

**ELUCIDATION OF CLINICAL AND LABORATORY  
FEATURES, COMORBIDITY RISKS, TREATMENT  
OPTIONS AND MOLECULAR PATHOPHYSIOLOGY  
OF ANTIPHOSPHOLIPID SYNDROME (APS)  
PATIENTS**

**MD ASIFUL ISLAM**

**UNIVERSITI SAINS MALAYSIA**

**2018**

**ELUCIDATION OF CLINICAL AND  
LABORATORY FEATURES, COMORBIDITY  
RISKS, TREATMENT OPTIONS AND  
MOLECULAR PATHOPHYSIOLOGY OF  
ANTIPHOSPHOLIPID SYNDROME (APS)  
PATIENTS**

by

**MD ASIFUL ISLAM**

**Thesis submitted in fulfilment of the requirements**

**for the degree of**

**Doctor of Philosophy**

**October 2018**

## ACKNOWLEDGEMENTS

Foremost, I would like to acknowledge the almighty Allah (God) for providing my non-stop provisions, keeping me amongst the privileged human beings and allowing me to continue my study up to a PhD level. I would like to express my sincere gratitude to my main supervisor Professor Dr. Gan Siew Hua for her continuous support to my PhD study and research, for her patience, motivation, enthusiasm and immense knowledge. Her guidance helped me all the way in my research and publications. This thesis would not have been possible without her active support. I could not have imagined having a better supervisor and mentor for my PhD study. Whenever I departed from the right path, she always patiently brought me back on track. She not only taught me how to be a researcher, but also how to be a good human being. I would also like to thank both of my co-supervisors - Associate Professor Dr. Teguh Haryo Sasongko and Dr. Rowani Mohd Rawi for their support, kindness and advices throughout my struggles.

I am grateful to all the staff and students of Human Genome Centre, Department of Pharmacology and Department of Immunology, School of Medical Sciences, Universiti Sains Malaysia (USM). During my PhD journey, I met some great research personalities and I would like to show my gratitude especially to Professor Dr. Md. Ibrahim Khalil (Bangladesh, Malaysia), Professor Dr. Mohammad Amjad Kamal (Australia, Saudi Arabia), Dr. Cinzia Cavestro (Italy), Professor Dr. Roger Levy (USA, Brazil) and Professor Dr. Doruk Erkan (USA) for their immense academic and research-oriented supports.

I owe my deepest gratitude to Malaysia International Scholarship (2012-2015) and USM Vice-Chancellor Award (2015-2018) for supporting my studentship and Bahagian Jaringan Industri & Masyarakat (BJIM) grant (PI-2012-012) for supporting this project. I would like to thank Dr. Xenia Specka and Mr. Md. Abdul Muqit Zoarder for assisting in translating an article in German language (Straube *et al.*, 1998); Dr. Suet Kee Loo, Ms. Zichen Zhang and Dr. Md. Ahsanul Kabir Khan for translating the articles in Chinese language (Meiping *et al.*, 1996 and Gong *et al.*, 2007) and Dr. Roopa Rajan and Dr. Cornelia Calcii for providing the raw data of Rajan *et al.*, 2014 and Calcii *et al.*, 2016, respectively.

I would like to dedicate this work to my mother - *Kazi Asma Begum*; my father - *Md. Rabiul Islam*, who passed away in November 2016 (1942-2016) and my only sister - *Masuma Akter Romani* who passed away in March 2017 at the age of 40 years. In addition, this work is dedicated to all those who have been suffering from or passed away due to antiphospholipid syndrome. Thanks to all of my great friends who's continuous supports, constructive criticisms have always made me feel that "I can do better".

## TABLE OF CONTENTS

Acknowledgements.....	ii
Table of Contents.....	iv
List of Tables.....	xvii
List of Figures.....	xix
List of Abbreviations.....	xxiv
Abstrak.....	xxx
Abstract.....	xxxii

### CHAPTER 1 : INTRODUCTION

1.0 Introduction.....	1
1.1 Antiphospholipid syndrome.....	1
1.2 Types of APS.....	1
1.3 Epidemiology.....	4
1.4 Major clinical features of APS.....	4
1.4.1 Thrombosis.....	4
1.4.2 Pregnancy complications.....	6
1.5 Non-criteria clinical features of APS.....	6
1.6 Molecular pathogenesis.....	9
1.6.1 Thrombosis.....	9
1.6.2 Pregnancy complications.....	10
1.7 Genetics of APS.....	11
1.8 Management.....	11
1.8.1 Thrombosis.....	11
1.8.1(a) Primary thromboprophylaxis.....	11

1.8.1(b) Secondary thromboprophylaxis.....	12
1.8.2 Pregnancy complications.....	13
1.9 Objectives.....	14
1.9.1 General objective.....	14
1.9.2 Specific objectives.....	14

## CHAPTER 2 : OPTIMISATION OF HIGH-QUALITY RNA

2.0 Optimisation of high-quality RNA extraction.....	16
2.1 Introduction.....	16
2.2 Materials and methods.....	17
2.2.1 RNA extraction from cell lines and lymphocytes.....	17
2.2.2 RNA extraction and purification: Protocol A.....	18
2.2.3 Genomic DNA removal and addition of RiboLock: Protocol B, C and D .....	19
2.2.4 RNA integrity number (RIN) check.....	20
2.3 Results.....	21
2.4 Discussion.....	32
2.5 Summary.....	33

## CHAPTER 3 : FAMILIAL AND RETROSPECTIVE STUDIES OF APS PATIENTS

3.1 Familial study of APS patients.....	35
3.1.1 Introduction.....	35

3.1.2 Case presentation.....	36
3.1.2(a) Case 1.....	36
3.1.2(b) Case 2.....	36
3.1.2(c) Case 3.....	37
3.1.3 Discussion.....	42
3.1.4 Future directions.....	43
3.1.5 Summary.....	44
3.2 Retrospective study of APS patients.....	45
3.2.1 Introduction.....	45
3.2.2 Materials and methods.....	46
3.2.2(a) Data acquisition.....	46
3.2.2(b) Statistical analyses.....	47
3.2.3 Results.....	47
3.2.3(a) Demographic characteristics.....	47
3.2.3(b) Clinical characteristics.....	48
3.2.3(c) Immunological features.....	55
3.2.3(d) Haematological features.....	55
3.2.3(e) Chemical pathological features.....	59
3.2.3(f) Treatment strategies.....	63
3.2.3(g) Patients for genetic analysis with RNA-Seq.....	63
3.2.4 Discussion.....	65
3.2.4(a) Demographic characteristics.....	65

3.2.4(b) Clinical characteristics.....	66
3.2.4(c) Other clinical characteristics.....	67
3.2.4(d) Immunological features.....	68
3.2.4(e) Haematological features.....	69
3.2.4(f) Chemical pathological features.....	70
3.2.4(g) Treatment strategies.....	70
3.2.5 Limitations and future directions.....	71
3.2.6 Summary.....	71

CHAPTER 4 : GENETIC RISK FACTORS IN THROMBOTIC PRIMARY ANTIPHOSPHOLIPID SYNDROME: A SYSTEMATIC REVIEW WITH BIOINFORMATIC ANALYSES

4.1 Introduction.....	73
4.2 Methods.....	74
4.2.1 Inclusion criteria.....	74
4.2.1(a) Type of studies.....	74
4.2.1(b) Type of participants.....	75
4.2.2 Search strategy.....	75
4.2.2(a) PubMed.....	75
4.2.2(b) Web of Science.....	76
4.2.2(c) Scopus.....	76
4.2.2(d) Google Scholar.....	77

4.2.2(e) ScienceDirect.....	77
4.2.3 Quality assessment.....	77
4.2.4 Bioinformatic analyses.....	78
4.2.4(a) Functional enrichment analysis.....	78
4.2.4(b) Molecular interaction analysis.....	78
4.2.4(c) Pathway analysis.....	78
4.2.4(d) Gene expression analysis: RNA-Seq data.....	79
4.3 Results.....	79
4.3.1 Selection of studies.....	79
4.3.2 Characteristics of the included studies.....	81
4.3.3 Quality assessment.....	92
4.3.4 Bioinformatic analyses.....	92
4.3.4(a) Functional enrichment analysis.....	92
4.3.4(b) Protein-protein interaction analysis.....	99
4.3.4(c) Pathway analysis.....	99
4.3.4(d) Gene expression analysis: RNA-Seq data.....	99
4.4 Discussion.....	105
4.4.1 Summary of the thrombotic APS-associated genes.....	105
4.4.1(a) PF4V1 (Platelet factor 4 variant 1).....	105
4.4.1(b) SELP (Selectin P).....	106
4.4.1(c) TLR2 (Toll like receptor 2).....	107
4.4.1(d) TLR4 (Toll like receptor 4).....	107

4.4.1(e) SERPINE1 (Serpin family E member 1).....	108
4.4.1(f) APOH (Apolipoprotein H).....	109
4.4.1(g) ITGA2 (Integrin subunit alpha-2).....	111
4.4.1(h) GP1BA (Platelet glycoprotein Ib alpha chain).....	112
4.4.1(i) F2R (Coagulation factor II thrombin receptor).....	112
4.4.1(j) F2RL1 (F2R like trypsin receptor 1).....	113
4.4.1(k) TFPI (Tissue factor pathway inhibitor).....	115
4.4.1(l) F3 (Coagulation factor III, tissue factor).....	115
4.4.1(m) VEGFA (Vascular endothelial growth factor A).....	116
4.4.1(n) FLT1 (FMS-related tyrosine kinase 1).....	117
4.4.1(o) F2 (Coagulation factor II, thrombin).....	118
4.4.1(p) TNF (Tumour necrosis factor).....	119
4.4.2 Bioinformatic analyses.....	119
4.5 Limitations and strengths.....	120
4.6 Future directions.....	121
4.7 Summary.....	121

**CHAPTER 5 : COMORBID RISK FACTORS: META-ANALYSES ON  
MIGRAINE, EPILEPSY AND DEMENTIA**

5.1 Migraine and aPLs.....	123
5.1.1 Introduction.....	123
5.1.2 Methods.....	124

5.1.2(a) Inclusion and exclusion criteria.....	125
5.1.2(b) Search strategy.....	125
5.1.2(c) Data collection and analysis.....	126
5.1.3 Results.....	128
5.1.3(a) Selection of studies.....	128
5.1.3(b) Characteristics of the included studies.....	130
5.1.3(c) Association between aPLs and migraine by meta-analysis.....	137
5.1.3(d) Subgroup analyses.....	141
5.1.3(e) Quality assessment.....	146
5.1.3(f) Publication bias.....	146
5.1.3(g) Heterogeneity.....	146
5.1.4 Discussion.....	154
5.1.4(a) Migraine and aPLs pathogenic relationships.....	154
5.1.4(b) Mediators of heterogeneity.....	157
5.1.4(c) Limitations and strengths.....	157
5.1.4(d) Future directions.....	158
5.1.5 Summary.....	159
5.2 Epilepsy and aPLs.....	160
5.2.1 Introduction.....	160
5.2.2 Methods.....	162
5.2.2(a) Eligibility criteria.....	162
5.2.2(b) Literature search.....	162

5.2.2(c) Data extraction.....	165
5.2.2(d) Heterogeneity and quality assessment.....	165
5.2.2(e) Statistical analyses.....	166
5.2.3 Results.....	167
5.2.3(a) Selection of studies.....	167
5.2.3(b) Study characteristics.....	169
5.2.3(c) Presence of aCL in epilepsy.....	170
5.2.3(d) Presence of anti- $\beta$ 2-GPI in epilepsy.....	170
5.2.3(e) Subgroup analysis of adult and paediatric subjects.....	176
5.2.3(f) Subgroup analysis of generalised and partial epilepsy.....	176
5.2.3(g) Subgroup analysis of studies from Asia, Europe and Africa.....	179
5.2.3(h) Heterogeneity and quality assessment.....	179
5.2.3(i) Publication bias assessment.....	186
5.2.4 Discussion.....	188
5.2.5 Summary.....	193
5.3 Dementia and aPLs.....	194
5.3.1 Introduction.....	194
5.3.2 Methods.....	196
5.3.2(a) Study selection criteria.....	196
5.3.2(b) Literature search.....	197
5.3.2(c) Data extraction, management and quality assessment.....	199
5.3.2(d) Exploration of heterogeneity and publication bias.....	200

5.3.2(e) Statistical analyses.....	200
5.3.3 Results.....	201
5.3.3(a) Study selection.....	201
5.3.3(b) Study characteristics and quality assessment.....	203
5.3.3(c) Assessment of aCL presence by meta-analysis.....	207
5.3.3(d) Subgroup analysis on VD and DAT.....	207
5.3.3(e) Subgroup analysis on different age ranges.....	207
5.3.3(f) Subgroup analysis on patients in different continents.....	208
5.3.3(g) Heterogeneity and publication bias.....	216
5.3.4 Discussion.....	219
5.3.5 Summary.....	224

CHAPTER 6 : COCHRANE SYSTEMATIC REVIEW AND META-ANALYSIS: ANTIPLATELET AND ANTICOAGULANT AGENTS FOR PREVENTING RECURRENCE OF PERIPHERAL VASCULAR THROMBOSIS IN PATIENTS WITH ANTIPHOSPHOLIPID SYNDROME

6.1 Introduction.....	225
6.1.1 Description of the condition.....	225
6.1.2 Description of the intervention.....	228
6.1.2(a) Warfarin.....	228
6.1.2(b) Heparin.....	229
6.1.2(c) Rivaroxaban.....	230

6.1.2(d) Apixaban.....	230
6.1.2(e) Edoxaban.....	231
6.1.2(f) Dabigatran.....	231
6.1.2(g) Aspirin.....	232
6.1.2(h) Clopidogrel.....	233
6.1.3 How the intervention works.....	234
6.1.3(a) Warfarin.....	234
6.1.3(b) Heparin.....	234
6.1.3(c) Rivaroxaban.....	235
6.1.3(d) Apixaban.....	235
6.1.3(e) Edoxaban.....	235
6.1.3(f) Dabigatran.....	236
6.1.3(g) Aspirin.....	236
6.1.3(h) Clopidogrel.....	236
6.1.4 Why it is important to do this review.....	237
6.1.5 Objectives.....	237
6.2 Methods.....	237
6.2.1 Criteria for considering studies for this review.....	237
6.2.1(a) Types of studies.....	237
6.2.1(b) Types of participants.....	238
6.2.1(c) Types of interventions.....	238
6.2.1(d) Types of outcome measures.....	238

6.2.2 Search methods for identification of studies.....	239
6.2.2(a) Electronic searches.....	239
6.2.2(b) Searching other resources.....	240
6.2.2(c) Search Strategy.....	240
6.2.3 Data collection and analysis.....	244
6.2.3(a) Selection of studies.....	244
6.2.3(b) Data extraction and management.....	245
6.2.3(c) Assessment of risk of bias in included studies.....	245
6.2.3(d) Measures of treatment effect.....	245
6.2.3(e) Unit of analysis issues.....	245
6.2.3(f) Dealing with missing data.....	246
6.2.3(g) Assessment of heterogeneity.....	246
6.2.3(h) Assessment of reporting biases.....	246
6.2.3(i) Data synthesis.....	246
6.2.3(j) Subgroup analysis and investigation of heterogeneity.....	247
6.3 Results.....	247
6.3.1 Description of studies.....	247
6.3.1(a) Results of the search.....	247
6.3.1(b) Included studies.....	247
6.3.2 Assessment of risk of bias in included studies.....	252
6.3.2(a) Allocation.....	252
6.3.2(b) Blinding.....	252

6.3.2(c) Incomplete outcome data.....	252
6.3.2(d) Selective reporting.....	253
6.3.3 Effects of interventions on primary outcomes (thrombosis).....	261
6.3.3(a) Moderate vs high-intensity of warfarin.....	261
6.3.3(b) Warfarin vs NOACs.....	261
6.3.3(c) Antiplatelet vs antiplatelet + anticoagulant.....	261
6.3.4 Effects of interventions on primary outcomes (bleeding).....	263
6.3.4(a) Major bleeding.....	263
6.3.4(b) Minor bleeding.....	263
6.3.5 Secondary outcomes.....	263
6.4 Discussion.....	267
6.4.1 Summary of main results.....	267
6.4.2 Overall completeness and applicability of evidence.....	267
6.4.3 Quality of the evidence.....	267
6.4.4 Potential biases in the review process.....	268
6.4.5 Agreements and disagreements with other studies or reviews.....	268
6.5 Summary.....	268
6.5.1 Implications for clinical practice.....	268
6.5.2 Implications for research.....	268
 CHAPTER 7 : CONCLUSION	
Conclusion.....	270

REFERENCES.....272

APPENDICES

APPENDIX A: HUMAN ETHICAL APPROVAL

APPENDIX B: PATIENT INFORMED CONSENT FORM

APPENDIX C: PRISMA & MOOSE CHECKLIST

APPENDIX D: R (METAFOR PACKAGE) CODES

APPENDIX E: AWARDS & SCHOLARSHIPS

LIST OF PUBLICATIONS AND PRESENTATIONS

## LIST OF TABLES

	<b>Page</b>
Table 1.1	Sydney classification criteria of APS 2
Table 1.2	Non-criteria manifestations of APS 7
Table 2.1	RNA quality and quantity in human lymphoma cell lines with un-optimised protocol 22
Table 2.2	Optimisation of the purification column spinning time 24
Table 2.3	Optimisation of the centrifuge speed 25
Table 2.4	Optimisation using different combinations of RNA purification protocols 26
Table 2.5	RIN values of RNA samples on days 0, 3 and 7 using the optimised RNA purification protocol 29
Table 3.1.1	Clinical and laboratory features of the three family members with primary APS during their initial diagnosis (2005-2009) 39
Table 3.1.2	Updated laboratory features of the three family members following a follow-up diagnosis in 2014 41
Table 3.2.1	Demographic characteristics of APS patients 49
Table 3.2.2	Clinical classification criteria and other manifestations of the investigated APS patients 51
Table 3.2.3	Immunological features of APS patients 56
Table 3.2.4	Haematological features of APS patients 58
Table 3.2.5	Chemical pathological features of APS patients (Kidney) 60
Table 3.2.6	Chemical pathological features of APS patients (Liver) 61
Table 3.2.7	Drug therapies received by the APS patients 64

Table 4.1	Major characteristics of the included studies	83
Table 4.2	Characteristics of thrombosis-associated primary APS genes	86
Table 4.3	Risk of bias assessment of the included studies according to the modified Newcastle-Ottawa Scale (NOS)	93
Table 4.4	Thrombotic primary APS-associated gene set enrichment analysis based on functional annotations and protein interactions network	95
Table 5.1.1	Major characteristics of the included studies	132
Table 5.1.2	Migraine diagnostic criteria, antiphospholipid antibody determination techniques and cut-off ranges used in the included studies	134
Table 5.1.3	Subgroup analysis	143
Table 5.1.4	Risk of bias assessment of the included studies according to the modified Newcastle-Ottawa Scale (NOS)	148
Table 5.2.1	Major characteristics of the included studies in this meta-analysis	171
Table 5.2.2	Risk of bias assessment of the included studies according to the modified Newcastle-Ottawa Scale (NOS)	183
Table 5.3.1	Major characteristics of the case-control studies mentioned in the meta-analysis	204
Table 5.3.2	Risk of bias assessment of the included studies according to the modified Newcastle-Ottawa Scale (NOS)	206
Table 6.1	Characteristics of the included studies	250
Table 6.2	Risk of bias of the included studies	254
Table 6.3	Summary of findings	265

## LIST OF FIGURES

		<b>Page</b>
Figure 1.1	Theoretical Framework of the study	15
Figure 2.1	RNA quality in human lymphoma cell lines	23
Figure 2.2	Absorbance ratios observed from different RNA quality optimising protocols indicating that protocol D is the best	27
Figure 2.3	Quality of RNA extracted using various protocols again indicating that protocol D is the best in terms of achieving optimum $A_{260/280} (\geq 1.8)$ , $A_{260/230} (\geq 1.8)$ and RNA yield ( $\geq 1.0 \mu\text{g}$ )	28
Figure 2.4	Gel images showing RIN values of RNA samples on days 0, 3 and 7 using TapeStation system	30
Figure 2.5	Electropherograms showing RNA integrity on day 0 (A), day 3 (B) and day 7 (C) via intact 18S and 28S RNAs	31
Figure 3.1.1	Family pedigree showing three family members (patients A, B and C) are confirmed, while three other members are suspected to have primary APS	38
Figure 3.2.1	Major clinical manifestations observed in APS patients of HUSM	53
Figure 3.2.2	Non-criteria clinical manifestations observed in APS patients from HUSM	54
Figure 4.1	PRISMA diagram of the study selection process	80

Figure 4.2	Protein-protein interactions among the identified thrombosis-associated primary APS gene-encoded proteins	101
Figure 4.3	Haemostasis pathway: platelet adhesion, activation; fibrin clot formation, dissolution and cell surface interactions at the vascular wall are affected by the APS thrombosis-associated gene-encoded proteins identified in the present study	102
Figure 4.4	Immune system: Interleukins and NF-Kappa B pathways are affected by the APS thrombosis-associated gene-encoded proteins identified in the present study	103
Figure 4.5	Gene expression levels of the identified genes in this study from RNA-Seq data on 32 different organs	104
Figure 5.1.1	Flow diagram on the study selection process	129
Figure 5.1.2A	Forest plot of the presence of aCL antibody in migraine patients as compared to healthy controls	138
Figure 5.1.2B	Forest plot of the presence of anti- $\beta$ 2-GPI antibody in migraine patients as compared to healthy controls	139
Figure 5.1.2C	Forest plot on the presence of LA in migraine patients as compared to healthy controls	140
Figure 5.1.3	Sensitivity analyses of the studies assessing aCL (A), anti- $\beta$ 2-GPI (B) and LA (C) antibodies	150

Figure 5.1.4	Funnel plot on the risk of publication bias in the meta-analysis assessing the presence of (A) aCL, (B) anti- $\beta$ 2-GPI antibody and (C) LA in migraine patients	151
Figure 5.1.5A	L'Abbé plot for aCL antibody suggesting no substantial heterogeneity	152
Figure 5.1.5B	L'Abbé plot for anti- $\beta$ 2-GPI antibody suggesting no substantial heterogeneity	152
Figure 5.1.5C	L'Abbé plot for LA suggesting possible heterogeneity	153
Figure 5.2.1	Flow diagram on the study selection process	168
Figure 5.2.2A	Forest plot on the presence of aCL antibodies in epilepsy patients	175
Figure 5.2.2B	Forest plot on the presence of anti- $\beta$ 2-GPI antibodies in epilepsy patients	175
Figure 5.2.3A	Subgroup analysis on the presence of aCL antibodies in adult and paediatric epilepsy patients	177
Figure 5.2.3B	Subgroup analysis on the presence of anti- $\beta$ 2-GPI antibodies in adult and paediatric epilepsy patients	177
Figure 5.2.4A	Subgroup analysis on the presence of aCL antibodies in generalised and partial epilepsy patients	178
Figure 5.2.4B	Subgroup analysis on the presence of anti- $\beta$ 2-GPI antibodies in generalised and partial epilepsy patients	178
Figure 5.2.5A	Subgroup analysis on the presence of aCL antibodies in European, African and Asian epilepsy patients	180
Figure 5.2.5B	Subgroup analysis on the presence of anti- $\beta$ 2-GPI antibodies in European and African epilepsy patients	181

Figure 5.2.6A	L'Abbé plot for aCL antibody suggests absence of substantial heterogeneity	182
Figure 5.2.6B	L'Abbé plot for anti- $\beta$ 2-GPI antibody suggests absence of substantial heterogeneity	182
Figure 5.2.7	Sensitivity analysis of the studies assessing aCL (A) and anti- $\beta$ 2-GPI (B) antibodies	185
Figure 5.2.8A	Contour-enhanced funnel plot assessing publication bias reporting aCL antibodies	187
Figure 5.2.8B	Contour-enhanced funnel plot assessing publication bias reporting anti- $\beta$ 2-GPI antibodies	187
Figure 5.3.1	Flow diagram on the study selection process	202
Figure 5.3.2	Forest plot representing the presence of aCL antibodies in dementia patients as compared to controls	209
Figure 5.3.3A	Subgroup analysis of aCL in patients with VD	210
Figure 5.3.3B	Subgroup analysis of aCL in patients with DAT	211
Figure 5.3.4A	Subgroup analysis of aCL in dementia patients with age range of 60 to 70 years	212
Figure 5.3.4B	Subgroup analysis of aCL in dementia patients with above 70 years old	213
Figure 5.3.5A	Subgroup analysis of aCL in Asian and European dementia population	214
Figure 5.3.5B	Subgroup analysis of aCL in North and South American dementia population	215

Figure 5.3.6	Sensitivity analysis of the studies assessing aCL antibodies	216
Figure 5.3.7	L'Abbé plot suggests no substantial heterogeneity for the assessment of aCL antibodies	217
Figure 5.3.7	Funnel plot showing the risk of publication bias assessing the presence of aCL antibodies in dementia patients	218
Figure 6.1	Flow diagram on the study selection process	249
Figure 6.2	Risk of bias graph: review authors' judgements about each risk of bias item presented as percentages across all included studies	259
Figure 6.3	Risk of bias summary: review authors' judgements about each risk of bias item for each included study	260
Figure 6.4	Forest plot showing moderate vs high-intensity of warfarin in preventing recurrent thrombosis	262
Figure 6.5	Forest plot showing moderate vs high-intensity of warfarin in assessing incidences of major bleeding	264
Figure 6.6	Forest plot showing moderate vs high-intensity of warfarin in assessing incidences of minor bleeding	264

## LIST OF ABBREVIATIONS

aCL	Anticardiolipin antibody
AD	Alzheimer's disease
ADP	Adenosine diphosphate
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANA	Antinuclear antibody
Anti- $\beta$ 2-GPI	Anti- $\beta$ 2-glycoprotein I
aPLs	Antiphospholipid antibodies
APOH	Apolipoprotein H
APS	Antiphospholipid syndrome
APTT	Activated partial thromboplastin time
AST	Aspartate aminotransferase
ATIII	Antithrombin III
AU	Arbitrary units
BBB	Blood-brain barrier
B&H	Benjamini-Hochberg
BD	Binswanger's disease
BGI	Beijing genomics institute
BIC	Benign infantile convulsion
CE	Cryptogenic epilepsy
CGRP	Calcitonin gene-related peptide
CI	Confidence interval
CIS	The Cochrane vascular information specialist

CNS	Central nervous system
CRS	The Cochrane register of studies
CT	Computed tomography
CTE	Controllable epilepsy
DALYs	Disability-adjusted life years
DAT	Dementia of the Alzheimer's type
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DRVVT	Dilute Russell's viper venom time
dsDNA	Double stranded DNA
DSM	Diagnostic and statistical manual of mental disorders
DVT	Deep vein thrombosis
ELISA	Enzyme-linked immunosorbent assay
F2	Coagulation factor II
F2R	Coagulation factor II thrombin receptor
F3	Coagulation factor III
F2RL1	F2R like trypsin receptor 1
FDR	False discovery rate
FLT1	FMS-related tyrosine kinase 1
GE	Generalised epilepsy
GO	Gene ontology
GP1BA	Platelet glycoprotein Ib alpha chain
GPL	Immunoglobulin G phospholipids
GI	Gastrointestinal
HR	Hazard ratio

HUSM	Hospital Universiti Sains Malaysia
$I^2$	Inconsistency (heterogeneity)
ICC	Immunocytochemistry
ICHD	International classification of headache disorders
IEMA	Immunoenzymometric assay
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
INR	International normalised ratio
IL	Interleukin
ILAE	International league against epilepsy
IRAK 1	Interleukin-1 receptor-associated kinase 1
ITGA2	Integrin subunit alpha-2
KCT	Kaolin clotting time
LA	Lupus anticoagulant
LLOD	Lower limit of detection
LMWH	Low molecular weight heparin
MA	Migraine with aura
MD	Mixed dementia
MD	Mean difference
MeSH	Medical subject heading
MHC	Major histocompatibility complex
MI	Myocardial infarction
MMSE	Mini mental state examination
MOA	Migraine without aura

MOOSE	Meta-analysis of observational studies in epidemiology
MPL	Immunoglobulin M phospholipids
mRNA	Messenger RNA
MS	Multiple seizure
NDE	Newly diagnosed epilepsy
NF- $\kappa$ B	Nuclear factor kappa B
NGS	Next-generation sequencing
NINCDS-ADRDA	National institute of neurological and communicative disorders and stroke and the Alzheimer's disease and related disorders association
NK	Natural killer
NOACs	New oral anticoagulants
NOS	Newcastle-Ottawa scale
OR	Odds ratio
PAI-1	Plasminogen activator inhibitor-1
PAMP	Pathogen-associated molecular pattern
PAR	Proteinase-activated receptor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
PE	Pulmonary embolism
PE	Partial epilepsy
PF4	Platelet factor 4

PF4V1	Platelet factor 4 variant 1
PGE2	Prostaglandin E2
PLT	Platelet
PRISMA	Preferred reporting items for systematic review and meta-analysis
RCT	Randomised controlled trial
RE	Refractory epilepsy
RevMan	Review manager
RF	Rheumatoid factor
RFLP	Restriction fragment length polymorphism
RIN	RNA integrity number
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
rpm	Revolutions per minute
RR	Risk ratio
RT-PCR	Reverse transcription-polymerase chain reaction
RU	Relative unit
RVVT	Russell's viper venom time
SELP	Selectin P
SERPINE1	Serpin family E member 1
SGU	Standard IgG unit
SLE	Systemic lupus erythematosus
SMD	Standardised mean difference
SNP	Single nucleotide polymorphism
STRING	Search tool for the retrieval of interacting genes

T <sub>c</sub>	T-cytotoxic
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
T <sub>h</sub>	T-helper
TIA	Transient ischaemic attack
TLE	Temporal lobe epilepsy
TLR	Toll-like receptor
TNF- $\alpha$	Tumour necrosis factor- $\alpha$
TNFR2	Tumour necrosis factor receptor 2
tPA	Tissue plasminogen activator
TPM	Transcripts per kilobase million
T <sub>reg</sub>	T-regulatory
tRNA	Transfer RNA
TXA <sub>2</sub>	Thromboxane A <sub>2</sub>
UE	Unclassified epilepsy
VD	Vascular dementia
VEGFA	Vascular endothelial growth factor A
Vitamin KO	Vitamin K epoxide
VKOR	Vitamin KO reductase
VTE	Venous thromboembolism
vWF	von Willebrand factor
WBC	White blood cell

**PENJELASAN CIRI-CIRI KLINIKAL DAN MAKMAL, RISIKO-RISIKO  
KOMORBIDITI, PILIHAN RAWATAN DAN PATOFISIOLOGI  
MOLEKULAR PESAKIT-PESAKIT SINDROM ANTIFOSFOLIPID (APS)**

**ABSTRAK**

Sindrom antifosfolipid (APS) adalah penyakit autoimun sistemik yang disifatkan dengan kehadiran peredaran antibodi antifosfolipid (aPLs) seperti antikoagulan lupus (LA), antikardiolipin (aCL) dan anti- $\beta$ 2 glikoprotein I antibodi ( $\beta$ 2-GPI) fosfolipid yang mengikat kepada protein. Walaupun penyakit ini telah wujud kira-kira 35 tahun lalu, kriteria diagnostik, faktor-faktor risiko, patogenesis, aspek-aspek genetic dan strategi rawatan kurang difahami serta belum dibangunkan sepenuhnya. Dalam kajian komprehensif ini, ciri-ciri klinikal dan makmal, faktor-faktor risiko genetic, risiko komorbidity, patofisiologi molekular dan strategi rawatan optimum kepada pesakit-pesakit APS dikaji. Kaedah pengekstrakan dan penulenan RNA yang berkualiti dan berintegriti tinggi diperolehi daripada sel mononuklear darah periferal manusia (PBMC) yang telah dioptimumkan (kelajuan pengempar: 14000rpm + masa putaran: 75 saat + rawatan DNase + perencat ribolock RNase perencat + pembersih RNA) yang boleh digunakan untuk menghantar RNA pesakit APS untuk tujuan penjujukan RNA. Dalam usaha mencari pesakit-pesakit APS, dua kajian telah dijalankan. Pertama, kes APS primer di kalangan keluarga dari Sarawak Malaysia; walaubagaimanapun pesakit-pesakit telah menghidap seronegatif and menerima rawatan warfarin dalam tempoh masa yang lama. Kes yang lain yang melibatkan subjek APS adalah dari Hospital Universiti Sains Malaysia (HUSM) yang telah disiasat secara retrospektif dengan menyiasat strategi klinikal, makmal dan rawatan. Kejadian morbiditi kehamilan yang tinggi, dan juga ciri klinikal yang luar biasa seperti ketidakfungsian hati dan buah pinggang secara berterusan, menorrhagia and sista ovari

telah dikaji. Penggunaan warfarin secara berintensiti sederhana telah berjaya menghalang thrombosis daripada berulang. Tambahan pula, disebabkan pesakit-pesakit HUSM tidak berminat untuk menyertai kajian ini secara sukarela, kami tidak dapat menghantar sample RNA ke BGI untuk penjujukan RNA. Satu ulasan sistematik menggunakan analisis bioinformatik telah dijalankan untuk mengenalpasti faktor-faktor risiko genetik kepada pesakit APS trombotik, dimana 16 gene dikaitkan dengan kesan thrombosis yang kebanyakan mempengaruhi laluan pembekuan darah dan sistem imun yang dikaitkan dengan APS. Secara keseluruhannya, tiga ulasan sistematik dan metaanalisis telah dijalankan bagi menentukan pengaruh aPLs pada pesakit-pesakit migrain, epilepsi dan dementia tanpa penyakit autoimun dan dibandingkan dengan individual kawalan, di mana aPLs dikaitkan dengan komorbid yang berkemungkinan bermanifestasi. Oleh itu, ciri-ciri neurologi telah dapat dikenalpasti pada peringkat awal klinikal manifestasi sebelum berkembang sepenuhnya kepada APS. Satu ulasan sistematik Cochrane telah dibangunkan untuk meneroka strategi rawatan yang optimum untuk subjek-subjek APS trombotik dimana pengambilan warfarin secara intensiti sederhana adalah lebih baik daripada pengambilan warfarin yang berintensiti tinggi. Keseluruhannya, kajian komprehensif ini meneroka ciri-ciri klinikal dan makmal, faktor-faktor risiko genetik, risiko komorbiditi, patofisiologi molekular dan strategi rawatan optimum untuk pesakit-pesakit APS telah berjaya dibangunkan.

**ELUCIDATION OF CLINICAL AND LABORATORY FEATURES,  
COMORBIDITY RISKS, TREATMENT OPTIONS AND MOLECULAR  
PATHOPHYSIOLOGY OF ANTIPHOSPHOLIPID SYNDROME (APS)**

**PATIENTS**

**ABSTRACT**

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterised by the presence of circulating antiphospholipid antibodies (aPLs) such as lupus anticoagulant (LA), anticardiolipin (aCL) and anti- $\beta$ 2-glycoprotein I ( $\beta$ 2-GPI) antibodies to phospholipid binding proteins. Although the disease has been in existence for approximately 35 years, the diagnostic criteria, risk factors, pathogenesis, genetic aspects, treatment strategies are poorly understood and have yet to be fully developed. In this study, the clinical and laboratory features, genetic risk factors, comorbidity risks, molecular pathophysiology and optimal treatment strategy of APS patients are explored. Human peripheral blood mononuclear cell (PBMC)-derived high-quality and integrity RNA extraction and purification method was optimised (centrifugal speed: 14000 rpm + spin time: 75 seconds + DNase treatment + Ribolock RNase inhibitor + RNA clean-up) which could be used to send APS patients' RNA for RNA-Seq. In quest of APS patients, two studies were conducted. Firstly, on a familial primary APS cases from Sarawak, Malaysia, patients however became seronegative following long warfarin therapy. Another one with APS subjects from Hospital Universiti Sains Malaysia (HUSM) were retrospectively investigated by exploring the clinical, laboratory and treatment strategies. High occurrence of pregnancy morbidity, as well as some unusual clinical features such as persistent dysfunction of liver and kidneys; menorrhagia and ovarian cyst were observed. The use of medium-intensity warfarin was successful in preventing thrombosis recurrence. Additionally, since the

HUSM patients were unwilling to participate in this study, we were unable to send the RNA samples for RNA-Seq to BGI. A systematic review with bioinformatic analyses was conducted to identify the genetic risk factors in thrombotic APS subjects where 16 genes were significantly associated with thrombosis affecting mostly the blood coagulation pathway and the immune system related to APS. Overall, three systematic reviews and meta-analyses were conducted to determine the influence of aPLs in patients with migraine, epilepsy and dementia without autoimmune disease as compared to controls, where aPLs were significantly comorbid with the said manifestations. Therefore, the neurologic features were early clinical manifestations before the development of full-blown APS. A single Cochrane systematic review was developed to explore the optimum treatment strategy for thrombotic APS subjects, where, moderate-intensity warfarin was superior than high-intensity warfarin. Overall, a comprehensive study exploring the clinical and laboratory features, genetic risk factors, comorbidity risks, molecular pathophysiology and optimal treatment strategy of APS patients was successfully established.

# CHAPTER 1

## INTRODUCTION

### 1.0 Introduction

#### 1.1 Antiphospholipid syndrome

Antiphospholipid syndrome (APS) or Hughes syndrome is a systemic autoimmune disease that was first described in 1983 (Hughes). Clinically, it is characterised by vascular thrombosis (venous and/or arterial) and/or pregnancy complications besides the presence of serum antiphospholipid antibodies (aPLs) such as lupus anticoagulant (LA), anticardiolipin (aCL) antibodies and anti- $\beta$ 2-glycoprotein I ( $\beta$ 2-GPI) antibodies. According to the latest classification criteria (Sydney criteria, Table 1.1), patients can only be confirmed as having APS when at least one laboratory marker and a clinical feature are present (Miyakis *et al.*, 2006). The major difference between the updated Sydney criteria and the first criteria (Sapporo) is the introduction of anti- $\beta$ 2-GPI and the addition of the cut-off values of LA, aCL and anti- $\beta$ 2-GPI in laboratory diagnosis (Wilson *et al.*, 1999).

#### 1.2 Types of APS

Broadly, there are three types of APS. APS is considered as primary when the patient has core clinical and laboratory features of definitive APS without the existence of another autoimmune disease (Asherson *et al.*, 1989). APS is secondary when at least one autoimmune disorder co-exists with the disease besides the core clinical and laboratory features of APS. In secondary APS, systemic lupus erythematosus (SLE) is the mostly observed coexisting autoimmune disease (Alarcón-Segovia *et al.*, 1989) while catastrophic APS is the most aggressive form of APS. Besides the clinical and laboratory features of APS, it is characterised by thrombi development in the small

blood vessels of multiple organs that result in multiple organ failure. Therefore, it is considered as the most common reason for the high mortality rate of APS (Cervera *et al.*, 2009).

**Table 1.1: Sydney classification criteria of APS**

---

<b>Clinical criteria</b>
<ul style="list-style-type: none"><li>• Vascular thrombosis<ul style="list-style-type: none"><li>- <math>\geq 1</math> arterial, venous or small vessel thromboses in any tissue or organ (excluding superficial thrombosis), as confirmed by appropriate imaging or histopathology.</li></ul></li><li>• Pregnancy morbidity<ul style="list-style-type: none"><li>- <math>\geq 1</math> unexplained deaths of a morphologically normal foetus at or beyond the 10<sup>th</sup> week of gestation.</li><li>- <math>\geq 1</math> premature births or a morphologically normal neonate before the 34<sup>th</sup> week of gestation owing to eclampsia, severe pre-eclampsia or placental insufficiency.</li><li>- <math>\geq 3</math> unexplained consecutive spontaneous abortions before the 10<sup>th</sup> week of gestation, with hormonal, chromosomal or maternal anatomical causes excluded.</li></ul></li></ul>
<b>Laboratory criteria</b>
<ul style="list-style-type: none"><li>- LA present in plasma, detected according to the guidelines of the International Society on Thrombosis and Haemostasis.</li></ul>

---

- 
- IgG and/or IgM isotype aCL present in medium to high titre [*i.e.* >40 IgG phospholipid (GPL) or IgM phospholipid (MPL) units] as measured by standard enzyme-linked immunosorbent assay (ELISA).
  - IgG and/or IgM isotype anti-β2-GP1 antibody in serum or plasma, present in medium/high titre (*i.e.* >99<sup>th</sup> percentile).

Any of the above three must be present on two or more occasions for at least 12 weeks apart and <5 years.

---

To fit the classification, one feature from each set of the clinical and laboratory criteria is required.

### **1.3 Epidemiology**

The incidence of APS is approximately five new cases per 100,000 persons per year with an estimated prevalence of approximately 40-50 patients per 100,000, and females are 3.5 times more likely to be affected than males (Cervera *et al.*, 2002). The total mortality rate was estimated as 9.3%, although it has been reported to be as high as 55.6% in catastrophic APS in which severe thrombosis is the most frequent cause of deaths (Cervera *et al.*, 2015). Though aPLs is observed in APS patients; in the general population, the prevalence of aPLs was estimated as 1-5% (Biggioggero and Meroni, 2010).

### **1.4 Major clinical features of APS**

#### **1.4.1 Thrombosis**

Among the acquired thrombophilic conditions, APS is the most frequently observed in young adults and is associated with both arterial and venous thrombosis (Nalli *et al.*, 2014). According to Cervera *et al.*, (2015), since APS affects at least 1% of the general population, aPL-induced vascular events exert a robust clinical impact and burden worldwide. According to the recent 10-year multicentre prospective study on 1000 APS patients (53.1% primary APS and 36.2% secondary APS), the most frequently reported causes of death were severe thrombosis (36.5%) and thrombotic events [stroke, transient ischemic attack (TIA), deep vein thrombosis (DVT), pulmonary embolism (PE), myocardial infarction (MI) and superficial thrombophlebitis] occurring in 21.4% of the patients (Cervera *et al.*, 2015). In APS patients, thrombosis (both venous and arterial) is the most common clinical manifestation and contributes to the high mortality rate (Santamaria *et al.*, 2005). Approximately 55% of APS patients suffer from venous thromboembolism (VTE)

including DVT and PE (Hanly, 2003), while cerebrovascular accidents and TIAs are the commonest (50%) arterial thrombotic manifestations (Nalli *et al.*, 2014).

In the general population, it has been estimated that aPLs are present in patients with stroke (13.5%), MI (11%) and DVT (9.5%) (Andreoli *et al.*, 2013). In fact, almost 20% of the patients (age <50 years) with stroke or VTE are APS patients (Roldan *et al.*, 2009; Ruiz-Irastorza *et al.*, 2011). Despite treatment with antiplatelet and/or anticoagulant agents, recurrent thrombosis in APS is a common phenomenon with a high occurrence rate (approximately 10%) (Ruiz-Irastorza *et al.*, 2011; Cervera *et al.*, 2015), possibly because of the lack of appropriate specific targeted regimens against the thrombotic pathogenesis of APS. In fact, in a primary APS cohort of Mexican ethnics, triple positivity for aPLs was the major independent risk factor for recurrent thrombosis (Hernandez-Molina *et al.*, 2013).

In APS patients, thrombi can appear in any tissues and/or organs as confirmed by imaging, ultrasound or histopathology studies, except in the case of superficial venous thrombosis (Miyakis *et al.*, 2006). Several key factors associated with cell activation (Atsumi *et al.*, 1998b; Forastiero *et al.*, 1998; Joseph *et al.*, 2001), coagulation pathway (Adams *et al.*, 2004; Giannakopoulos *et al.*, 2012; Dharma *et al.*, 2015), fibrinolytic pathway (Takeuchi *et al.*, 2002; Bu *et al.*, 2009), immune system (Hattori *et al.*, 2000; Simonin *et al.*, 2015) and genetic factors (Galli *et al.*, 2000; Caliz *et al.*, 2001; Karassa *et al.*, 2003) are affected by the interference of aPLs which further contribute to the development of thrombotic manifestations in APS.

### **1.4.2 Pregnancy complications**

According to the APS Alliance for Clinical Trials and International Networking (APS ACTION) group, aPLs were present in 6% of patients experiencing pregnancy morbidity (Andreoli *et al.*, 2013). In a multicentre prospective cohort study, Cervera *et al.* (2002) observed early miscarriages in 35.4% of cases, foetal death in 16.9%, premature birth in 10.6%, PE in 9.5%, eclampsia in 4.4% and abruption placentae in 2% of pregnant APS women (n=590) with 1580 pregnancies. According to the European Registry on Obstetric APS, recurrent miscarriage was the most frequently observed (approximately 54%) obstetrical complication in women with APS (Alijotas-Reig *et al.*, 2015). In APS, foetal death is observed due to the possible consequences of placental dysfunction which is firmly associated with the presence of aPLs (Silver *et al.*, 1993; Abou-Nassar *et al.*, 2011). From the analysis of Stillbirth Collaborative Research Network enrolling 512 stillbirths (2006-2008), aPLs were positive in 11.1% of the women [95% confidence interval (CI) 8.4-14.4] (Page *et al.*, 2017).

### **1.5 Non-criteria clinical features of APS**

Besides the definitive clinical criteria of APS, some other clinical manifestations are rather frequently observed in APS patients. Since these manifestations have not yet been recognised to be included in the criteria clinical features of APS, they are often known as non-criteria clinical features and are often observed to comorbid with APS. Among the non-criteria features; besides thrombocytopenia; different neurological (*i.e.*, migraine, epilepsy and dementia), cardiac, pulmonary, dermatological and renal manifestations are frequently observed to comorbid in patients with APS (Table 1.2). Nevertheless, the comorbidity risks of these non-criteria features in APS still needs to be investigated.

**Table 1.2: Non-criteria manifestations of APS**

<b>Non-criteria manifestations</b>	<b>References</b>
<ul style="list-style-type: none"> <li>• <b>Thrombocytopenia</b></li> </ul>	(Krause <i>et al.</i> , 2005; Cervera <i>et al.</i> , 2015)
<ul style="list-style-type: none"> <li>• <b>Neurological</b></li> </ul>	
<ul style="list-style-type: none"> <li>- Headache and migraine</li> </ul>	(Cervera <i>et al.</i> , 2009; Zhu <i>et al.</i> , 2014; Abreu <i>et al.</i> , 2015)
<ul style="list-style-type: none"> <li>- Epilepsy</li> </ul>	(Shoenfeld <i>et al.</i> , 2004; Stojanovich <i>et al.</i> , 2013)
<ul style="list-style-type: none"> <li>- Dementia</li> </ul>	(Mosek <i>et al.</i> , 2000; Chapman <i>et al.</i> , 2002)
<ul style="list-style-type: none"> <li>- Multiple sclerosis</li> </ul>	(Stosic <i>et al.</i> , 2010; Koudriavtseva <i>et al.</i> , 2014)
<ul style="list-style-type: none"> <li>- Psychosis</li> </ul>	(Cardinal <i>et al.</i> , 2009; Paz-Silva <i>et al.</i> , 2014)
<ul style="list-style-type: none"> <li>- Chorea and other movement disorders</li> </ul>	(Avcin <i>et al.</i> , 2008; da Silva and de Carvalho, 2014)
<ul style="list-style-type: none"> <li>- Cognitive Impairment</li> </ul>	(Jacobson <i>et al.</i> , 1999; Tektonidou <i>et al.</i> , 2006)

- Bipolar disorder	(Raza <i>et al.</i> , 2008; Avari and Young, 2012)
- Transverse myelitis	(D'Cruz <i>et al.</i> , 2004; Rodrigues and de Carvalho, 2011)
<ul style="list-style-type: none"> <li>• <b>Cardiac</b></li> </ul>	
- Myocardial infarction	(Cervera <i>et al.</i> , 2015)
- Cardiac valve abnormalities	(Cervera <i>et al.</i> , 2011)
<ul style="list-style-type: none"> <li>• <b>Pulmonary</b></li> </ul>	
- Pulmonary emboli and infarction	(Cervera <i>et al.</i> , 2015)
- Pulmonary hypertension and intra-alveolar haemorrhage	(Stojanovich <i>et al.</i> , 2012)
<ul style="list-style-type: none"> <li>• <b>Dermatological</b></li> </ul>	
- Livedo reticularis	(Toubi and Shoenfeld, 2007)
<ul style="list-style-type: none"> <li>• <b>Renal</b></li> </ul>	
- Renal insufficiency, acute renal failure and hypertension	(Tektonidou, 2014)

## 1.6 Molecular pathogenesis

### 1.6.1 Thrombosis

Although the complete thrombotic pathophysiology of APS is still unclear, generally, the formation of thrombi in APS is mediated by aPL-induced dysregulation of cell activation, coagulation, the fibrinolytic pathway, the immune system, genetics and some other factors (Negrini *et al.*, 2017). Activation of platelet markers, such as CD63, procaspase activating compound-1 (Joseph *et al.*, 2001), CD62 (Fanelli *et al.*, 1997), and CD62P (Jy *et al.*, 2007), the formation of a thrombogenic complex between homotetrameric platelet factor 4 (PF4) and two dimerised molecules of  $\beta$ 2-GPI (Vlachoyiannopoulos and Routsias, 2010), activation, elevation of the von Willebrand factor (vWF) levels (Lindsey *et al.*, 1993; Cugno *et al.*, 2010), dysfunction of ADAMTS13 (a protease enzyme) (Amoura *et al.*, 2004; Crawley *et al.*, 2011), dysregulation of prostaglandins [prostacyclin (PGI<sub>2</sub>), thromboxane A<sub>2</sub> (TXA<sub>2</sub>)] (Carreras and Vermlyen, 1982; Årfors *et al.*, 1990; Lindsey *et al.*, 1993), annexin A2 (Ao *et al.*, 2011) and toll-like receptors (TLRs) (Benhamou *et al.*, 2014) are involved in platelet activation, adhesion and aggregation, leading to the generation of thrombi in APS patients. In addition to impaired fibrinolysis (Atsumi *et al.*, 1998a) in the coagulation pathway, the involvement of tissue factors (TFs) (López-Pedreira *et al.*, 2006), annexin A5 (Rand *et al.*, 2008), proteins C and S (Keeling *et al.*, 1993; Erkan *et al.*, 2002), factor XI (Giannakopoulos *et al.*, 2012), tissue factor pathway inhibitor (TFPI) (Adams *et al.*, 2004) was also observed in patients with thrombotic APS. The activation of the complement system (Pierangeli *et al.*, 2005; Breen *et al.*, 2012), the involvement of B and T cells (Conti *et al.*, 2014; Simonin *et al.*, 2015) and tumour necrosis factor alpha (TNF- $\alpha$ ) (Swadzba *et al.*, 2011; Bećarević *et al.*, 2012) were

reported to be the major immune-mediated phenomena contributing to thrombosis in APS patients.

### **1.6.2 Pregnancy complications**

The pathogenesis of aPL-associated recurrent early pregnancy loss (first-trimester) is different from that of late pregnancy loss (Derksen and de Groot, 2008). During aPLs-derived first-trimester pregnancy loss, aPLs have direct inhibitory effects on proliferation of trophoblast cells (Chamley *et al.*, 1998; Di Simone *et al.*, 2000). The late pregnancy complications of APS such as pre-eclampsia, intrauterine growth restriction and stillbirths are the consequence of placental dysfunction by failure of extravillous trophoblasts to adequately remodel the spiral arteries, reduced maternal blood flow to the placenta resulting hypoxic injury, inadequate supply of nutrients to the foetus and high-velocity and high-pressure blood flow that can damage the placenta (Burton *et al.*, 2009). aPLs play roles in reducing proliferation and invasion of extravillous trophoblasts and triggering inflammation at the maternal-foetal interface, which together drive impaired placentation (Abrahams, 2009; Mulla *et al.*, 2009; Mulla *et al.*, 2010).  $\beta$ 2-GPI is constitutively expressed on the cell surface of placental trophoblasts as well as on maternal decidual endothelial cells (De Groot *et al.*, 2012). Interestingly, anti- $\beta$ 2-GPI antibodies can bind with human trophoblasts and the endothelium via the domain 5 phospholipid binding site of  $\beta$ 2-GPI (Di Simone *et al.*, 2005). Both *in vitro* and *in vivo* studies have reported that aPLs can inhibit spontaneous trophoblast migration, increase trophoblast antiangiogenic soluble endoglin secretion and disrupt trophoblast-endothelial interactions via low-density lipoprotein receptor-related protein 8 (LRP8) (Mulla *et al.*, 2009; Mulla *et al.*, 2010; Carroll *et al.*, 2011; Alvarez *et al.*, 2015; Ulrich *et al.*, 2016). Injection of immunoglobulin G (IgG)

antibodies from an APS patient to pregnant mice resulted in foetal resorption and growth restriction (Holers *et al.*, 2002). aPLs is usually localised to the placenta and is generated in developing inflammatory responses via different components such as complement activation and recruitment and stimulation of neutrophils which result in placental insufficiency, foetal loss and growth restriction in APS (Girardi *et al.*, 2003).

## **1.7 Genetics of APS**

The presence of aPLs was reported in family members even before the development of autoimmune diseases. Additionally, higher incidence of aPLs has been reported among first degree relatives of primary APS (Exner *et al.*, 1980; Mackworth-Young *et al.*, 1987; Goldberg *et al.*, 1995). Both family and non-family studies were reported to be linked with major histocompatibility complex (MHC) genes (Willis *et al.*, 2012; Sebastiani *et al.*, 2016) in addition to thrombotic genetic markers (Namjou, 2003; Castro-Marrero *et al.*, 2009). Taken together, these scenarios indicate the strong possibility of genetic predisposition in APS.

## **1.8 Management**

### **1.8.1 Thrombosis**

#### **1.8.1(a) Primary thromboprophylaxis**

Primary thromboprophylaxes are regimens used to prevent thrombosis in those without history of previous clots. Asymptomatic APS subjects with very high antibody titres, triple positive subjects (LA, aCL and anti- $\beta$ 2-GPI antibodies) along with the presence of cardiovascular risk factors can be considered for treatment with low-dose aspirin or hydroxychloroquine as primary thromboprophylactic agents (Ruiz-Irastorza *et al.*, 2011). According to a meta-analysis of observational studies, low-dose aspirin

efficiently reduced 50% risk of primary thrombosis development in patients with APS (Arnaud *et al.*, 2014), albeit with an increased risk of bleeding (Baigent *et al.*, 2009). As an alternative to low-dose aspirin, hydroxychloroquine is successfully applied in clinical setting on the basis of findings from *in vitro* experiments (Wallace *et al.*, 2012).

### **1.8.1(b) Secondary thromboprophylaxis**

Secondary thromboprophylaxes are regimens which are used to prevent recurrent thrombosis following the first thrombotic event. Selecting an optimum secondary thromboprophylaxis has been one of the major challenges in managing thrombotic APS patients. Only a few randomised controlled trials (RCTs) have attempted to determine the best secondary thromboprophylaxis such as anticoagulants (*i.e.*, warfarin) or antiplatelet agents (*i.e.*, aspirin) in preventing recurrence of thrombosis in APS (Crowther *et al.*, 2003; Finazzi *et al.*, 2005; Cuadrado *et al.*, 2014; Cohen *et al.*, 2016), albeit with dissimilar findings. Two systematic reviews (including prospective, retrospective studies and RCTs) published in 2006 (Lim *et al.*) and 2007 (Ruiz-Irastorza *et al.*) have tried to explore the best secondary prophylaxis for thrombotic APS with the conclusion of moderate intensity warfarin [international normalised ratio (INR): 2-3] to be recommended for recurrent thrombotic APS patients. Recently, non-vitamin K antagonist oral anticoagulants (NOAC) have been utilised in the management of secondary thrombosis in APS (Son *et al.*, 2015) and some RCTs on NOACs are also currently ongoing (*i.e.*, NCT02926170, NCT02157272 and NCT02295475).

### **1.8.2 Pregnancy complications**

To prevent pregnancy complications in individuals with obstetrical APS, low-dose aspirin, intermediate-dose of low molecular weight heparin (LMWH) or unfractionated heparin have been reported to be efficient (Bouvier *et al.*, 2013; Andreoli *et al.*, 2016). Pregnant APS women with primary thrombotic history require intermediate or full-dose anticoagulation (usually LMWH) throughout their pregnancies to prevent further thrombotic events (Rey *et al.*, 2009). Based on a Cochrane systematic review, 54% of the pregnancy loss could be reduced when treating obstetric APS subjects with unfractionated heparin in combination with low-dose aspirin (Empson *et al.*, 2005).

Although the disease has been known for more than 30 years, the diagnostic criteria, risk factors, molecular pathogenesis, genetic aspects and treatment strategies are poorly understood and yet to be fully developed. In Malaysia, to date, there are only a few studies on APS (Ong *et al.*, 2002; Wan Ali *et al.*, 2011; Hong-Kee *et al.*, 2014), however, none has retrospectively evaluated the clinical, laboratory characteristics and management strategies of APS patients. Additionally, there is a substantial absence of information on risk factors, pathophysiology, genetics and optimal treatment strategies for APS patients worldwide.

Therefore, the current study will embark on a widespread exploration of APS towards elucidating the clinical and laboratory features of Malaysian APS patients, explore the genetic aspects, identify the potential risk factors, molecular pathogenesis, current and optimal treatment strategies for better management of APS patients globally.

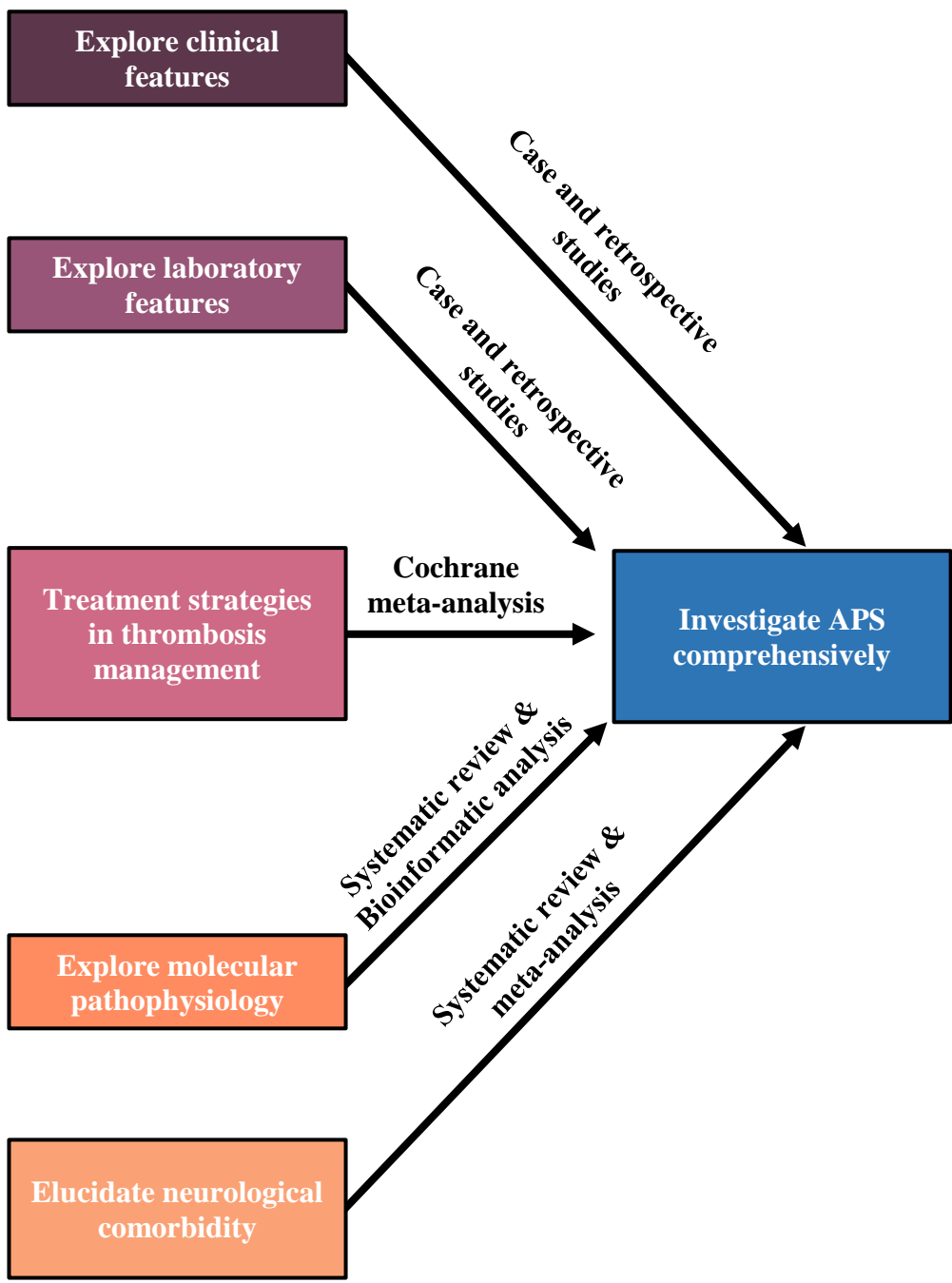
## **1.9 Objectives**

### **1.9.1 General objective**

To explore the clinical and laboratory features, genetic risk factors, comorbidity risks, molecular pathophysiology and optimal treatment strategy for APS patients.

### **1.9.2 Specific objectives**

1. To establish an optimised protocol for extracting high-quality and integrity RNA from human peripheral blood mononuclear cells (PBMCs) and additionally perform Ribonucleic acid (RNA)-Sequencing (RNA-Seq) of APS patients from Hospital Universiti Sains Malaysia (HUSM).
2. To retrospectively examine the criteria and non-criteria clinical and laboratory features, as well as treatment strategies of Malaysian APS patients.
3. To explore the genetic risk factors in thrombotic APS by evolving a systematic review with bioinformatic analysis (*i.e.*, functional enrichment, molecular interaction, pathway and gene expression).
4. To elucidate the comorbidity risks of non-criteria manifestations (*i.e.*, migraine, epilepsy and dementia) associated with aPLs by developing three systematic reviews and meta-analyses.
5. To identify the optimal treatment strategy in managing thrombotic APS patients by developing a Cochrane systematic review and meta-analysis.



**Figure 1.1: Theoretical Framework of the study**

## CHAPTER 2

### OPTIMISATION OF HIGH-QUALITY RNA

#### 2.0 Optimisation of high-quality RNA extraction

##### 2.1 Introduction

RNA-Seq is a high-throughput next-generation sequencing (NGS) technology which is highly efficient to differentially analyse gene sequencing and expression profile of both healthy and diseased subjects. The data generated from RNA-Seq offers knowledge on pathogenic mechanisms and signalling pathways associated with a disease as compared to healthy controls (Ozsolak and Milos, 2011; Tarazona *et al.*, 2011). Nevertheless, for generating a reliable data from RNA-Seq, it is mandatory to have high-quality RNA representing with 1) high purity and 2) high integrity. RNA purity is evaluated by determining absorbance<sub>260/280</sub> ( $A_{260/280}$ ) and  $A_{260/230}$  ratios, which should ideally be  $\geq 1.8$  for high-quality RNA samples. RNA integrity is assessed by RNA integrity number (RIN) ranging between 1 and 10, where 1 indicates highly degraded RNA samples while 10 indicates highly integrated RNA samples (Schroeder *et al.*, 2006). RNA samples with a RIN value of  $\geq 7$  is normally considered as integrated RNA which is usually appropriate for unbiased RNA-Seq (Madabusi *et al.*, 2006; Kiewe *et al.*, 2009). RNA required for RNA-Seq should be free from contamination of deoxyribonucleic acid (DNA), nucleases and other proteins, salts, polysaccharides and organic compounds. Nevertheless, high-quality RNA is a challenge to obtain because RNA is an unstable molecule susceptible to be degraded by endogenous RNases which is ubiquitous in the environment (Becker *et al.*, 2010).

PBMCs are peripheral blood cells [approximately 35% of the white blood cells (WBCs)] having a spherical nucleus consisting of lymphocytes [T-cells (70%), B-cells

(15%), natural killer (NK) cells (10%)] and monocytes (5%) (Autissier *et al.*, 2010; Murphy *et al.*, 2016). PBMCs are considered as ideal cell types to extract RNA from, since the cells interact with all organs and tissues in the body and consequently present gene expression profiles that can reflect the pathophysiological status, behaviours, growth stage and lifestyle of subjects (Mohr and Liew, 2007; Peters *et al.*, 2015).

The objective of this study was to establish an optimised protocol for extracting high-quality RNA from human PBMCs so that high-quality RNA ( $A_{260/280}$  and  $A_{260/230}$  ratios  $\geq 1.8$ ; RIN  $\geq 7$ ; total RNA  $\geq 1 \mu\text{g}$  and concentration  $\geq 65 \text{ ng}/\mu\text{l}$ ) of APS patients (following selection and confirmation) can be sent to Beijing Genomics Institute (BGI), Hong Kong for RNA-Seq.

## **2.2 Materials and methods**

### **2.2.1 RNA extraction from cell lines and lymphocytes**

Before starting with human PBMCs drawn from peripheral blood, first, cultured lymphoma cell lines (RL-60, RL-75, HT-60 and HT-75) were used to run the un-optimised protocol. Later, from a volunteer, 20 ml of peripheral blood was collected in K2EDTA tubes (BD Vacutainer, New Jersey, United States). Then, the blood sample was immediately transferred into a 50 ml leucosep tube (Greiner Bio-One, Kremsmunster, Austria) with porous barrier containing Ficoll-Paque via 5 ml sterile transfer pipette. After that the tube containing 20 ml blood was centrifuged (5810R, Eppendorf, Hamburg, Germany) for 15 min at 2000 revolutions per min (rpm) (switching brakes off) at room temperature. After centrifugation, from the three-layered sequence, the thick whitish buffy coat was transferred by a Pasteur pipette (Sigma-Aldrich, Missouri, United States) to a 50 ml polypropylene tube (BD

Biosciences, California United States). Then, the cell fraction was mixed with 25 ml of 1x phosphate buffered saline (PBS) (Sigma-Aldrich, Missouri, United States) followed by a 10-min centrifugation (5810, Eppendorf, Hamburg, Germany) at 1100 rpm (brake  $\pm$  5). The supernatant was discarded and another 2 ml of 1x PBS was re-suspended. From there, 10  $\mu$ L cell suspensions was mixed with 10  $\mu$ L trypan blue and viable cells were counted by a haemocytometer using a microscope (IMT-2, Olympus, Tokyo, Japan).

### **2.2.2 RNA extraction and purification: Protocol A**

RNA extraction and purification were conducted following the protocol of GeneJET RNA purification kit (Thermo Scientific, Massachusetts, United States) with slight modifications. Before starting the experiments, RNase Zap<sup>®</sup> was sprayed and wiped on microcentrifuge tubes, glassware, plastic surfaces, countertops, pipettes and inside the hood to eliminate RNase contamination. Not more than 10 million cell pellets were taken into a 2.0 ml eppendorf tube and washed with 1.5 ml of 1x PBS to remove residual growth medium. Following 2000 rpm centrifugation (MiniSpin, Eppendorf, Hamburg, Germany), the supernatant was removed. Lysis buffer (600  $\mu$ l) supplemented with  $\beta$ -mercaptoethanol (12  $\mu$ l) was added with the pellet and vortexed at 1400 rpm for 10 seconds to mix thoroughly. As cell debris were observed, the sample was then centrifuged at 14000 rpm (MiniSpin, Eppendorf, Hamburg, Germany) for 5 min followed by the transfer of the supernatant into an RNase-free microcentrifuge tube. Subsequently, 360  $\mu$ l of 100% ethanol was added into the sample and mixed by pipetting (15-20 times). Then, 700  $\mu$ l of lysate was transferred into a GeneJET RNA purification column (inserted in a collection tube) and centrifuged for 75 seconds at 14000 rpm (MiniSpin, Eppendorf, Hamburg, Germany). The flow-

through was discarded and the step was repeated so that all of the lysate can be transferred into the column. Then, the collection tube was discarded with flow-through and was replaced with a new collection tube. Then, the column was washed subsequently with 700  $\mu$ l wash buffer 1 and 600  $\mu$ l wash buffer 2 and centrifuged at both 13000 and 14000 rpm for 60, 75 and 90 seconds. Again, the column was washed with 250  $\mu$ l of wash buffer 2 followed by another 2-min centrifugation at both 13000 and 14000 rpm. After this, as residual solution was seen in the purification column, again the column was centrifuged for 1 min at 14500 rpm. Then, the collection tube containing the flow-through was discarded and replaced with a new 1.5 ml RNase-free microcentrifuge tube. For eluting RNA, 50  $\mu$ l of nuclease free water was placed at the middle of the column membrane and centrifuged for 1 min at both 13000 and 14000 rpm. The quality of the extracted RNA was estimated (from 2  $\mu$ l RNA sample) by using the Epoch Microplate Spectrophotometer (BioTek, Vermont, United States). Nuclease free water (2  $\mu$ l) was used as the blank sample. The protocol was the modified version of the manufacturer's recommended protocol (Ni *et al.*, 2015; Song *et al.*, 2016).

### **2.2.3 Genomic DNA removal and addition of RiboLock: Protocol B, C and D**

RapidOut DNA removal kit (Life Technologies, California, United States) was used for removing genomic DNA from the sample. For 1  $\mu$ g of RNA, 1  $\mu$ l 10x reaction buffer with magnesium chloride ( $MgCl_2$ ) and 1  $\mu$ l DNase I (RNase-free) was used. To scale up the solution to 10  $\mu$ l, diethylpyrocarbonate (DEPC)-treated water was used in this step (Protocol B). Additionally, RiboLock RNase inhibitor (Life Technologies, California, United States) was mixed at 1 U/ $\mu$ l. Then, the mixture was incubated in a water bath for 30 min at 37°C (Protocol C). After the incubation, the RNA sample was

re-purified by following the RNA clean-up protocol by using a GeneJET RNA purification kit (Protocol D). Here, RNA was taken and the volume was adjusted to 100  $\mu$ l by adding nuclease free water followed by adding 300  $\mu$ l lysis buffer without  $\beta$ -mercaptoethanol and mixed thoroughly by pipetting. Then, 180  $\mu$ l ethanol (100%) was added and mixed and the mixture was transferred in the GeneJET RNA purification column inserted in a collection tube and centrifuged for 1 min at 14000 rpm (MiniSpin, Eppendorf, Hamburg, Germany). The flow-through was discarded and the step was repeated so that all of the lysate can be transferred into the column. Then, the collection tube was discarded with flow-through and was replaced with a new 2 ml collection tube. Subsequently, the column was washed with 700  $\mu$ l wash buffer 1 and 600  $\mu$ l wash buffer 2 and centrifuged at both 13000 and 14000 rpm for 60, 75 and 90 seconds. Again, the column was washed with 250  $\mu$ l of wash buffer 2 followed by another 2-min centrifugation at both 13000 and 14000 rpm. Then, the collection tube containing the flow-through was discarded and replaced with a new 1.5 ml RNase-free microcentrifuge tube. Then, the collection tube containing the flow-through was discarded and replaced with a new 1.5 ml RNase-free microcentrifuge tube. The quality of the purified intact RNA (without genomic DNA and RNase) was estimated (from 2  $\mu$ l RNA sample) by using the Epoch Microplate Spectrophotometer (BioTek, Vermont, United States). Nuclease free water (2  $\mu$ l) was used as blank. The protocol was the modified version of the manufacturer's recommended protocol (Rocha *et al.*, 2015; Boguslawska *et al.*, 2016)

#### **2.2.4 RNA integrity number (RIN) check**

RIN of the purified RNA was checked by using a 2200 TapeStation (Agilent, California, United States). The R6K ScreenTape (Agilent, California, United States)

was used for the quality assessment of total RNA. R6K sample buffer (4  $\mu$ l) was mixed with 1  $\mu$ l RNA sample followed by heating the samples at 72°C (Thermal cycler, Bio-Rad Laboratories, California United States) for 3 min. Immediately after heating, the sample was placed on ice for 2 min. Then, the sample was loaded in the ScreenTape and analysed the RIN by using the Agilent 2200 TapeStation software (version A.01.04).

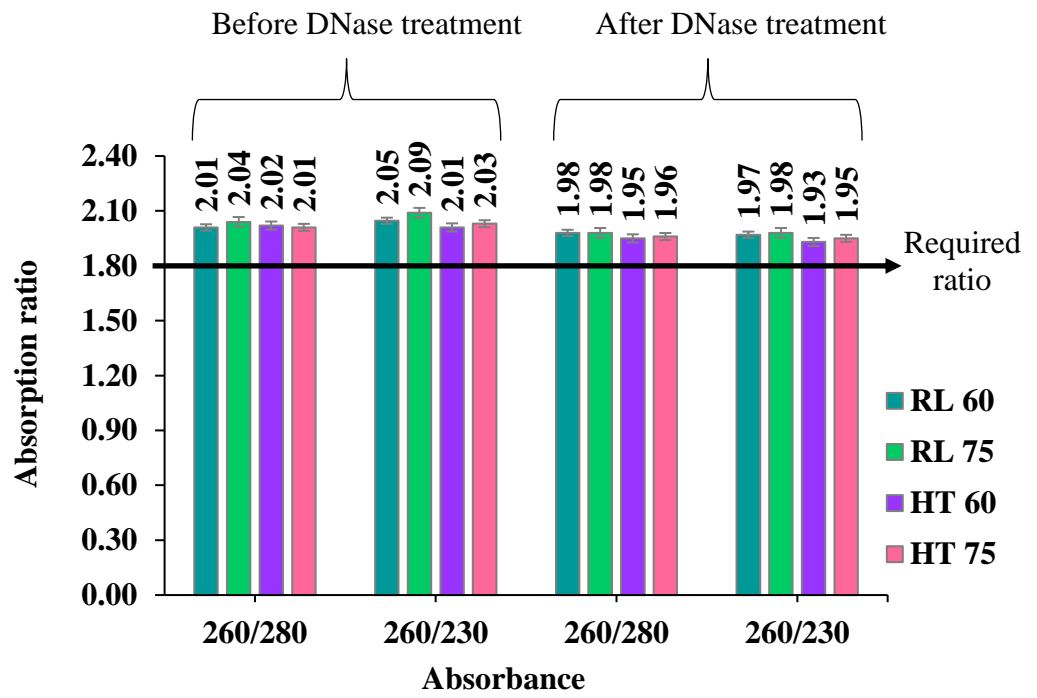
### **2.3 Results**

In case of the quality of RNA extracted from cell lines, both  $A_{260/280}$  and  $A_{260/230}$  ratios were  $>1.8$  for all the four cell lines (Table 2.1 and Figure 2.1) indicating high purity of RNA. Based on the optimised protocol of RNA extraction, the used kit (GeneJET RNA purification kit) was able to extract and purify high-quality RNA from PBMCs. The optimised spinning time for the purification column was 75 seconds (Table 2.2), while optimum centrifuge rpm was selected as 14000 (Table 2.3). Combining the optimised parameters, both  $A_{260/280}$  and  $A_{260/230}$  ratios were  $>1.8$  (Table 2.4 and Figure 2.2) indicating that the method (Protocol D) is ideal. There was optimum RNA yield ( $3.71 \pm 0.41$   $\mu$ g) with high concentration ( $74.28 \pm 8.17$  ng/ $\mu$ L), therefore, the final protocol (Protocol D) was selected (Figure 2.3). RIN of the PBMC-extracted RNA was  $>8.5$  even after seven days (Table 2.5, Figures 2.4 and 2.5) which represents highly integrated RNA sample.

**Table 2.1: RNA quality and quantity in human lymphoma cell lines with un-optimised protocol**

Cell lines*	RNA purity		RNA concentration (ng/μl)
	A <sub>260/280</sub>	A <sub>260/230</sub>	
Before DNase treatment			
RL-60	2.01 ± 0.05	2.05 ± 0.04	156.06 ± 0.12
RL-75	2.04 ± 0.03	2.09 ± 0.04	476.05 ± 0.60
HT-60	2.02 ± 0.04	2.01 ± 0.05	148.14 ± 0.55
HT-75	2.01 ± 0.05	2.03 ± 0.03	405.06 ± 1.51
After DNase treatment			
RL-60	1.98 ± 0.03	1.97 ± 0.03	155.98 ± 0.12
RL-75	1.98 ± 0.02	1.98 ± 0.02	475.63 ± 0.60
HT-60	1.95 ± 0.02	1.93 ± 0.01	147.75 ± 0.55
HT-75	1.96 ± 0.04	1.95 ± 0.03	403.98 ± 1.51

\* Human promyelocytic leukaemia cell line derived from PBMCs.



**Figure 2.1: RNA quality in human lymphoma cell lines**

RL-60, R-75, HT-60 and HT-75: Human promyelocytic leukaemia cell lines.

**Table 2.2: Optimisation of the purification column spinning time**

Spinning time (seconds)	RNA purity		RNA concentration (ng/ $\mu$ l)	Ideal condition
	$A_{260/280}$	$A_{260/230}$		
Before DNase treatment				
60	$1.86 \pm 0.03$	$1.82 \pm 0.03$	$72.33 \pm 5.86$	X
<b>75</b>	<b><math>1.95 \pm 0.04</math></b>	<b><math>1.87 \pm 0.03</math></b>	<b><math>104.33 \pm 4.16</math></b>	<b>✓</b>
90	$1.69 \pm 0.04$	$1.75 \pm 0.03$	$87.33 \pm 6.03$	X
After DNase treatment				
60	$1.76 \pm 0.02$	$1.74 \pm 0.03$	$54.67 \pm 3.51$	X
<b>75</b>	<b><math>1.89 \pm 0.04</math></b>	<b><math>1.84 \pm 0.02</math></b>	<b><math>78.67 \pm 2.52</math></b>	<b>✓</b>
90	$1.65 \pm 0.02$	$1.70 \pm 0.01$	$63.33 \pm 1.53$	X