

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

AYESHA JAVED

**School of Life Sciences
Faculty of Science
University of Technology Sydney**

2018

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.

Production Note:

Signature: Signature removed prior to publication.

Date: 21-01-2019

Acknowledgements

I would first like to thank my thesis supervisor Dr Najah Nassif of the School of Life Sciences and Faculty of Science at the University of Technology Sydney. She was always available to answer any query pertaining to my research project and was very supportive. She consistently steered me in the right direction whenever she thought I needed it.

I would also like to thank the experts who taught me various techniques, procedures and provided guidance: Associate Professor Sarah Lal and Professor Fatima Shad, without their passionate participation and input I would not have been able to accomplish my goals in this project.

I would also like to acknowledge Lisa Windon for her support in completing tasks, providing encouragement and guidance and I am gratefully indebted to her for her very valuable suggestions.

Finally, I would like to express very profound gratitude to my parents and to my husband Naveed Ahmad for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them.

Table of Contents

Chapter 1 Introduction.....	18
1.1 Overview of LAM.....	18
1.2 LAM Prevalence.....	20
1.3 Differential Diagnosis.....	21
1.4 The genetic basis of LAM.....	21
1.5 Expression of Progesterone versus Oestrogen in LAM cell.....	23
1.6 PEcomas and Lymphangi leiomyomatosis.....	23
1.7 Clinical Features of LAM.....	25
1.8 Air Travel and Pregnancy for LAM Patients.....	26
1.9 Screening and Follow-up of LAM.....	27
1.10 Mortality Rate in LAM.....	27
1.11The Clinical Course of LAM.....	28
1.12 Treatment and Clinical Management of LAM.....	28
1.13 Diagnostic criteria for LAM.....	31
1.14 Biomarkers: an introduction.....	38
1.14.1 Development of non-invasive diagnostic and prognostic biomarkers for LAM.....	38
1.14.2 Previous work.....	39
(A) Validated differentially expressed Proteins.....	42

(B) Differentially expressed Proteins yet to be validated.....	42
1.15 Need for a Biomarker and Background to the Current Project.....	45
1.15.1 Limitations of current diagnostic procedures for LAM.....	45
1.15.2 Developing new serum-based diagnostic biomarkers for LAM	46
1.16 Hypotheses and Aims of the Project.....	46
Chapter 2 Materials and Methods.....	48
2.1 Materials.....	48
2.1.1 General Materials and Reagents.....	48
2.1.2 Materials for ELISA analysis.....	48
2.1.3 Materials used for Western Analysis.....	49
2.1.4 Protein standards for electrophoresis.....	50
2.2 Methods.....	51
2.2.1 Blood biobank establishment and sample collection.....	51
2.2.2 Study Population.....	51
2.2.3 Study cohorts and sample processing.....	52
2.2.4 Study Design and Plan.....	53
2.2.5 ELISA Protocols.....	57
2.2.6 Western blot protocols for optimization and validation.....	61
2.2.7 Data handling and data analysis.....	66
Chapter 3 Results 1: Validation of potential serum biomarkers for LAM diagnosis.....	67
3.1 Introduction.....	69

3.2 Selection of differentially expressed proteins and validation of differential expression using ELISA and western analysis.....	70
3.2.1 Optimisation of ELISA and Western Blot prior to validation of altered protein expression.....	71
3.2.2 Validation of LBP as a potential biomarker for LAM.....	82
3.2.3 Validation of Fetuin-B as a potential biomarker for LAM.....	84
3.2.4 Validation of plasma serine protease inhibitor as a potential biomarker for LAM.....	86
3.2.5 Validation of PLTP as a potential biomarker for LAM.....	90
3.2.6 Validation of SHBG as a potential biomarker for LAM.....	92
3.2.7 Validation of APO A4 as a potential biomarker for LAM.....	94
3.2.8 Validation of APO A1 as a potential biomarker for LAM.....	96
3.2.9 Validation of Heparin cofactor 2 as a potential biomarker for LAM.....	98
3.2.10 Validation of N-acetylmuramoyl-L-alanine amidase (PGLYRP2) as a potential biomarker for LAM.....	101
3.2.11 Validation of Talin 1 as a potential biomarker for LAM.....	103
Chapter 4 Results 2: Comparison between Biomarker levels and Clinical severity of disease.....	110
4.1 Correlation between the severity of lung functions and APO A4 levels in LAM and ILD...	111
4.1.1 Correlation between clinical severity of ILD and APO A4 levels.....	111
4.1.2 Correlation between clinical severity of LAM and APO A4 levels.....	113
4.2 Correlation between the severity of lung functions and levels of Heparin cofactor 2 in LAM and ILD.....	115
4.2.1 Correlation between clinical severity of LAM and Heparin cofactor 2 levels.....	115

4.2.2 Correlation between clinical severity of ILD and Heparin cofactor 2 levels.....	117
4.3 Correlation between the severity of lung functions and levels of PGLYRP2 in LAM and ILD.....	118
4.3.1 Correlation between clinical severity of ILD and PGLYRP2 levels.....	118
4.3.2 Correlation between clinical severity of LAM and PGLYRP2 levels.....	120
4.4 Correlation between the severity of lung functions and levels of plasma serine protease inhibitor in ILD.....	121
4.5 Correlation between the severity of lung functions and levels of Fetuin-B in LAM and ILD.....	123
Chapter 5 Discussion.....	124
5.1 Validation of differential protein expression in LAM and ILD.....	126
5.1.1 Protein candidates showing differential expression in LAM and/or ILD.....	126
5.1.2 Proteins showing no significant change in expression in LAM and/or ILD.....	130
5.2 Correlation between candidate biomarker protein levels and clinical severity of LAM and ILD.....	131
5.3 Conclusion.....	133
5.4 Future Directions.....	135
5.5 Novelty and contribution to LAM diagnostics.....	136
REFERENCES.....	140

LIST OF FIGURES

Figure 1.1: Schematic diagram of the mTOR pathway showing the different sites of action of various drugs used to treat LAM.....	31
Figure 1.2: Lung section of a LAM patient.....	33
Figure 1.3: Histological features of LAM.....	34
Figure 1.4: Computerised tomography (HRCT) of the chest in LAM.....	36
Figure 1.5: Abdominal CT scanning in LAM.....	37
Figure 2.1: Collection and biobanking of samples from the 3 study groups used in this study...	53
Figure 2.2: Methodological plan for the current project.....	54
Figure 2.3 Overall project plan.....	55
Figure 2.4: Serial dilutions for the LBP standard.....	58
Figure 2.5: Protocol for preparing serial dilutions of standard for Fetuin-B	59
Figure 3.1: Optimisation of detection and western analysis of plasma serine protease inhibitor at 1/500 serum dilution and 1/2000 antibody dilution.....	73
Figure 3.2: Optimisation of detection and western analysis of PLTP at 1/1000 serum dilution and 1/2000 antibody dilution.....	74
Figure 3.3: Optimisation of detection and western analysis of PLTP at varying serum dilutions with 1/2000 antibody dilution.....	75
Figure 3.4: Optimisation of detection and western analysis of SHBG at varying serum dilutions with 1/2000 antibody dilution.....	75
Figure 3.5: Optimisation of detection and western analysis of SHBG at varying serum dilutions with 1/3000 antibody dilution.	76

Figure 3.6: Optimisation of detection and western analysis of SHBG at varying serum dilutions with 1/3000 antibody dilution	77
Figure 3.7 Optimisation of detection and western analysis of APO4 at varying serum dilutions with 1/2000 antibody dilution	77
Figure 3.8: Optimisation of detection and western analysis of APOA4 at varying serum dilutions with 1/1000 antibody dilution.....	78
Figure 3.9: Optimisation of detection and western analysis of APOA1 at varying serum dilutions with 1/2000 antibody dilution	79
Figure 3.10: Optimisation of detection and western analysis of APOA1 at varying serum dilutions with 1/1000 antibody dilution.....	79
Figure 3.11: Determining LBP concentration in the serum of LAM patients relative to ILD patients and normal subjects.....	83
Figure 3.12: Concentration of LBP in the serum of LAM patients as compared to ILD patients and normal subjects.....	84
Figure 3.13: Determining the concentration of Fetuin-B in the serum of LAM patients relative to ILD patients and normal subjects.....	85
Figure 3.14: Concentration of Fetuin-B in the serum of LAM patients as compared to ILD patients and normal subjects.....	86
Figure 3.15: Determining the concentration of plasma serine protease inhibitor in the serum of LAM patients, ILD patients and normal subjects.....	87
Figure 3.16: Concentration of plasma serine protease inhibitor in the serum of LAM patients as compared to ILD patients and normal subjects.....	88
Figure 3.17: Serum proteins were electrophoresed on SDS PAGE gels and transferred to a membrane for western analysis of PLTP.....	91

Figure 3.18: Serum proteins were electrophoresed on SDS PAGE gels and transferred to a membrane for western analysis of PLTP.....	92
Figure 3.19: Serum SHBG levels in LAM patients as compared to ILD patients and normal subjects.....	93
Figure 3.20: Concentration of SHBG in serum of LAM patients as compared to ILD patients and Normal subjects.....	94
Figure 3.21: Serum APO A4 levels in LAM patients as compared to ILD patients and Normal subjects.....	95
Figure 3.22: Concentration of APO A4 in serum of LAM patients as compared to ILD patients and Normal subjects.....	96
Figure 3.23: Serum APO A1 levels in LAM patients as compared to ILD patients and Normal subjects.....	97
Figure 3.24: Concentration of APO A1 in serum of LAM patients as compared to ILD patients and Normal subjects.....	98
Figure 3.25: Serum Heparin cofactor 2 levels in LAM patients as compared to ILD patients and Normal subjects.....	99
Figure 3.26: Concentration of Heparin cofactor 2 in serum of LAM patients as compared to ILD patients and Normal subjects.....	100
Figure 3.27: Serum PGLYRP2 levels in LAM patients as compared to ILD patients and Normal subjects.....	101
Figure 3.28: Concentration of PGLYRP2 in serum of LAM patients as compared to ILD patients and Normal subjects.....	102
Figure 3.29: Serum Talin 1 levels in LAM patients as compared to ILD patients and Normal subjects.....	104

Figure 3.30: Concentration of Talin 1 in serum of LAM patients as compared to ILD patients and Normal subjects.....	105
Figure 4.1: A graph showing a correlation between the lung functions and levels of APO A4 in ILD patients.....	112
Figure 4.2: A graph to show a correlation between the lung functions and levels of APO A4 in LAM patients.....	114
Figure 4.3: A graph showing a correlation between the lung functions and levels of Heparin cofactor 2 in LAM patients.....	116
Figure 4.4: A graph showing a correlation between the lung functions and levels of Heparin cofactor 2 in ILD patients.....	117
Figure 4.5: A graph showing a correlation between the lung functions and levels of PGLYRP2 in ILD patients.....	119
Figure 4.6: A graph showing a correlation between the lung functions and levels of PGLYRP2 in LAM patients.....	120
Figure 4.7: A graph showing a correlation between the lung functions and levels of plasma serine protease inhibitor in ILD patients.....	122

LIST OF TABLES

Table 1.1: Differentially expressed proteins identified in serum samples of LAM patients.....	41
Table 1.2: Differentially expressed proteins identified in serum samples of LAM patients as compared to normal individuals and validated by ELISA.....	41
Table 2.1: A list of some of the shortlisted ELISA kits available.....	56
Table 2.2: Serial dilutions carried out in order to obtain the various serum concentrations for ELISA.....	60
Table 2.3 Preparation of the various primary antibody dilutions used in the optimisation of the western blotting experiments for each protein.....	63
Table 2.4: Secondary antibodies used for each primary antibody being validated.....	64
Table 3.1 Summary of the target proteins to be validated for differential expression and the techniques employed for validation of each candidate protein.....	69
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.....	81
Table 3.3: Clinical characteristics of the ILD Patients from which serum samples have been obtained for this study.....	89
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.....	106
Table 3.5 It shows the levels of each protein in LAM as compared to ILD patients as validated using reliable methods.....	107
Table 3.6 shows the p value of each protein in one group as compared to the other groups.....	108
Table 4.1 Shows Pearson coefficient r showing statistical association between APO A4 levels and lung function tests in ILD.....	112

Table 4.2: Shows Pearson coefficient r showing statistical association between APO A4 levels and lung function tests in LAM patients.....	114
Table 4.3: Shows Pearson coefficient r showing statistical association between heparin cofactor 2 levels and lung function tests in ILD patients.....	116
Table 4.4: Shows Pearson coefficient r showing statistical association between heparin cofactor 2 levels and lung function tests in ILD patients.....	118
Table 4.5: Shows Pearson coefficient r showing statistical association between PGLYRP2 levels and lung function tests in ILD patients.....	119
Table 4.6: Shows Pearson coefficient r showing statistical association between PGLYRP2 levels and lung function tests in LAM patients.....	121
Table 4.7 Shows Pearson coefficient r showing statistical association between plasma serine protease inhibitor levels and lung function tests in ILD patients.....	122
Table 5.1 Clinical characteristics of the LAM Patients from whom serum samples were obtained for this study.....	132
Table 5.2 Clinical characteristics of the normal individuals from whom serum samples obtained for this study.....	133
Table 5.3: Summary of findings and contribution made by this study.....	137

.

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 lymphangiomyomatosis (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.