500 dilution); Lane 7-8 contain Normal samples (1/1000 dilution) Lane M, protein molecular weight marker

3.2.1.2.4 Optimisation of APOA4 for Western blotting

In order to find out the optimal conditions for western blotting, a series of experiments were performed using different concentrations of serum/plasma and with different primary antibody concentrations (Figures 3.6, 3.7 and 3.8). The most optimal conditions estimated were the one with serum concentration of 1/1000 and primary antibody concentration of 1/3000 for (Figure 3.6).

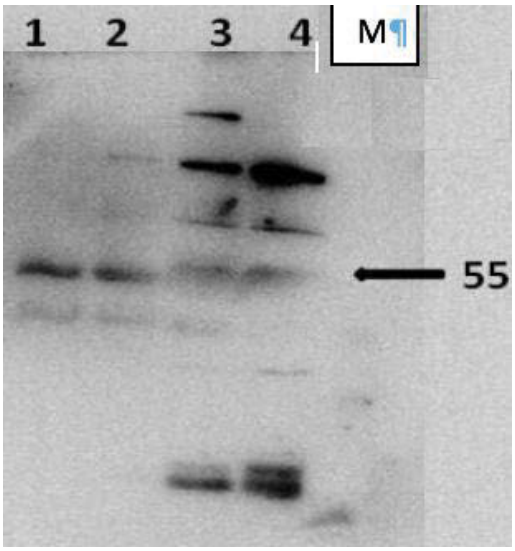

Figure 3.6: Optimisation of detection and western analysis of APOA4 at varying serum dilutions with 1/3000 antibody dilution. Serum proteins were electrophoresed on SDS PAGE gels and transferred to a membrane for western analysis of APOA4. Equal volumes of serum (diluted to 1/1000, 1/500, 1/100, 1/50) were subjected to protein electrophoresis and western analysis using an antibody for APOA4 (1/3000 dilution) with expected molecular weight of 45-55 kDa. Key: Lane 1, normal sample (1/1000 dilution); Lane 2, normal sample (1/500 dilution); Lane 3, normal sample (1/100 dilution); Lane 4, normal sample (1/50 dilution); Lane M, protein molecular weight marker.

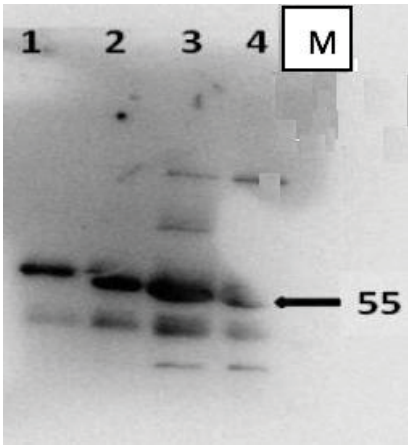

Figure 3.7: Optimisation of detection and western analysis of APOA4 at varying serum dilutions with 1/2000 antibody dilution. Serum proteins were electrophoresed on SDS PAGE gels and transferred to a membrane for western analysis of APOA4. Equal volumes of serum (diluted to 1/1000, 1/500, 1/100, 1/50) were subjected to protein electrophoresis and western analysis using an antibody for APOA4 (1/2000 dilution) with expected molecular weight of 45-55 kDa. Key: Lane 1, normal sample (1/1000 dilution); Lane 2, normal sample (1/500 dilution); Lane 3, normal sample (1/100 dilution); Lane 4, normal sample (1/50 dilution); Lane M, protein molecular weight marker.

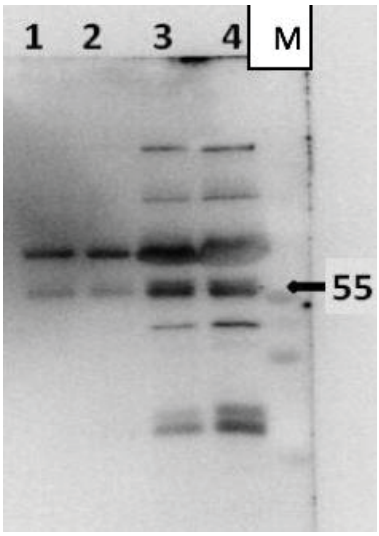

Figure 3.8: Optimisation of detection and western analysis of APOA4 at varying serum dilutions with 1/1000 antibody dilution. Serum proteins were electrophoresed on SDS PAGE gels and transferred to a membrane for western analysis of APOA4. Equal volumes of serum (diluted to 1/1000, 1/500, 1/100, 1/50) were subjected to protein electrophoresis and western analysis using an antibody for APOA4 (1/1000 dilution) with expected molecular weight of 45-55 kDa. Key: Lane 1, normal sample (1/1000 dilution); Lane 2, normal sample (1/500 dilution); Lane 3, normal sample (1/100 dilution); Lane 4, normal sample (1/50 dilution); Lane M, protein molecular weight marker.

3.2.1.2.5 Optimisation of APO A1 for Western blotting

In order to find out the optimal conditions for western blotting, a series of experiments were performed using different concentrations of serum/plasma and with different primary antibody concentrations (Figure 3.9, 3.10). All the conditions mentioned above were used in order to obtain the final optimal conditions. The most optimal conditions estimated were the one with serum concentration of 1/1000 and primary antibody concentration of 1/2000 (Figure 3.10).

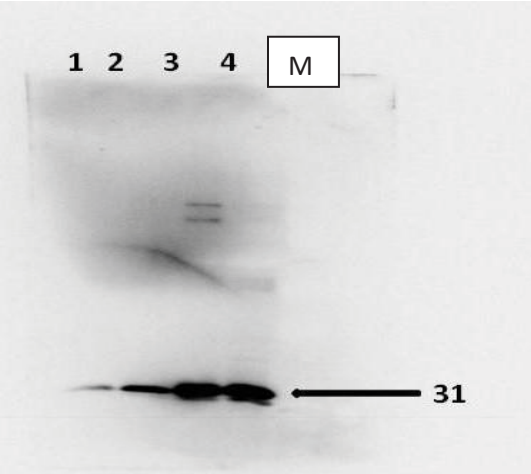

Figure 3.9: Optimisation of detection and western analysis of APOA1 at varying serum dilutions with 1/2000 antibody dilution. Serum proteins were electrophoresed on SDS PAGE gels and transferred to a membrane for western analysis of APOA1. Equal volumes of serum (diluted to 1/1000, 1/500, 1/100, 1/50) were subjected to protein electrophoresis and western analysis using an antibody for APOA1 (1/2000 dilution) with expected molecular weight of 31 kDa. Key: Lane 1, normal sample (1/1000 dilution); Lane 2, normal sample (1/500 dilution); Lane 3, normal sample (1/100 dilution); Lane 4, normal sample (1/50 dilution); Lane M, protein molecular weight marker.

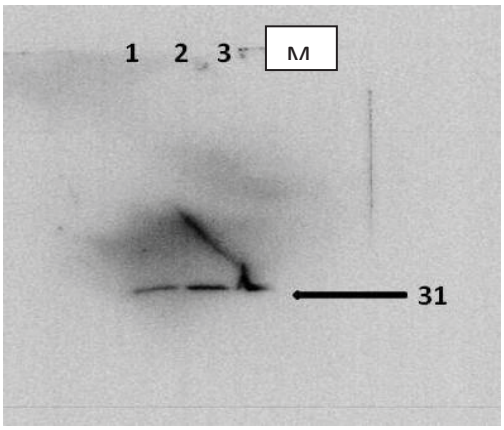

Figure 3.10: Optimisation of detection and western analysis of APOA1 at varying serum dilutions with 1/1000 antibody dilution. Serum proteins were electrophoresed on SDS PAGE gels and transferred to a membrane for western analysis of APO A1. Equal volumes of serum (diluted to 1/1000, 1/500, 1/100, 1/50) were subjected to protein electrophoresis and western analysis using an antibody for APOA1 (1/1000 dilution) with expected molecular weight of 31 kDa. Key: Lane 1, normal sample (1/1000 dilution); Lane 2, normal samples (1/500 dilution); Lane 3, normal sample (1/100 dilution); Lane 4, normal sample (1/50 dilution); Lane M, protein molecular weight marker.

Similar optimisation experiments were carried out with all the above mentioned serum dilutions (1/50, 1/100, 1/500, 1/1000) and primary antibody concentrations (1/1000, 1/2000, 1/3000). All the optimisation electrophoretograms are not included in this section but the final results obtained have been discussed. If all the optimisation results were included in this section it would be a lot of repetition of optimization steps. Above mentioned are just those few which are sufficient to give the idea regarding what had been done in optimization and how some different optimization results were obtained. The optimal conditions identified for each protein is mentioned in the table 3.2.

Table 3.2 This shows the most optimal conditions identified as a result of series of optimization experiments that were carried out using western blotting technique. Key: LBP, Lipopolysaccharide binding protein; APO A1, apolipoprotein A1; APO A4, apolipoprotein A4; plasma serine protease inhibitor; PGLYRP2, N-acetylmuramoyl-L-alanine amidase; SHBG, sex hormone binding globulin.

Protein validated using Western Blotting	Optimal Serum dilution	Optimal primary antibody concentration	Optimal secondary antibody concentration	Optimal Voltage (V) and current (mA) for transfer of proteins
plasma serine protease inhibitor	1/1000	1/2000	1/2000	200 mA, 1 tub (2 h) 400 mA, 2 tubs (4 h) 25 V, 2 h
SHBG	1/100	1/3000	1/2000	200 mA, 1 tub (2 h) 400 mA, 2 tubs (4 h) 25 V, 2 h
APO A4	1/1000	1/3000	1/2000	200 mA, 1 tub (2 h) 400 mA, 2 tubs (4 h) 25 V, 2 h
APO A1	1/1000	1/2000	1/2000	200 mA, 1 tub (2 h) 400 mA, 2 tubs (4 h) 25 V, 2 h
Heparin cofactor 2	1/1000	1/3000	1/2000	200 mA, 1 tub (2 h) 400 mA, 2 tubs (4 h) 25 V, 2 h
PGLYRP2	1/100	1/3000	1/2000	200 mA, 1 tub (2 h) 400 mA, 2 tubs (4 h) 25 V, 2 h
Talin 1	1/50	1/1000	1/2000	200 mA, 1 tub (4 h) 400 mA, 2 tubs (8 h) 25 V, 2 h

3.2.2 Validation of LBP as a biomarker for LAM

The LBP marker was validated using ELISA to determine quantitative differences between normal and patient serum samples. Overall, there were 10 samples tested in each group (n=10 for LAM, ILD and normal). The results of the ELISA analysis show that LBP levels are highly similar in both the LAM (890 ng/mL) and ILD (802 ng/mL) samples, but were marginally lower in the samples from normal subjects (700 ng/mL). There was, however, no significant difference seen in LBP levels in LAM as compared to ILD and normal serum samples ($p > 0.05$) (Figure 3.11, 3.12). These results point towards the potential exclusion of LBP as a potential biomarker for LAM, and indicate that it is not as important as a marker for differentiating between LAM and idiopathic pulmonary fibrosis (interstitial lung disease). Students T tests were performed for determining statistical significance and the results are recorded in table 3.4.

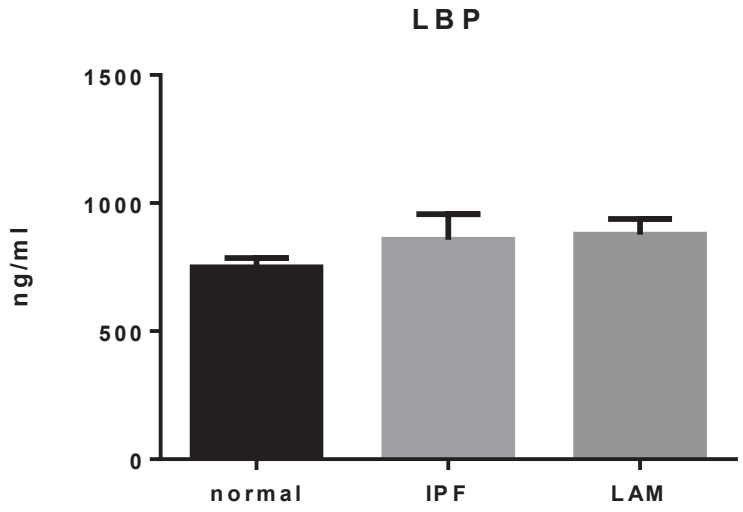

Figure 3.11: LBP concentration in the serum of LAM patients as compared to ILD patients and normal subjects. Each bar in the graph corresponds to a different subject group as identified by the labels under the bars (n=10 in each group). Concentration of LBP (in ng/ml) in serum specimens is given along the vertical axis. Key: ILD, interstitial lung disease; LAM, lymphangioleiomyomatosis. All values are expressed as mean ±SD.

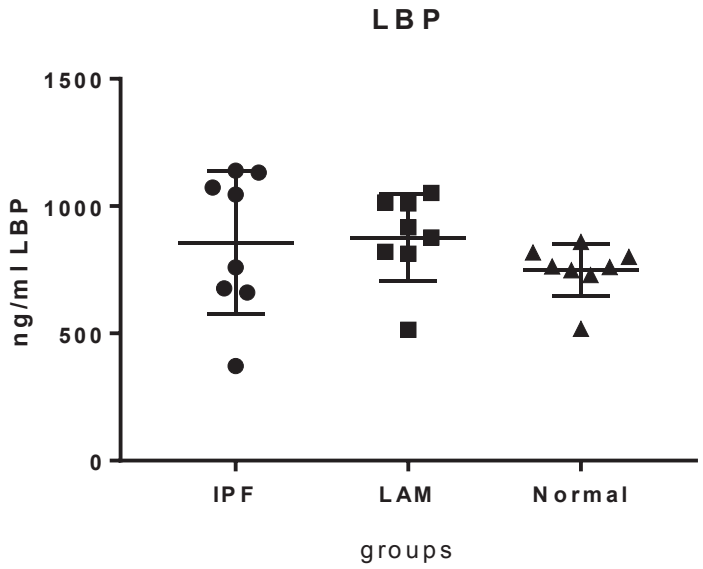

Figure 3.12: Concentration of LBP in serum of LAM patients as compared to ILD patients and Normal subjects. Data points show the individual values (here the concentrations in serum), protein quantification is done in terms of densitometry (arbitrary units) (n=10 in each group). Key: ILD, interstitial lung disease, LAM, (lymphangioleiomyomatosis). All values expressed as mean ±SD.

LBP levels were chosen for analysis as they appeared to show promising potential in prior preliminary study, however the validation experiments carried out as part of this work have shown otherwise. Even the individual value graphs showing data points did not show notable difference among different groups (Figure 3.12). In order for a biomarker to be of any potential significance, the p value ought to be less than 0.05 which was not the case here. There was no notable difference detected between the LAM and ILD groups either ($P>0.05$). LBP does not appear to be a potential diagnostic blood biomarker for LAM or any other condition clinically resembling LAM.

3.2.3 Validation of Fetuin-B as a biomarker for LAM

Fetuin-B was validated using ELISA analysis to determine the levels of this protein in serum samples from LAM patients, ILD patients and normal individuals (n=10 in each group). The

analysis showed that concentrations of Fetuin-B were lower in the serum of LAM patients ($3.0x10^6$ pg/ml) compared to ILD patients ($4.9x10^6$ pg/ml) and normal samples ($3.9x10^6$ pg/ml). However, these differences did not reach statistical significance ($p<0.05$) when ILD and LAM groups were compared with normal. But LAM group when compared with ILD group did show statistical significance with p value =0.023 and hence a potential biomarker. (Figure 3.13). Additionally, at this stage of analysis, Fetuin-B is showing significant distinction between ILD and LAM. Students T test were performed to detect the significance of the results as given in table 3.4.

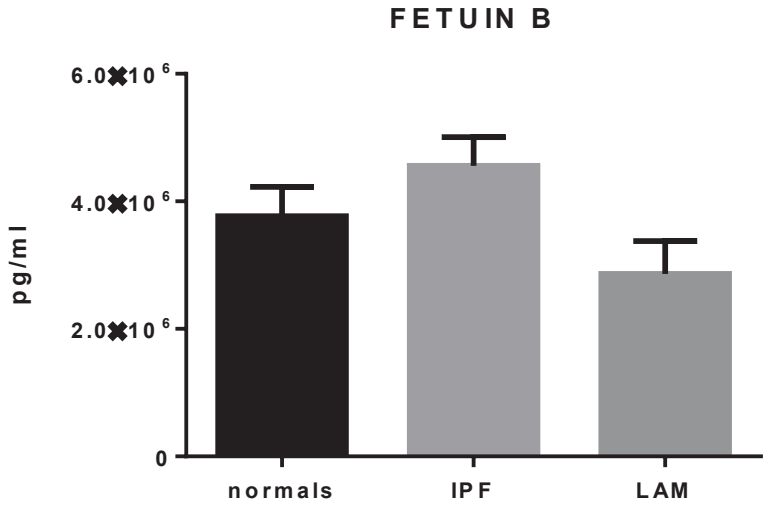

Figure 3.13: Concentration of Fetuin-B in the serum of LAM patients as compared to ILD patients and normal subjects. Each bar shows a different group of patients as identified by the labels under the bars (n=10 in each group). Concentration of Fetuin-B in pg/ml in the serum specimen is given along the vertical axis. Key: ILD, interstitial lung disease; LAM, (lymphangioleiomyomatosis). All values expressed as mean ±SD.

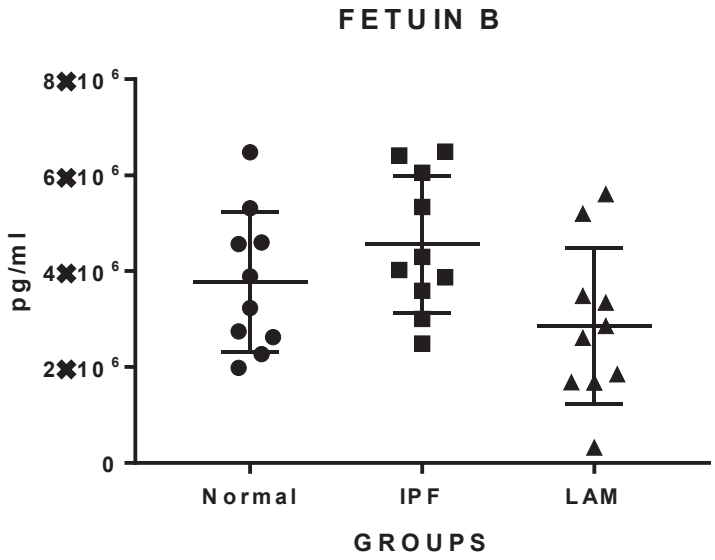

Figure 3.14: Concentration of Fetuin-B in serum of LAM patients as compared to IL (ILD) patients and Normal subjects. Data points show the individual values (here the concentrations in serum), protein quantification is done in terms of densitometry (arbitrary units) (n=10 in each group). Key: ILD, interstitial lung disease, LAM, (lymphangioleiomyomatosis). All values expressed as mean ±SD.

Individual value graph showing data points showed that most of the patients in LAM group had significantly lower values of Fetuin-B as compared to ILD patients (Figure 3.14). Fetuin-B levels although remained different in each subject group being lowest in the LAM group and highest in ILD group with normal group values staying at middle level in between the two other groups, but difference here has p value greater than 0.05. This proves its potential significance as a diagnostic biomarker for LAM as compared to other clinically similar respiratory disease being investigated here.

3.2.4 Validation of plasma serine protease inhibitor as a potential biomarker for LAM

The potential marker plasma serine protease inhibitor was validated using western blot analysis of protein derived from serum of LAM patients (n=10), ILD patients (n=10) and normal control individuals (n=10). In all cases the same amount of protein was loaded in each gel lane. The

analysis of this marker showed that plasma serine protease inhibitor levels were slightly increased in the LAM samples as compared to the normal samples but were significantly lower in the LAM samples as compared to the ILD patient samples (Figure 3.13). Levels of plasma serine protease inhibitor were found to be highest in the ILD samples as compared to the other 2 groups. Of the ILD samples, only 3 had values significantly different from the rest of the ILD group and the other groups (Figure 3.15). As information regarding the severity of clinical symptoms of the ILD patient cohort was available, the values of the three samples in particular (number 23, 28 and 30) were compared against the severity of clinical symptoms to determine if there was any correlation between the two in these patients. In this case, the severity of the clinical features appeared to correlate with the elevated plasma serine protease inhibitor levels. Three of the 10 ILD patients who suffered from severe lung function deterioration clinically, also had elevated levels of this protein.

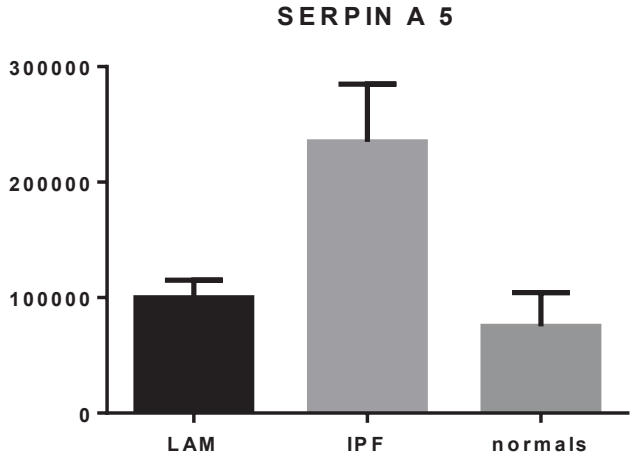

Figure 3.15: Concentration of plasma serine protease inhibitor in serum of LAM patients, ILD patients and normal subjects. Each bar in the graph shows a different group of patients as identified by the labels under the bars (n=10 in each group). The serum concentrations are given along the vertical axis, protein quantification is done in terms of densitometry (arbitrary units). Key: ILD, interstitial lung disease; LAM, lymphangioleiomyomatosis. All values are expressed as mean ±SD.

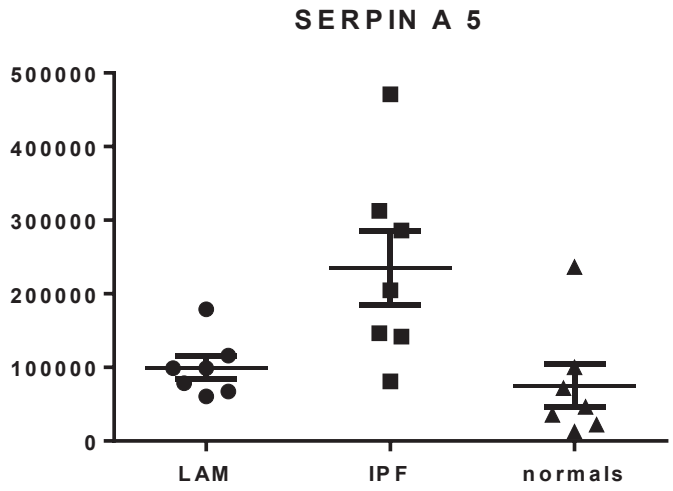

Figure 3.16: Concentration of plasma serine protease inhibitor in serum of LAM patients as compared to ILD patients and normal subjects. Data points show the individual values (here the concentrations in serum), protein quantification is done in terms of densitometry (arbitrary units) (n=10 in each group). Key: ILD, interstitial lung disease, LAM, (lymphangioleiomyomatosis). All values expressed as mean ±SD.

The plasma serine protease inhibitor levels showed clear differences in all the three subject groups under investigation. Overall, normal samples exhibited the lowest levels of this protein with the ILD group having the highest values in most of the subjects. Almost all LAM samples had values concentrated around a certain level. All the three groups differed from each other but the ILD group had most different values than the other two groups but the p value was greater than 0.05.

The data was further evaluated to look for any correlation with the clinical features of the disease in the ILD group. Clinical features that were recorded for evaluation in this study were forced vital capacity (FVC) and forced expiratory volume (FEV). These are two tests that clinically evaluate the lung function of the patients with incapacitating respiratory conditions and are good determinants of the residual lung function and severity of the lung disease. FVC is the amount of air that a person can forcefully exhale at the end of a deep breath and FEV is the amount of air

that a person can forcefully exhale after a deep breath in one second. Both tests are commonly used to clinically evaluate the severity of the pulmonary disease.

Clinically severe ILD disease was noted in some patients and these patients were shown to have raised plasma serine protease inhibitor levels compared to the other patients in the same group, as well as those in the other two groups. This result is a valuable finding in terms of clinical assessment of ILD. Although the sample size is small here in relevance to ILD but still carries some degree of information that may lead towards a new research area if further investigated with a larger cohort of patients with ILD and comparing them with the normal subjects. If it comes out as something significant in ILD with a sufficient sample size, that might help identify a good clinical biomarker

Table 3.3. Clinical characteristics of the ILD Patients from which serum samples have been obtained for this study. Lung function test results are shown and the highlighted values indicate severe lung function deterioration.

ILD Sample number	ILD Sample	Forced Vital Capacity (FVC) %[#]	Forced Expiratory Volume 1 (FEV1) %
001	Serum/plasma	85	88
002	Serum/plasma	75	82
003	Serum/plasma	73	68
012	Serum/plasma	73.5	72
020	Serum/plasma	70.9	77.1
023	Serum/plasma	60*	57*
024	Serum/plasma	109.9	116.2
026	Serum/ Plasma	104	104
028	Serum/Plasma	24*	24*
030	Serum/Plasma	45*	50*

#Reference ranges: Mild incapacity (70-79%), Moderate incapacity (60-69%), Severe incapacity (less than 60%). *Abnormal results

3.2.5 Validation of PLTP as a potential biomarker for LAM

The potential marker PLTP was tested in the samples from the 3 cohorts (LAM, ILD and normal) using western analysis. The antibody used allowed the detection of a band of the expected molecular weight for PLTP (55 kDa) but additional bands were also detected alongside the required bands, most prominent at 76 kDa and 50 kDa (Figures 3.17 and 3.18). Optimal conditions for PLTP had been selected based on the presence of a minimal number of additional bands, and those bands, when present, being much fainter in intensity to the target band.

In general, in western analysis, the use of higher concentrations of primary antibody cause the appearance of multiple bands, additional to the band of interest, due to non-specific binding. This issue was dealt with by lowering the serum concentration used by diluting to 1/1000 to 1/2000 and also reducing the primary antibody concentration by making dilutions at 1/2000 and 1/3000. Using a 1/2000 dilution of primary antibody and 1/1000 dilution of the serum led to multiple bands still being detected, however no bands appeared at all when analysed using 1/2000 and 1/3000 serum dilutions. Therefore, the working concentration for the serum samples was determined to be a 1/1000 dilution. Neither set of dilutions of primary antibody and/or serum samples was not able to resolve the issue and a single specific band corresponding to the protein of interest was not detected.

Further measures to avoid the appearance of multiple bands, including increasing the number of washes after incubation with the primary and secondary antibodies, did not eliminate the appearance of multiple bands. It is most likely that the protein of interest is present in low quantities in the serum and therefore a greater amount of serum may need to be loaded. However, the use of a 1/50 dilution of the serum, combined with varying the number and duration of the washes, did not eliminate the non-specific binding.

Elimination of the primary antibody in the western procedure led to the disappearance of the multiple bands, indicating that the primary antibody was the causative factor, most likely a manifestation of poor quality. Due to the questionable quality of the PLTP primary antibody, an alternative PLTP antibody was sourced.

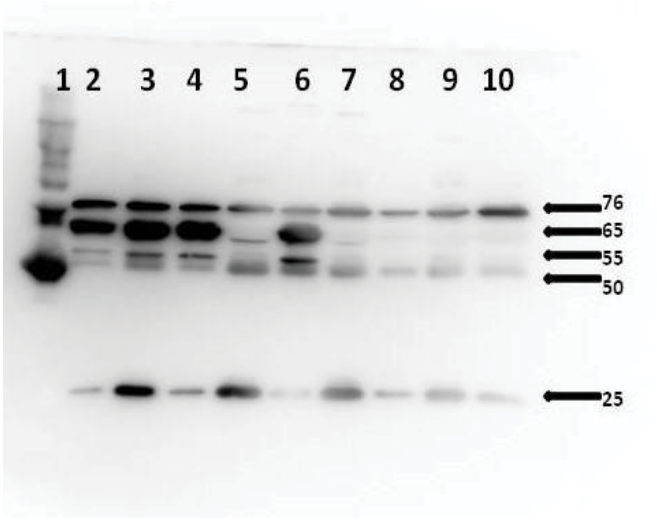

Figure 3.17: Serum proteins were electrophoresed on SDS PAGE gels and transferred to a membrane for western analysis of PLTP. All patient serum samples were diluted to 1/1000 concentration and electrophoresed for western analysis. Significant fractions are identified by labels along the sides (in kDa). PLTP antibody concentration was 1/2000 and the corresponding protein band is present at 55-65 kDa. Key: Lane 1, protein molecular weight marker; Lanes 2, 3 and 4, LAM patient serum samples; Lanes 5, 6 and 7, ILD patient samples; Lanes 8, 9 and 10, Normal samples.

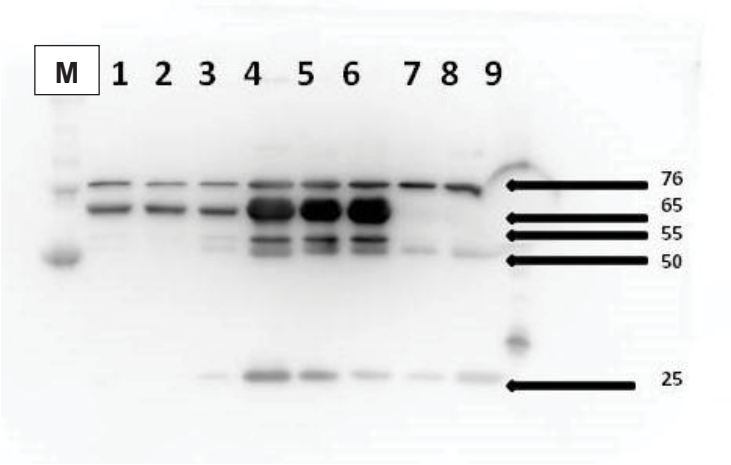

Figure 3.18: Serum proteins were electrophoresed on SDS PAGE gels and transferred to a membrane for western analysis of PLTP. All serum samples were diluted to 1/1000 concentration and the PLTP antibody concentration used was 1/2000. The corresponding target protein band is present at 55-65 kDa. The significant fractions are identified by labels along the sides (in kDa). Key: Lane M, protein molecular weight marker; Lanes 1, 2 and 3, LAM patient samples; Lanes 4, 5 and 6, ILD patient samples; Lanes 7, 8 and 9, Normal samples.

An alternate antibody for PLTP was used for the experiments and after a series of optimisation experiments, the optimal conditions for western analysis were with a serum dilution of 1/1000 and a primary antibody dilution of 1/2000. However, the use of the most optimal conditions still led to multiple bands appearing.

One of the reasons for getting lower molecular bands could be that the samples have degraded resulting in formation of protein degradation products, however all steps had been taken to prevent this. Due to these difficulties, and the lack of alternative antibodies for PLTP that could be tested, no further work was carried out on this biomarker.

3.2.6 Validation of SHBG as a potential biomarker for LAM

The potential biomarker SHBG was examined by western analysis and levels of this protein were compared across the 3 study cohorts (LAM, ILD and controls). Western analysis indicated increased levels of SHBG in the LAM serum samples compared to the ILD samples but lower

than in the normal individuals (Figure 3.19). Levels of SHBG were found to be highest in the normal group as compared to the other 2 groups. Even in individual value data point graph most of the values in different patient groups were scattered around similar average value (Figure 3.20). Overall the differences in SBHG levels between the different sample groups was not statistically significant ($p>0.05$).

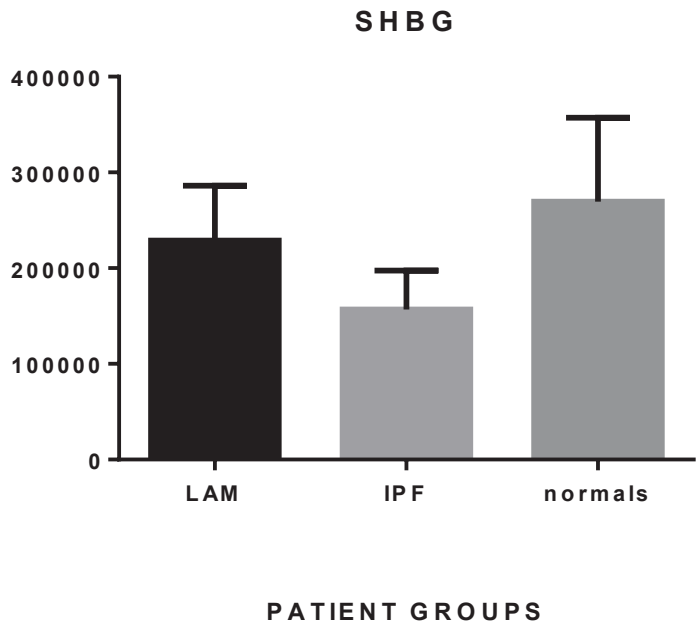

Figure 3.19: Serum SHBG levels in LAM patients as compared to ILD patients and Normal subjects. Each bar represents a different group of patients as identified by the labels under the bars (n=10 in each group). The serum concentrations are given along the vertical axis, protein quantification is done in terms of densitometry (arbitrary units). Key: ILD, interstitial lung disease; LAM, lymphangioleiomyomatosis. All values are expressed as mean ±SD. P>0.05.

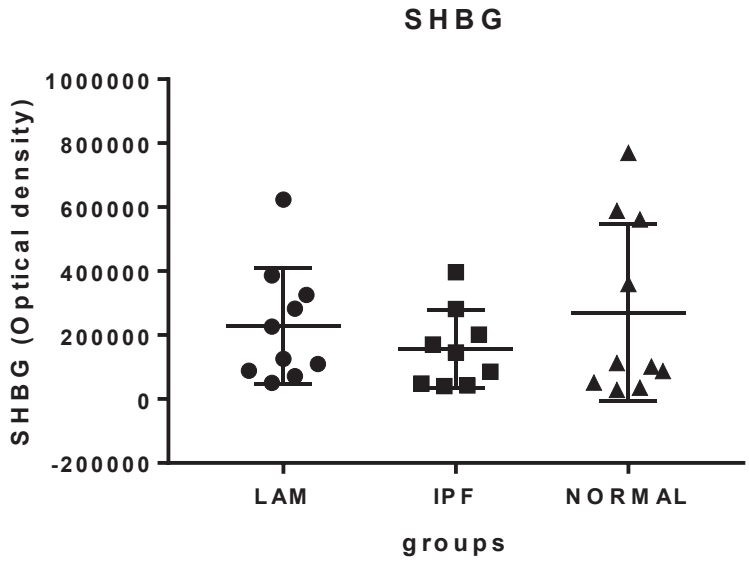

Figure 3.20: Concentration of SHBG in serum of LAM patients as compared to ILD patients and Normal subjects. Data points show the individual values (here the concentrations in serum), protein quantification is done in terms of densitometry (arbitrary units) (n=10 in each group). Key: ILD, interstitial lung disease, LAM, (lymphangioleiomyomatosis). All values expressed as mean ±SD.

3.2.7 Validation of APO A4 as a potential biomarker for LAM

The potential biomarker, APO A4 was examined in the 3 sample cohorts (LAM, ILD and normal) using western analysis. Analysis of APO A4 showed that levels of this protein are highest in the normal sample cohort and present at similar levels in the LAM and ILD groups (Figure 3.21). The level appears to be almost twice as high in the normal group as compared to the LAM group. But looking at data point graph it becomes very clear that most of the values fell within the same range in almost all the three groups (Figure 3.22). However, no significant difference was identified between LAM and ILD group in terms of difference in expression of APO A4. This potentially excludes APO A4 as diagnostic biomarker for LAM.

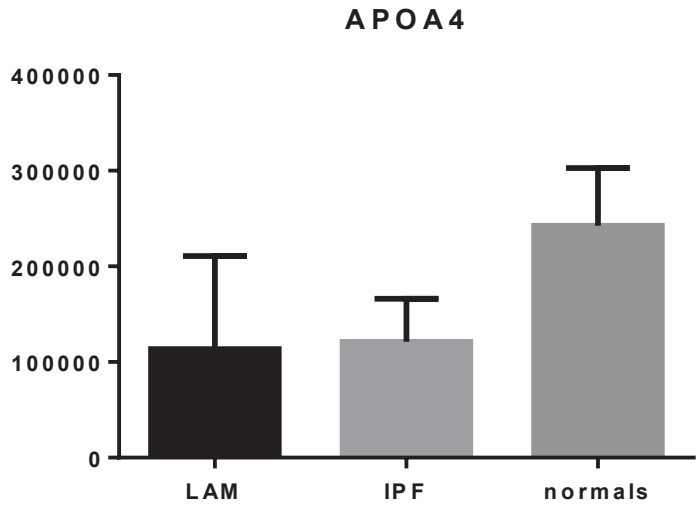

Figure 3.21. Serum APO A4 levels in LAM patients as compared to ILD patients and Normal subjects. Each bar represents a different group of patients as identified by the labels under the bars (n=10 in each group). The serum concentrations are given along the vertical, protein quantification is done in terms of densitometry (arbitrary units). Key: ILD, interstitial lung disease, idiopathic pulmonary fibrosis; LAM, lymphangioleiomyomatosis. All values are expressed as mean ±SD. P>0.05.

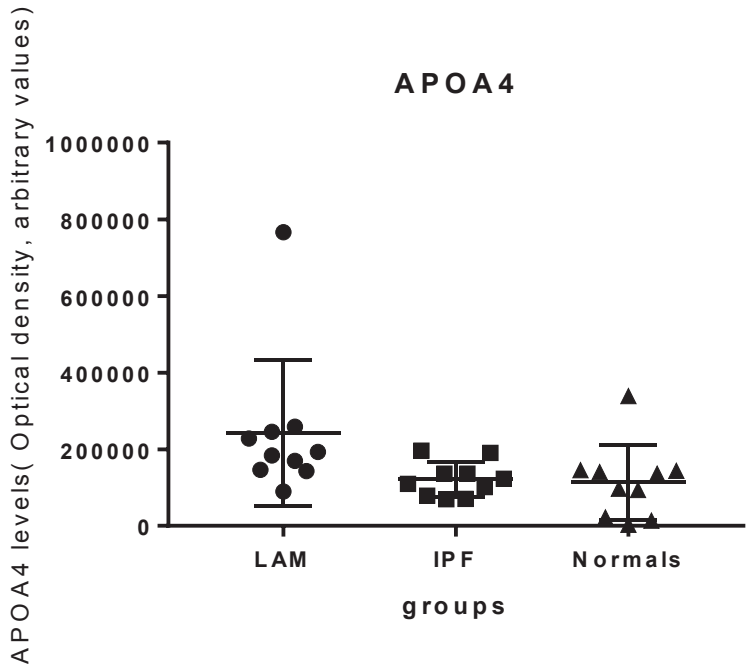

Figure 3.22: Concentration of APO A4 in serum of LAM patients as compared to ILD patients and normal subjects. Data points show the individual values (here the concentrations in serum, protein quantification is done in terms of densitometry (arbitrary units) (n=10 in each group). Key: ILD, interstitial lung disease, LAM, (lymphangioleiomyomatosis). All values expressed as mean ±SD, p value from the T tests is >0.05

This result indicates that APO A4 may have potential clinical significance. Although the values in the LAM group are clearly lower as compared to the normal group, the deviation from the mean standard is notable (Figure 3.22). Some values usually do not impact the overall mean value of the whole group and the mean value is considered significant. In this case sample size was significant for LAM, but given the size the outliers are impacting the deviation of the whole group and this cannot be ignored entirely.

3.2.8 Validation of APO A1 as a potential biomarker for LAM

APO A1 levels in all the three groups (LAM, ILD, Normal) are different with normal and LAM groups being very close to each other in terms of value (Figure 3.23). ILD on the other hand clearly stands at a distinct level from the rest of the two groups. The overall mean average concentration of APO A1 in ILD is half as that much in the other two groups.

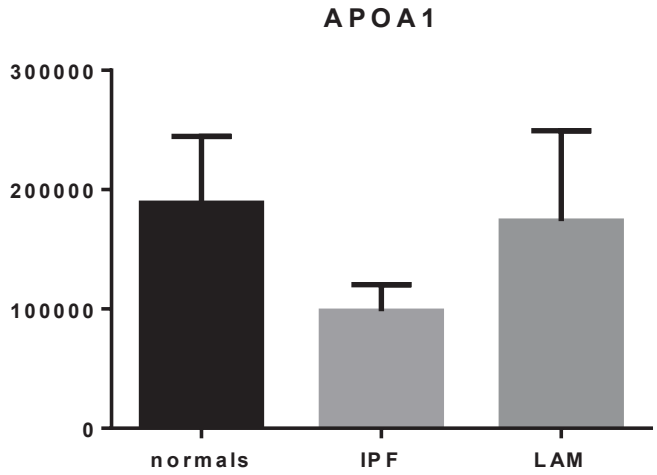

Figure 3.23: Serum APO A1 levels in LAM patients as compared to ILD patients and normal subjects. Each bar represents a different group of patients as identified by the labels under the bars (n=10 in each group). The serum concentrations are given along the vertical axis, protein quantification is done in terms of densitometry (arbitrary units). Key: ILD, interstitial lung disease; LAM, lymphangioleiomyomatosis. All values are expressed as mean ±SD. P>0.05.

The individual values of each sample in each group lie roughly around the same average value with only one sample lying outside the average mean levels in each of the LAM and normal groups (Figure 3.24). These results indicate that APO A1 levels could provide a prospective potential biomarker for diagnosing and differentiating ILD from LAM and normal individuals but not to distinguish LAM patients from normal individuals and vice versa. These results are obtained without taking into consideration gender, age and lifestyle factors in each group, which may ultimately affect the findings and when given due consideration, and may provide more valuable insights. Some data points that are missing in a few results including the results shown

here is due to a fewer sample number used in those experiments due to limited quantity of LAM or ILD serum available. Given the fact that many experiments were performed for optimization steps and often repeat experiments some of the sample serum were all used up and therefore a lesser number of data points in that particular set of experiment.

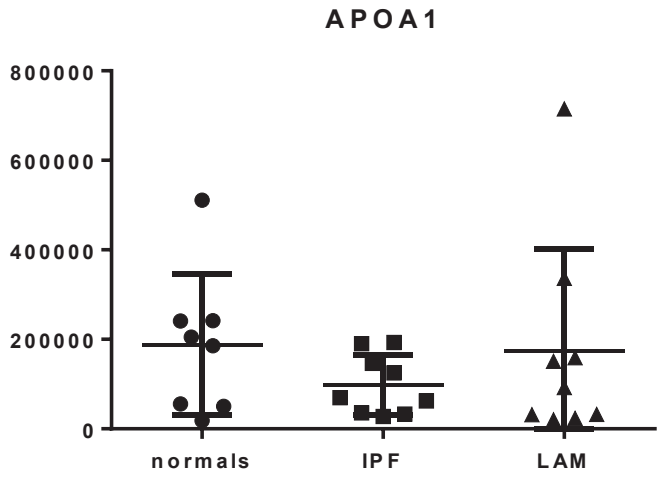

Figure 3.24: Concentration of APO A1 in serum of LAM patients as compared to ILD patients and normal subjects. Data points show the individual values (protein quantification in terms of densitometry (arbitrary units) (n=10 in each group). Key: ILD, interstitial lung disease, LAM, (lymphangioleiomyomatosis). All values expressed as mean ±SD. p >0.05 (from T-test)

3.2.9 Validation of Heparin cofactor 2 as a potential biomarker for LAM

Heparin cofactor 2 levels were tested in the 3 sample cohorts using western analysis. This analysis showed that heparin cofactor 2 levels are highest in the normal group; followed by the ILD group and then the LAM group (Figures 3.25 and 3.26). Lowest mean value are found present in the LAM group with ILD being in the middle in terms of the mean average value.

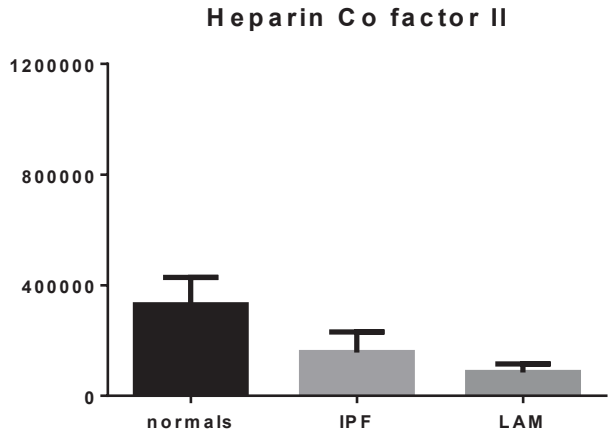

Figure 3.25: Serum Heparin cofactor 2 levels in LAM patients as compared to ILD patients and normal subjects. Each bar represents a different group of patients as identified by the labels under the bars (n=10 in each group). The serum concentrations are given along the vertical axis, protein quantification is done in terms of densitometry (arbitrary units). Key: ILD, interstitial lung disease; LAM, lymphangioleiomyomatosis. All values are expressed as mean ±SD.

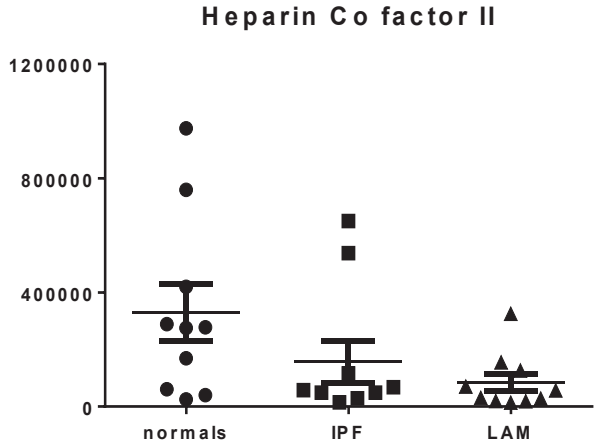

Figure 3.26: Concentration of Heparin cofactor 2 in serum of LAM patients as compared to ILD patients and normal subjects. Data points show the individual values (here the concentrations in serum, protein quantification is done in terms of densitometry (arbitrary units)(n=10 in each group). Key: ILD, interstitial lung disease, LAM, (lymphangioleiomyomatosis). All values expressed as mean ±SD.

A scatter plot of the individual values for each individual in each cohort shows that most of the values in the LAM group are very close to the average mean standard deviation. The numbers of samples that lie outside the average mean standard deviation in LAM are insignificant. In the normal group, nearly half of the group has values far above the LAM average mean standard deviation. The difference between the ILD and LAM group is not significant enough to further evaluate heparin cofactor 2 as a differentiating biomarker between the two disease groups. Heparin cofactor 2 has shown some promise in this study, it has a differentiating potential between the LAM and normal groups. The p value was 0.0303 when LAM group was compared with the normal group for the quantification of heparin cofactor 2. LAM and ILD average mean values lie close to each other and do not show a significant difference (p value 0.377) for further consideration. In other words heparin cofactor 2 levels may be able to differentiate LAM and ILD patients from normal individuals. This proves that heparin cofactor 2 is a potential biomarker of significance when differentiating LAM from normal using blood samples.

3.2.10 Validation of N-acetylmuramoyl-L-alanine amidase (PGLYRP2) as a potential biomarker for LAM

The potential biomarker PGLYRP2 was tested in the 3 sample cohorts (LAM, ILD and normal) using western analysis. This analysis showed that serum PGLYRP2 levels are almost similar in the normal and ILD groups (Figure 3.27) with only a minute difference in the mean average value (Figure 3.28). The mean average concentration of PGLYRP2 in the LAM group is considerably low compared to the other two groups.

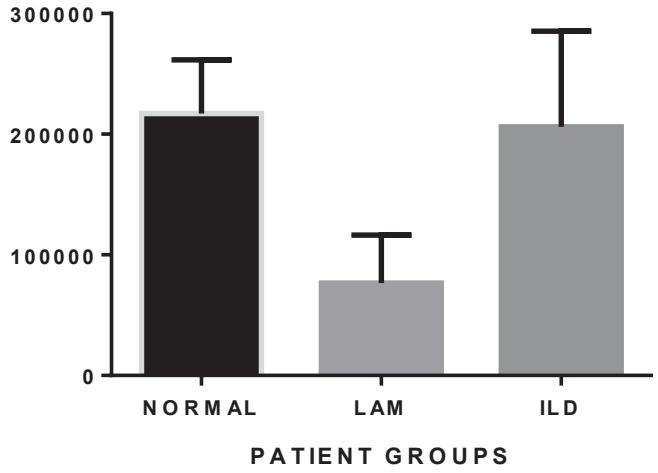

Figure 3.27: Serum PGLYRP2 levels in LAM patients as compared to ILD patients and normal subjects. Each bar represents a different group of patients as identified by the labels under the bars (n=10 in each group). The serum concentrations are given along the vertical axis, protein quantification is done in terms of densitometry (arbitrary units). Key: ILD, interstitial lung disease; LAM, lymphangioleiomyomatosis. All values are expressed as mean ±SD.

This protein (PGLYRP2) has shown some promising results in this study and may be effective in diagnosing LAM. The statistical significance was determined by calculating p value which was found out to be showing significance when LAM group was compared to each of the other groups (LAM compared to normal p value is 0.0061) and (LAM as compared to ILD p value is 0.00047) This protein may be shown to be diagnostic biomarker for LAM as compared to ILD and normal human subjects.

The individual values of the samples an individual value bar graph (a scatter plot) was evaluated, almost all the LAM samples were found closely around the same mean average value. The samples in the normal group clearly had higher PGLYRP2 levels than in the LAM group.

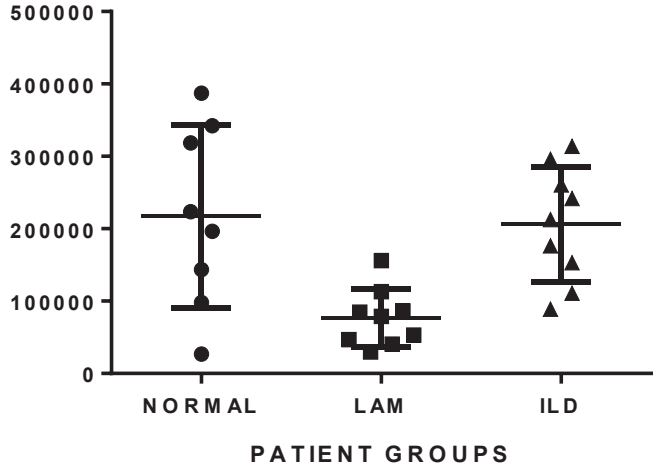

Figure 3.28: Concentration of PGLYRP2 in serum of LAM patients as compared to ILD patients and normal subjects. Data points show the individual values (here the concentrations in serum) protein quantification is done in terms of densitometry (arbitrary units). (n=10 in each group). Key: ILD, interstitial lung disease, LAM, (lymphangioleiomyomatosis). All values expressed as mean ±SD.

This has proven that PGLYRP2 is a potential diagnostic biomarker for diagnosing LAM as compared to clinically similar ILDs and normal individuals. Additionally, the severity of the clinical symptoms of the individual LAM and ILD samples were assessed against their individual values in the graph. The outcome was obtained to determine any association between the serum concentrations of the potential biomarkers and clinical severity. This has been discussed in detail in the subsequent sections.

3.2.11 Validation of Talin 1 as a potential biomarker for LAM

Talin one levels were determined in all three groups namely LAM, ILD and normal. The graph shows the relative mean average value of Talin 1 in each of the groups as compared to the others. LAM group showed lowest levels of Talin 1 followed closely by the normal group which had slightly higher value, but ILD group had the highest value of all groups. These differences were calculated to determine the p value which was found out to be greater than 0.05 and thus

insignificant. This proves that Talin 1 is not a potential biomarker for diagnosing LAM or for differentiating between LAM and ILD.

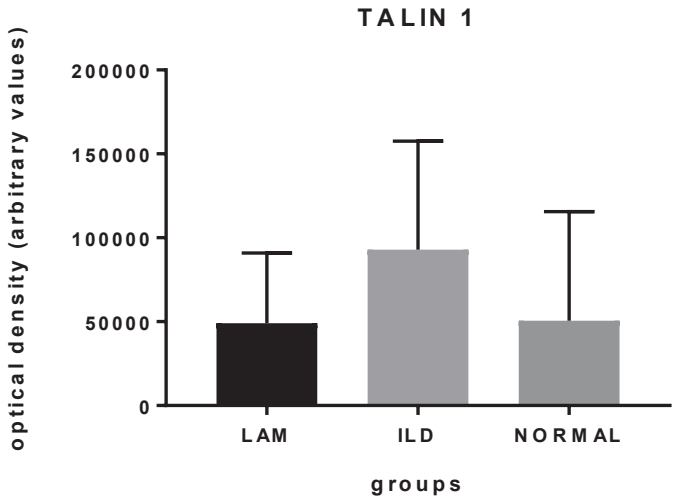

Figure 3.29: Serum Talin 1 levels in LAM patients as compared to ILD patients and normal subjects. Each bar represents a different group of patients as identified by the labels under the bars (n=10 in each group). The serum concentrations are given along the vertical axis, protein quantification is done in terms of densitometry (arbitrary units). Key: ILD, interstitial lung disease; LAM, lymphangioleiomyomatosis. All values are expressed as mean ±SD.

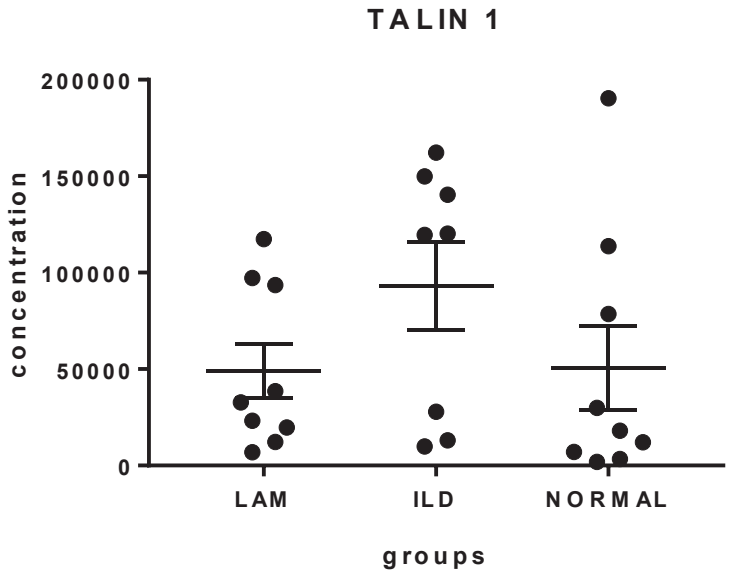

Figure 3.30: Concentration of Talin 1 in serum of LAM patients as compared to ILD patients and normal subjects. Data points show the individual values (here the concentrations in serum) protein quantification is done in terms of densitometry (arbitrary units). (n=10 in each group). Key: ILD, interstitial lung disease, LAM, (lymphangioleiomyomatosis). All values expressed as mean ±SD

Table 3.4 A table showing comparison between the results of the preliminary study that used mass spectrometric analysis method and this validation study conducted using reliable methods. Key: LBP, Lipopolysaccharide binding protein; APO A1, apolipoprotein A1; APO A4, apolipoprotein A4; plasma serine protease inhibitor; PGLYRP2, N-acetyl-muramoyl-L-alanine amidase; SHBG, sex hormone binding globulin.

Protein showing Altered expression	**Preliminary result (levels in LAM as compared to normal)**	**Validated result (levels in LAM as compared to normal)**
Fetuin-B	Decreased	Decreased
LBP	Decreased	Marginally Increased
plasma serine protease inhibitor	Increase	Marginally Increased
PLTP	Increase	Indeterminate
SHBG	Decreased	Marginally decreased
APO A4	Increase	Decreased
APO A1	Increase	Marginally decreased
Heparin cofactor 2	Decreased	Significantly Decreased
PGLYRP2	Increase	Significantly Decreased
Talin 1	Increase	Decreased

Table 3.5 It shows the levels of each protein in LAM as compared to ILD patients as validated using reliable methods. Key: LBP, Lipopolysaccharide binding protein; APO A1, apolipoprotein A1; APO A4, apolipoprotein A4; plasma serine protease inhibitor (SERPIN A5); PGLYRP2, N-acetylmuramoyl-L-alanine amidase; SHBG, sex hormone binding globulin.

Protein showing Altered expression	**Validated result (levels in LAM as compared to ILD)**
Fetuin-B	Significantly decreased
LBP	Almost similar
plasma serine protease inhibitor	Decreased
PLTP	Indeterminate
SHBG	Increased
APO A4	Almost similar
APO A1	Increased
Heparin cofactor 2	Decreased
PGLYRP2	Significantly decreased
Talin 1	Decreased

Table 3.6 shows the p value of each protein in one group as compared to the other groups. Items marked with * indicate significant values. Key: LBP, Lipopoly saccharide binding protein; APO A1, apolipoprotein A1; APO A4, apolipoprotein A4; plasma serine protease inhibitor (SERPIN A5); PGLYRP2, N-acetyl-muramoyl-L-alanine amidase; SHBG, sex hormone binding globulin

PROTEIN NAME	P value LAM Vs ILD	P value ILD Vs Normal	P value LAM Vs Normal
LBP	0.9322	0.2349	0.0790
Fetuin-B	0.023*	0.1108	0.206
APO A1	0.56637	0.7711	0.6994
APO A4	0.06645	0.8235	0.0732
Heparin cofactor 2	0.3774	0.18162	0.030*
Plasma serine protease inhibitor	0.8530	0.4868	0.3753
PGYLRP2	0.00047*	0.8303	0.0061*
SHBG	0.3295	0.2766	0.7010
Talin 1	0.1138	0.5275	0.357

CHAPTER 4

RESULTS 2

COMPARISON BETWEEN BIOMARKER LEVELS AND CLINICAL SEVERITY OF THE DISEASES

4.1 Correlation between the severity of lung functions and APO A4 levels in LAM and ILD

4.1.1 Correlation between clinical severity of ILD and APO A4 levels

Altered levels of APO A4 were found in the ILD group as compared to the normal group and this is described in chapter 3. Although the altered expression of APO A4 was not found to be significant, this candidate protein was evaluated to determine if there was any relationship between altered expression and disease severity. APO A4 levels were found to be decreased in ILD patients as compared to the normal subjects and correlation of APO A4 levels with disease severity (based on lung function test measures) revealed a gradual decline in APO A4 levels with deteriorating lung function (Figure 4.1 and Table 4.1). Lung function measures can be further split into two categories. Lung functions between the ranges of 60-69% are considered moderately incapacitating and those above 70 are considered mildly incapacitating. The group of patients studied as part of this work fell mostly in the range of mild to moderate impairment with only three patients having severe lung function impairment. The same trend in APO A4 expression levels was observed in both lung function groups. Thus with increasing severity or impairment of lung function in ILD, there was a corresponding decrease in APO A4 levels. Patients with normal, or near normal, lung function had significantly greater APO A4 levels. Thus it would appear that there is a relation between altered APO A4 expression and the extent of lung function impairment in patients suffering from ILD.

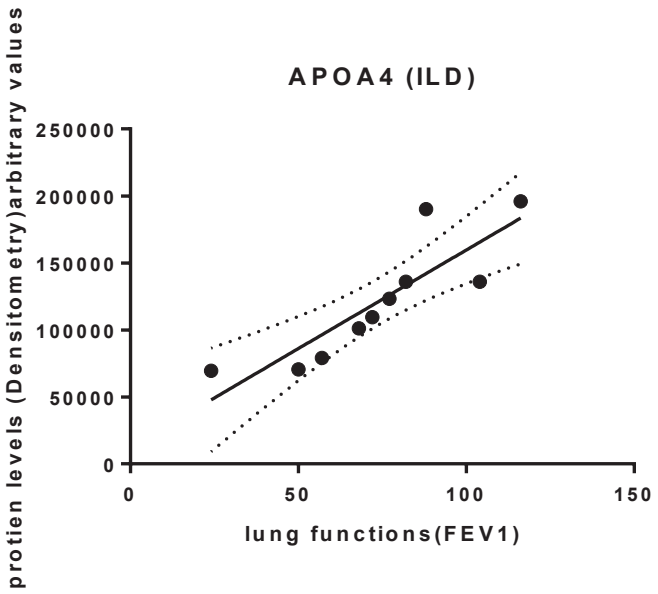

Figure 4.1: A graph showing a correlation between the lung functions and levels of APO A4 in ILD patients. Lung function values (in %) are plotted along the x axis and the corresponding value of APO A4 levels is plotted along y axis. y axis shows densitometry in arbitrary values. Key: APO A4, apolipoprotein A4; ILD, interstitial lung disease.

Table 4.1 Shows Pearson coefficient r showing statistical association between APO A4 levels and lung function tests in ILD patients and its significance. Key: r: Pearson correlation coefficient. Here $p<0.05$

Pearson r	
r	0.8663
P value	
P (two-tailed)	0.0012
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	10

Further explanation of the different values for "r'', and the interpretation of these values are shown below.

Value of r	Strength of relationship
-1.0 to -0.5 or 1.0 to 0.5	Strong
-0.5 to -0.3 or 0.3 to 0.5	Moderate
-0.3 to -0.1 or 0.1 to 0.3	Weak
-0.1 to 0.1	None or very weak

4.1.2 Correlation between clinical severity of LAM and APO A4 levels

As already discussed in the results section, values of APO A4 also apparently seemed to be altered in LAM group as compared to normal group although this did not show significance but was chosen to further evaluate because of its apparent difference in ILD and LAM as compared to the normal group. This apparent difference was more pronounced in ILD. The APO A4 levels in terms of densitometry (arbitrary values) were plotted along y axis and the corresponding lung function test values in percentage were plotted along x axis Figure 4.2. Lung functions ranged from mild to moderate and severe impairment. The corresponding APO A4 levels in these LAM patients did not show any regular trend or pattern. There was no correlation observed when comparing the APO A4 levels and the severity of the lung functions in LAM patients in contrast to one observed in ILD group (Table 4.2).

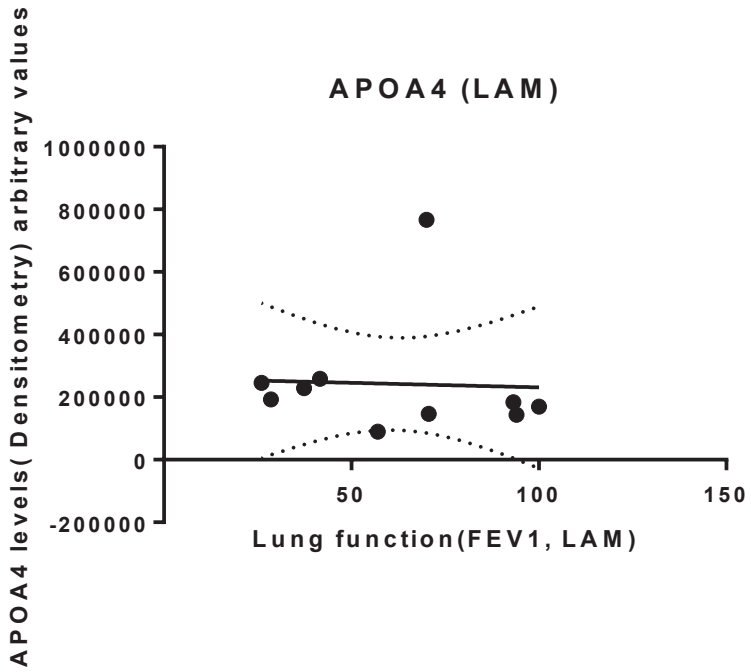

Figure 4.2: A graph to show a correlation between the lung functions and levels of APO A4 in LAM patients. Lung function values (in %) are plotted along the x axis and the corresponding value of APO A4 level is plotted along y axis. The serum concentrations are plotted along y axis in terms of densitometry (arbitrary values). Key: APO A4, apolipoprotein A4; LAM, lymphangioeiomyomatosis

Table 4.2 shows the values related to the Pearson ‘’r’’ correlation as calculated for determining the significance of the correlation between APO A4 levels and LAM severity.

Table 4.2: Shows Pearson coefficient r showing statistical association between APO A4 levels and lung function tests in LAM patients and its significance. Key: r: Pearson correlation coefficient. Here p>0.05

Pearson r	
r	-0.04275
P value	
P (two-tailed)	0.9067
P value summary	ns
Number of XY Pairs	10

4.2 Correlation between the severity of lung functions and levels of Heparin cofactor 2 in LAM and ILD

4.2.1 Correlation between clinical severity of LAM and Heparin cofactor 2 levels

Heparin cofactor 2 levels were significantly reduced in LAM group as compared to the normal group however there seemed to be apparent difference in LAM and ILD group as well which was considered here as basis for comparing clinical features with ILD group too. The protein levels were seen to be decreasing with the increasing severity of the disease as depicted through declining lung function tests values. Here the patients with normal lung functions had clearly higher levels of heparin cofactor 2 in their blood. A sharp increase in heparin cofactor 2 value was seen in the LAM patient with normal lung function value. The patients with moderate impairment of lung function had lower levels of heparin cofactor 2, whereas the patients with severe lung function impairment as depicted by lung function tests had the lowest values heparin cofactor 2. The fall in the levels of heparin cofactor 2 showed a steady decline as the lung functions deteriorated (Figure 4.3 and Table 4.3).

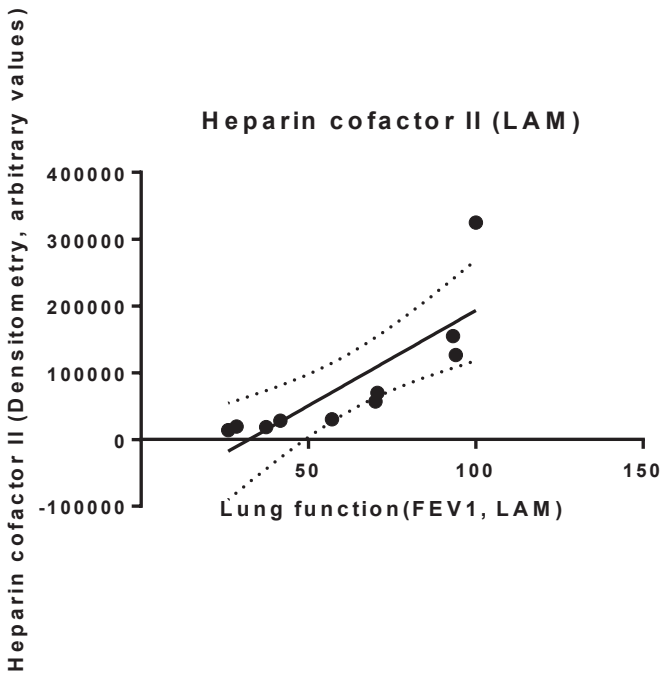

Figure 4.3: A graph showing a correlation between the lung functions and levels of Heparin cofactor 2 in LAM patients. Lung function values (in %) are plotted along the x axis and the corresponding value of Heparin cofactor 2 level is plotted along y axis. The serum concentrations are plotted along y axis in terms of densitometry (arbitrary values). Key: LAM, lymphangioleiomyomatosis

The statistical association between the lung function tests and thus the severity of the disease is calculated using Pearson's correlation coefficient. It determines the strength of association between two variables, here lung function tests and protein levels. The significance is also calculated in table 4.3.

Table 4.3: Shows Pearson's 'r' coefficient showing statistical association between heparin cofactor 2 levels and lung function tests in ILD patients and its significance. Key: r: Pearson correlation coefficient. Here $p<0.05$

Pearson r	
r	0.8201
P value	
P (two-tailed)	0.0037
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	10

4.2.2 Correlation between clinical severity of ILD and Heparin cofactor 2 levels

Severity of the lung functions did not relate to the increasing or decreasing values of heparin cofactor 2 in ILD patients. This is also shown in the figure 3.32. There is no particular trend or pattern when the heparin cofactor 2 values were compared with the lung functions of the ILD patients. Although in the results, it was seen that heparin cofactor 2 levels in ILD group were lower as compared to the normal group. Here in this graph lung function values were plotted along the x axis and heparin cofactor 2 concentrations were plotted along the y axis. The lung function test values were measured in percentage (%) and the serum concentrations are measured in terms of densitometry (arbitrary values). A dot graph pattern was plotted to determine the relation which was found out to be absent (Figure 4.4 and Table 4.4).

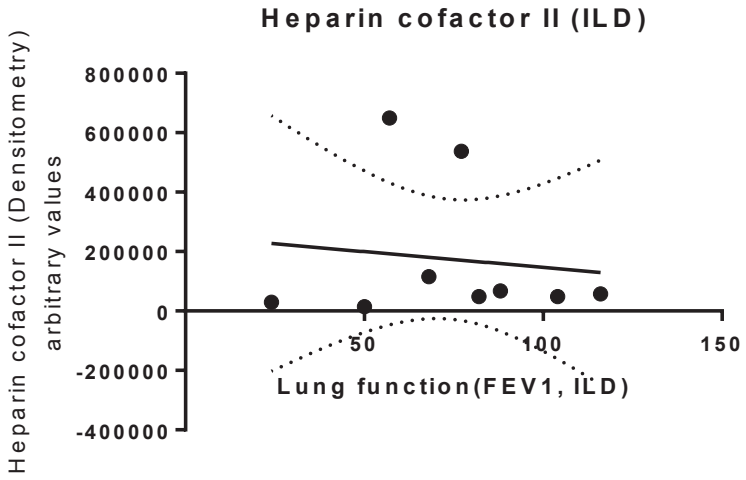

Figure 4.4: A graph showing a correlation between the lung functions and levels of Heparin cofactor 2 in ILD patients. Lung function values (in %) are plotted along the x axis and the corresponding value of Heparin cofactor 2 level is plotted along y axis. The serum concentrations are plotted along y axis in terms of densitometry (arbitrary values). Key: ILD, interstitial lung disease.

Table 4.4: Shows Pearson coefficient r showing statistical association between heparin cofactor 2 levels and lung function tests in ILD patients and its significance. Key: r: Pearson correlation coefficient. Here p>0.05.

Pearson r	
R (strength of association between two variable)	-0.1236
P value	
P (two-tailed)	0.7513
P value summary	ns
Significant? (alpha = 0.05)	No
Number of XY Pairs	9

4.3 Correlation between the severity of lung functions and levels of PGLYRP2 in LAM and ILD

PGLYRP2 levels in LAM and ILD patients were compared to the lung functions below. PGLYP2 had shown significant decrease in patients suffering from LAM as compared to those suffering from ILD and normal healthy subjects as described in the results section.

4.3.1 Correlation between clinical severity of ILD and PGLYRP2 levels

Although the PGLYRP2 values in ILD did not differ significantly from normal group but yet the difference was significant as compared to the LAM group. Therefore it was chosen to be evaluated for determining any correlation between protein levels and disease severity. The comparison between the severity of lung functions and PGLYRP2 in ILD was done by plotting the lung functions of each ILD patient along the x axis and lung function severity along the y axis. The lung functions were expressed in percentage and protein serum levels were expressed in terms of densitometry (arbitrary values). The result as shown in the figure 3.34 showed depicted a particular trend. Although the patients with less severe lung disease and values above 80%, tended to have PGLYRP2 values towards higher side and those with lung functions below 80% generally had lower values but this finding would not support diagnostic value of this biomarker, however in diagnosed cases of ILD would potentially show severity trend (Figure 4.5 and Table 4.5).

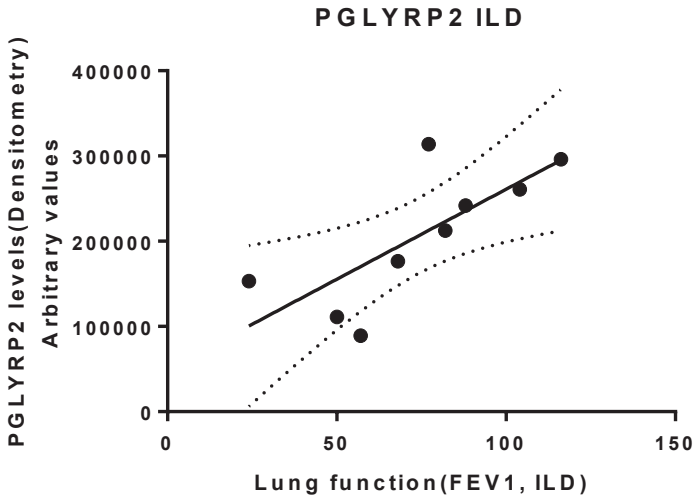

Figure 4.5: A graph showing a correlation between the lung functions and levels of PGLYRP2 in ILD patients. Lung function values (in %) are plotted along the x axis and the corresponding value of PGLRP2 level is plotted along y axis. The serum concentrations are plotted along y axis in terms of densitometry (arbitrary values). Key: PGLYRP2, N-acetylmuramoyl-L-alanine amidase; ILD, interstitial lung disease.

Table 4.5: Pearson coefficient 'r' showing statistical association between PGLYRP2 levels and lung function tests in ILD patients and its significance. Key: r: Pearson correlation coefficient. Significance: $p<0.05$

Pearson r	
r	0.7496
P value	
P (two-tailed)	0.0200
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	9

4.3.2 Correlation between clinical severity of LAM and PGLYRP2 levels

PGLYRP2 levels were shown to be significantly altered/ decreased in LAM patients as compared to the normal and ILD groups. This has been described in the results section. The lung functions of the LAM patients were compared the PGLYRP2 levels in LAM patients a graph was plotted (Figure 3.35). Again the lung function was plotted along x axis and PGLYRP2 levels were plotted along y axis. It was seen that PGLYRP2 values showed a decline as the lung disease became severer. The lower the value of lung function, in other words the greater the impairment of lung function the lower the value of PGLYRP2 was seen in LAM patients. The patients who suffered from severe disease, with lung function test values below 50% had the lowest values of PGLYRP2. A particular trend was observed here which was seen in most patients. It can therefore be deduced that PGLYRP2 levels are significantly reduced in LAM patients as compared to normal individuals and ILD patients the decreased levels also correspond with the severity of the disease showing a relationship (Figure 4.6 and Table 4.6).

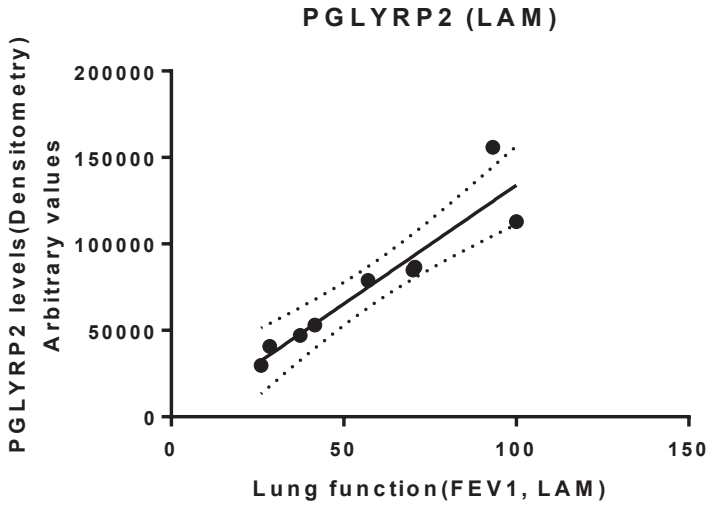

Figure 4.6: A graph showing a correlation between the lung functions and levels of PGLYRP2 in LAM patients. Lung function values (in %) are plotted along the x axis and the corresponding value of PGLYRP2 level is plotted along y axis. The serum concentrations are plotted along y axis in terms of desitometry (arbitrary values). Key: PGLYRP2, N-acetylmuramoyl-L-alanine amidase; ILD, interstitial lung disease.

Table 4.6: Pearson coefficient 'r' showing statistical association between PGLYRP2 levels and lung function tests in LAM patients and its significance. Key: r: Pearson correlation coefficient. Significance: $p<0.05$

Pearson r	
r	0.9358
P value	
P (two-tailed)	0.0002
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	9

4.4 Correlation between the severity of lung functions and levels of plasma serine protease inhibitor in ILD

The plasma serine protease inhibitor levels were observed to be increased in ILD patients as compared to the normal subjects and the LAM patients and this has been described in the results section. Further, the increased levels were compared with the clinical severity of the disease. The values were plotted on a graph (figure 4.7) with lung functions along the x axis and plasma serine protease inhibitor levels along the y axis in terms of densitometry (arbitrary values). It was observed that the plasma serine protease inhibitor levels showed an increase with deteriorating lung functions in ILD patients (Table 4.7). The three patients with the lowest lung function values had the severest disease and maximally raised values of plasma serine protease inhibitor as compared to the rest of the group. Even in patients with moderate decline in lung functions there was a rise in the S plasma serine protease inhibitor levels. A trend of steady rise in plasma serine protease inhibitor levels with increasingly severe disease in ILD was seen. Thus it can be deduced that with severe disease plasma serine protease inhibitor levels get higher and can be useful indicator to determine disease severity in ILD in clinical settings in case of an established ILD patient.

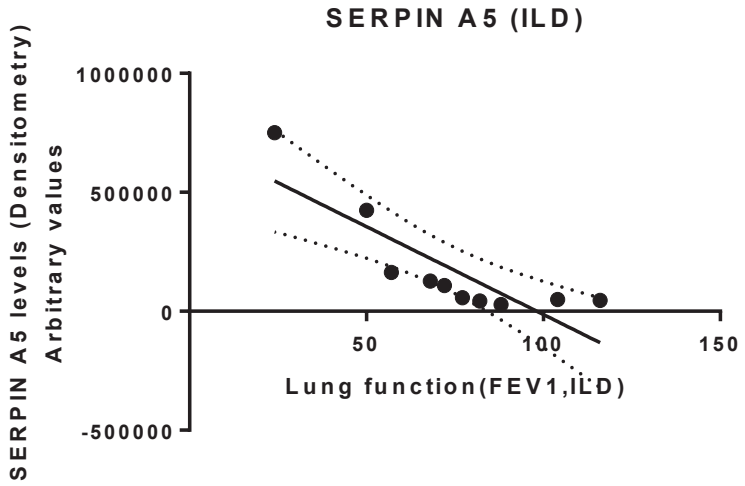

Figure 4.7: A graph showing a correlation between the lung functions and levels of plasma serine protease inhibitor in ILD patients. Lung function values (in %) are plotted along the x axis and the corresponding value of plasma serine protease inhibitor level is plotted along y axis. The serum concentrations are plotted along y axis in terms of densitometry (arbitrary values). Key: plasma serine protease inhibitor; ILD, interstitial lung disease.

Table 4.7 Shows Pearson coefficient r showing statistical association between plasma serine protease inhibitor levels and lung function tests in ILD patients and its significance. Key: r: Pearson correlation coefficient. Here $p<0.05$

Pearson r	
r	-0.8435
P value	
P (two-tailed)	0.0022
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	10

4.5 Correlation between the severity of lung functions and levels of Fetuin-B in LAM and ILD

The fetuin-B levels were seen to be significantly altered in LAM patients as compared to ILD group. It was further evaluated to look for any correlation if existed between its levels in blood and the clinical severity of LAM and ILD. The lung functions were plotted along x axis in percentage and Fetuin-B levels were plotted along y axis in terms of densitometry, to determine any relationship between the two. The resultant graph obtained did not show any particular pattern or trend eliminating the possibility of any relation between the disease severity and Fetuin-B levels in both LAM and ILD groups

CHAPTER 5

DISCUSSION

5.1 Validation of differential protein expression in LAM and ILD

The research described in this thesis has provided some promising data to support the potential for future diagnostic or prognostic markers for LAM, based on differential expression of particular serum proteins in patients with LAM compared to the normal individuals. This research has shown, for the first time that Fetuin-B is a potential diagnostic biomarker to differentiate LAM from ILD, heparin cofactor 2 is a potential diagnostic biomarker to differentiate LAM from normal individuals and PGLYRP2 is potential diagnostic biomarker for differentiating LAM from ILD and normal individuals. Additionally, it has also, for the first time, shown a correlation between lung functions tests and potential biomarkers in LAM and ILD, i.e. APO A4, PGLYRP2 and plasma serine protease inhibitor are potential biomarkers for determining clinical severity of lung disease in ILD whereas heparin cofactor 2 and PGLYRP2 are potential biomarkers of determining clinical severity of lung disease in LAM.

The findings for each of the protein candidates are discussed below. The discussion of the findings is divided in to 3 main parts, whereby the first part will focus on the protein markers that were validated as differentially expressed (either increased or decreased) in LAM patient serum, the second part will focus on the protein markers that, after validation, were shown not to be differentially expressed in LAM patients, and the third part will focus on the validated protein markers whose expression appears to be correlated with the severity of LAM and/or ILD.

There is very little, if any, information regarding the use of these proteins as biomarkers in other diseases, and very little information addressing diagnostic biomarkers for LAM in general, hence the novelty and importance of this work in validating some potential future diagnostic biomarkers for LAM.

Although the sample size for this study was not very large, if considered for ILD, which belongs to a very diverse and large group of interstitial lung diseases and occurs more frequently than LAM in the general population, this is in contrast to LAM which is an extremely rare condition and the results obtained with even a small sample number would be of significance.

5.1.1 Protein candidates showing differential expression in LAM and/or ILD

Of the 10 proteins selected for validation from the previous proteomics analysis, 1 was shown to be increased in expression in LAM and 2 were increased in ILD samples compared to controls. Interestingly, 2 were decreased in LAM, compared to ILD patient samples.

Fetuin-B was validated to be differentially expressed (decreased in LAM as compared to ILD) in LAM as compared to ILD. Heparin cofactor 2 was validated to be differentially expressed (decreased in LAM as compared to normal individuals) in LAM as compared to normal individuals. PGLYRP2 was validated to be differentially expressed (decreased in LAM compared to ILD patients and normal individuals) in LAM as compared to ILD and normal individuals.

5.1.1.2 Proteins showing increased expression in LAM and/or ILD

Of the ten proteins validated in this study, two showed increased expression in LAM and/or ILD compared to the normal cohort and this was APO A1 and plasma serine protease inhibitor.

In this study, APO A1 levels were shown to be almost similar in the LAM and normal control sample, with a very slight increase in the LAM patients, but were apparently clearly decreased in the ILD patients (Figure 3.23). This is of interest as the levels of this marker appear to differentiate ILD, one of the most common interstitial lung diseases, from the normal controls and from patients with LAM.

SEPRIN A5 levels were shown to be slightly increased in the LAM patient samples compared to the normal control cohort, but were less than the levels in the ILD sample group ($p>0.05$) who had markedly increased plasma serine protease inhibitor levels. The levels of this serum marker appear to be able differentiate LAM from ILD. Thus plasma serine protease inhibitor is one serum protein that should be selected for further study in a larger cohort to confirm these findings. Clinically, LAM (which is often mistaken for ILD) and ILD, a common interstitial lung disease, mostly present with similar features and thus a reliable diagnostic biomarker would substantially improve the quality of life by reducing the requirement of hospitalisation and invasive diagnostic techniques currently in use in order to determine the diagnosis of LAM.

Correlation analysis of plasma serine protease inhibitor levels with clinical severity of ILD in this sample population showed a significant negative correlation between increasing plasma serine protease inhibitor levels and worsening lung function or clinical severity ($p<0.05$, Pearson r =0.0012). These results together, paint a picture of plasma serine protease inhibitor as a potential biomarker of severity of ILD. Diagnosed ILD patients may benefit from the discovery of such a potential biomarker for determining the severity and of course of the disease as it may help to determine early therapeutic intervention time which may identify ways to improving the outcomes of therapeutic management of the disease.

5.1.1.2 Proteins showing decreased expression in LAM and/or ILD

Of the 10 proteins selected for validation, 6 were shown to be decreased in LAM and/or ILD patient samples compared to the normal cohort. These were (a) SHBG, (b) Fetuin-B, (c) heparin cofactor 2, (d) PGLYPR2, (e) Talin 1 and (f) APO A4. The results of each of these protein candidates are discussed below.

(a) SHBG levels, as shown in figure 3.19, are lower in the LAM patient group compared to the normal, but higher than the ILD group. The levels of this marker protein are somewhat lower in the ILD group compared to the LAM group. Similar to the APO A1 marker, SHBG levels could have potential as a means of differentiating ILD from normal individuals.

(b) Fetuin-B levels were found to be significantly decreased in the LAM patient cohort compared to ILD patient cohort ($p<0.05$). Thus Fetuin-B levels, as determined by ELISA, may have potential diagnostic value for LAM and for differentiating LAM from ILD. Thus Fetuin-B is one serum protein that should be selected for further study in a larger cohort to confirm these findings. Clinically, LAM (which is often mistaken for ILD) and ILD, a common interstitial lung disease, mostly present with similar features and thus a reliable diagnostic biomarker would substantially improve the quality of life by reducing the requirement of hospitalisation and invasive diagnostic techniques currently in use in order to determine the diagnosis of LAM.

(c) Heparin cofactor 2 levels were shown to be decreased in the LAM patient cohort compared to both the normal control and ILD patient cohorts although this decrease in LAM is significant only as compared to normal group only ($p<0.05$). Thus, this marker may prove to be useful in the diagnosis of LAM using a simple blood test. Heparin cofactor 2 is another protein which should

be followed up with further validation studies in a larger cohort to confirm the current findings. As there was no significant difference in the levels of this marker between the LAM and ILD groups, it would not be considered for potential use as a biomarker for diagnosing ILD or differentiating between LAM and ILD diagnoses.

Of interest, correlation analysis of heparin cofactor 2 levels with clinical severity of LAM showed that levels of this protein decreased with increasing severity of the disease. This trend was noted in the majority of the patients (about 80%). When similar clinical correlations were determined for ILD, no specific pattern or trend was observed. This is a very valuable finding and points towards a promising potential biomarker for determining the severity of LAM using a simple blood test.

(*d) PGLYRP2* levels were shown to be significantly decreased in the LAM sample cohort compared to both the normal control (p=0.0061) and ILD patient (p=0.00047) cohorts. These results are very promising for the potential use of PGLYRP2 for the diagnosis of LAM. This is therefore another marker that should be further validated in a larger cohort of patients to confirm the findings of this study.

Interestingly, when the levels of this protein were correlated with the clinical features of the LAM patients, a strong correlation was found between decreasing PGLYRP2 levels and decreasing lung function test results or increase in disease the severity (Pearson's r = 0.935 ,p value 0.0002), as determined by FEV1 and FVC. This information adds further to the potential utility of this biomarker as a diagnostic tool for diagnosing LAM and also for aiding in the determination of the severity of LAM using a simple blood test.

Correlation of PGLYRP2 levels with clinical features of ILD showed a trend of positive correlation, i.e. with the decreasing levels of PGLYRP2 a decrease in lung function tests was observed (in other words, an increase in severity of ILD) (Pearson's r = 0.749, p value 0.020). Therefore, this protein marker can be included as a potential serum biomarker for determining clinical severity of ILD.

(e) Talin-1 were slightly decreased in the LAM sample group compared to the control cohort (P>0.05), but were more markedly, decreased compared to the ILD patient cohort (p>0.05). The levels of Talin 1 do not differentiate the LAM group from the ILD or normal groups in this study population.

(***(f) APO A4*** levels were observed to be decreased in both the LAM and the ILD patient cohorts compared to the normal control group (P>0.05 in all cases). The levels of APO A4 were almost the same in both the LAM and ILD groups. Thus serum APOA4 levels do not appear to distinguish between LAM and ILD in this sample population. In this study, other parameters such as age, gender, lifestyle, treatment taken and severity or stage of the diseases have not been not considered.

When APO A4 levels were correlated with the severity of clinical features in ILD, a decrease in the levels of APO A4 in ILD patients was correlated with increasing severity of the disease as determined by the lung function tests FEV1 and FVC. Additionally, if studied at different stages of disease, this may yield information pertaining to path or clinical course of the disease.

5.1.2 Proteins showing no significant change in expression in LAM and/or ILD

The remaining 2 of the 10 candidate proteins selected for validation showed no change in expression among the three sample cohorts. These were (i) LBP and (ii) PLTP and are discussed below.

(i) Lipoplysaccharide binding protein (LBP) levels were not significantly different among the three groups studied. The LAM and ILD patients chosen were of differing disease stages and severity levels, hence correlations between LBP levels and disease severity or stage of disease were not possible in this case. Overall, serum LBP levels do not appear to be useful for diagnosis of LAM or ILD. In this study, age, gender and lifestyle factors were not considered when determining inclusion or exclusion criteria. These factors may have impacted the study outcomes in one or more ways. If investigated further, it may be possible that at a given level of severity a correlation (between LBP levels and clinical features) may exist.

(ii) PLTP levels were not able to be determined with accuracy, as in this validation study PLTP showed vague results with the appearance of multiple bands in the western blot analysis. With the

use of another antibody this issue was still not resolved. An ideal, or near ideal, primary antibody for western blotting could not be identified after thorough research and some interpretations were deduced from the results obtained. The source of the additional bands was either antibody non-specificity or the presence of post-translational modifications of PLTP. Experiments were carried out to determine the source of the additional bands but all experiments were inconclusive, and results from this marker have not been included.

5.2 Correlations between candidate biomarker protein levels and clinical severity of LAM and ILD

Some of the candidate biomarker proteins tested showed significant alterations in expression levels in either LAM or ILD patient's samples compared to the normal group. These candidates were further evaluated to determine if there were any correlations between marker expression changes and clinical disease severity. Clinical severity was determined by measurement of lung function including the measures FEV1 and FVC which have been briefly explained (section 3). These are standard tests used to determine the extent of lung function impairment in the clinical setting and provide a reasonable estimation of the severity of lung disease. Three of the 10 candidate proteins were found to have altered expression levels upon validation, two more showed a differential trend and were therefore selected to determine if the protein expression changes correlated with measures of disease severity in any of the patient groups. The proteins selected were APO A4, Heparin cofactor 2, plasma serine protease inhibitor and PGLYRP2. The lung function measures and hence clinical severity of disease, of the LAM patients (Table 5.1) and normal group (Table 5.2) are presented with additional information pertaining to treatments given in the case of the LAM patients. Clinical data for ILD patients whose blood samples have been analysed for the validation of differential protein expression have been provided in the results (chapter 3) (Table 3.6).

Table 5.1 Clinical characteristics of the LAM Patients from whom serum samples were obtained for this study. Lung function test results are shown and the highlighted values indicate severe lung function deterioration.

LAM Sample number	Patient Age	LAM Sample	Forced Expiratory Volume 1 (FEV1) %	Sirolimus Treatment given
001	37	Serum/plasma	37.4*	yes
003	48	Serum/plasma	100	No
004	67	Serum/plasma	41.6*	yes
005	42	Serum/plasma	70.6	No
006	48	Serum/plasma	28.5*	No
007	44	Serum/plasma	93.2	No
009	69	Serum/plasma	94	No
010	70	Serum/ Plasma	57*	No
011	36	Serum/Plasma	26*	No
013	37	Serum/Plasma	70	yes

#Reference ranges: Mild incapacity (70-79%), Moderate incapacity (60-69%), Severe incapacity (less than 60%).
*Abnormal results (severe)

Table 5.2 Clinical characteristics of the normal individuals from whom serum samples obtained for this study. Lung function test results are shown (all of which are normal).

Normal Sample number	Age	Normal Sample	Forced Expiratory Volume 1 (FEV1) %
001	51	Serum/plasma	100
002	46	Serum/plasma	100
004	41	Serum/plasma	100
005	36	Serum/plasma	100
006	33	Serum/plasma	100
007	26	Serum/plasma	100
008	25	Serum/plasma	100
009	54	Serum/ Plasma	100
011	59	Serum/Plasma	100
012	53	Serum/Plasma	100

5.3 Conclusions

5.3.1 General conclusions

Any work that aimed at advancing LAM diagnostics is a contribution towards better disease management. This work has provided some interesting and important novel data that may, in future, be useful in the early and quick diagnosis of LAM. Any single step taken towards a timely, non-invasive diagnostic test, will contribute towards increasing quality of life for LAM patients. The prime goal was to provide clinicians with an accurate and reliable test as a diagnostic tool able to differentiate LAM and IDL from each other and allowing early treatment

intervention, making the diagnostic journey less invasive and overall improving the quality of life of LAM sufferers.

From this study, some promising candidate proteins have been identified as potential markers for LAM diagnosis and for determining disease severity. Heparin cofactor 2 has shown promise in terms of diagnosing LAM as compared to normal individuals. Similarly, PGLYRP2 has shown promise in finding a diagnostic biomarker for LAM that can differentiate LAM from closely resembling conditions and normal healthy subjects by just a simple blood test. Fetuin-B has also shown a promising potential in diagnosing LAM as compared to ILD. Clinically LAM resembles ILD and requires a series of invasive diagnostic techniques in order to differentiate and diagnose it. It would become simple and quick to diagnose LAM or ILD just by a blood test.

Additionally, correlations between candidate protein levels and disease severity have shown some interesting and valuable correlations. Although this was not a primary aim of the study, it is a very interesting finding and will lead towards the invention of tools for monitoring disease progression and assessing severity. APO A4 levels showed a corresponding decrease with increasing severity in ILD. It can be further studied in ILD in order to identify a biomarker for determining the disease severity or measuring disease progression in diagnosed ILD patients. Heparin cofactor 2 and PGLYRP2 have also shown correlations with the severity of LAM. In addition to having shown a promising potential in diagnosing LAM, these proteins have shown an ability to assess severity of LAM and thus have a potential to also measure disease progression. plasma serine protease inhibitor and PGLYRP2 levels have shown a correlation with disease severity in ILD and thus shown a potential in determining the stage of disease and its progression in already diagnosed ILD patients.

The cost of the disease burden is also tremendous and studies such as this contribute towards lessening this pressure. Simple, easy, less invasive, less expensive and quick diagnostic tools for chronic debilitating diseases like LAM are the need of the hour. The usual time delays in disease identification impacts individuals and their families. Major invasive techniques put burden on patients with the morbidity associated with these procedures being an added disadvantage to the patients and economies. According to the statistics, the treatment given for the interventional

procedures side effects is a further cost that both patients and the governments pay. Additionally, the individual's contribution to the society as a healthy participating member is also compromised.

Lightening the burden of disease associated morbidity is something that can be achieved to a great degree by effective treatment plan. Discoveries that unfold the hidden dynamics of a disease process can aid in the finding effective treatments by targeting the exact mechanism involved in initiating the disease process. This particular study worked at finding the altered products of complex disease processes, thus pointing towards the possible underlying pathways or mechanisms. The area of LAM blood biomarkers identification is a multidimensional field because altered proteins in LAM are an expression of altered processes operating in such individuals which need to identified, and this identification would also contribute towards advancement in finding disease etiology, target treatments, measuring prognosis and thus reduction in overall morbidity associated with it or maybe in future mortality as well.

This study has been the beginning of a journey towards improving the management outcomes in LAM patients, which range from prevention, early diagnosis, and appropriate timely treatment to increased life span in such patients. The direction and findings that this study has provided is a valuable addition in this research area and to other closely resembling conditions in terms of disease processes, genetic resemblance and phenotypic behaviour (e.g. different cancers, interstitial lung diseases and other respiratory illnesses).

5.3.2 Novelty of the work and contribution to LAM diagnostics

The findings that have been discovered in this study are a way forward to advancement towards easier, quicker and reliable modes of diagnosis for LAM. Certain proteins have also shown promise towards finding potential diagnostic tools for ILD. Additionally, the correlation with the clinical severity of the disease is a very promising discovery as it would point towards a path of identifying biomarkers of severity for ILD and LAM. This in turn may also direct towards the identifying tools that can reliably detect the clinical course of the disease. Table 5.3 summarises the main contributions of the study.

Table 5.3: Summary of the findings and contribution made by this study. Potential biomarkers for diagnosing LAM and ILD and potential biomarkers for determining clinical severity of LAM and ILD are indicated.

Protein name	Differential expression	Clinical correlation	P value for differential expression	P value for clinical correlation	Outcome
Fetuin-B	Yes, In LAM as compared to ILD	None identified	0.023	None identified	Potential biomarker for diagnosing LAM as compared to ILD
APO A4	None identified	Yes, strong positive in ILD	None identified	0.0012	Potential biomarker for determining clinical severity in ILD.
Heparin cofactor 2	Yes, In LAM as compared to normal	Yes, strong positive in LAM	0.030	0.0037	Potential biomarker for diagnosing LAM as compared to normal Potential biomarker for determining clinical severity of LAM
PGLYRP2	Yes, In LAM as compared to ILD and normal subjects	Yes, strong positive in LAM and ILD	LAM vs. Normal 0.0061 LAM vs. ILD 0.00047	In LAM 0.0002 In ILD 0.020	Potential biomarker for diagnosing LAM as compared to ILD and normal subjects Potential biomarker for determining severity of LAM and ILD
Plasma serine protease inhibitor	None identified	Yes, negative in ILD	None identified	0.002	Potential biomarker for determining severity of ILD

This is a novel study that validated potential diagnostic biomarkers for LAM. The comparison of these differentially expressed proteins in LAM with other similar respiratory diseases has been completed for the first time in this study. There are several novel findings in this study (Table 5.3). Secondly, identification of a correlation between protein levels and the clinical features of the diseases has also yielded novel significant results. These findings have a great potential in terms of identifying a means of quickly diagnosing LAM by a simple blood test. This will prevent patients undergoing a great deal of stress and delayed management associated with undiagnosed debilitating conditions. Performing a simple blood test to measure differentially expressed proteins as diagnostic biomarkers is one of the least invasive, time efficient techniques to diagnose diseases. This study has identified potential diagnostic biomarkers for LAM. Clinically correlating biomarkers are the potential biomarkers for determining severity of a disease. Several potential biomarkers for determining severity of LAM and ILD have also been identified in this study.

5.4 Future Directions

There could be further discovery in this area following this initial pilot biomarker study and aspects of this study that could be followed up and expanded are discussed here. Firtsly, some promising results have shown the importance or potential of certain validated proteins as biomarkers for LAM diagnosis. While this study used a small sample cohort, the next step is to validate the utility of these biomarkers in a larger cohort of patients to confirm and further validate the work described in this thesis.

Additionally, the stability of the biomarker, if checked in LAM patients and clinically similar conditions over a period of time (e.g. three months) will help determine if any change can be identified in marker expression level may be correlated with disease progression, regression or stability in the disease process itself. These significantly altered proteins, if investigated further, could prove to be effective tools for determining LAM prognosis in the future. It may become possible to stage the disease by further studying the significantly altered proteins based on their quantitative expression in the blood. Additionally, altered protein levels, when measured as part

of clinical management, may also help in assessing the efficacy of therapeutic agents for LAM over time by measuring these protein levels and comparing the response to the therapeutic agent.

Ideal randomisation and optimal selection of patients is also an important aspect to be considered when testing biomarkers for diagnosis. In future, lifestyle risk factors should also be well matched in order to obtain reliable results. These include: 1) tobacco and alcohol consumption, 2) body mass index and physical activity and 3) use of over the counter drugs. Stratification of patients according to genetic risk is also pivotal to ideal randomisation. This could be considered in the future studies and would lead to determining near ideal blood biomarkers.

Finally, in terms of realising aim 3, a diagnostic algorithm may yet be developed with the support of a greater body of data and statistically significant data. This work represented a preliminary study to determine the potential for developing diagnostic and prognostic biomarkers for LAM from proteomics data showing differential protein expression in the sera of LAM patients and normal individuals. Since LAM is an extremely rare disease, gathering a number of subjects for a statistically significant cohort will be a challenging factor for future studies.

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