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

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LIST OF ABBREVIATIONS

aCL	Anticardiolipin antibody
AD	Alzheimer’s disease
ADP	Adenosine diphosphate
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANA	Antinuclear antibody
Anti-β2-GPI	Anti-β2-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-κB	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_c	T-cytotoxic
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
T_h	T-helper
TIA	Transient ischaemic attack
TLE	Temporal lobe epilepsy
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
TNFR2	Tumour necrosis factor receptor 2
tPA	Tissue plasminogen activator
TPM	Transcripts per kilobase million
T_{reg}	T-regulatory
tRNA	Transfer RNA
TXA2	Thromboxane A2
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-β2 glikoprotein I antibodi (β2-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 genetik, risiko komorbiditi, 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. Penggunan warfarin secara berintensi 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 kebanyakkan mempengaruhi laluan pembekuan darah dan sistem immun 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 manifastasi 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 intensity 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-β2-glycoprotein I (β2-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-β2-glycoprotein I (β2-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-β2-GPI and the addition of the cut-off values of LA, aCL and anti-β2-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

Clinical criteria
• Vascular thrombosis - ≥1 arterial, venous or small vessel thromboses in any tissue or organ (excluding superficial thrombosis), as confirmed by appropriate imaging or histopathology. • Pregnancy morbidity - ≥1 unexplained deaths of a morphologically normal foetus at or beyond the 10^{th} week of gestation. - ≥1 premature births or a morphologically normal neonate before the 34^{th} week of gestation owing to eclampsia, severe pre-eclampsia or placental insufficiency. - ≥3 unexplained consecutive spontaneous abortions before the 10^{th} week of gestation, with hormonal, chromosomal or maternal anatomical causes excluded.
Laboratory criteria
- LA present in plasma, detected according to the guidelines of the International Society on Thrombosis and Haemostasis.

- 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.* >99th 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

Non-criteria manifestations	References
• **Thrombocytopenia**	(Krause *et al.*, 2005; Cervera *et al.*, 2015)
• **Neurological**	
- Headache and migraine	(Cervera *et al.*, 2009; Zhu *et al.*, 2014; Abreu *et al.*, 2015)
- Epilepsy	(Shoenfeld *et al.*, 2004; Stojanovich *et al.*, 2013)
- Dementia	(Mosek *et al.*, 2000; Chapman *et al.*, 2002)
- Multiple sclerosis	(Stosic *et al.*, 2010; Koudriavtseva *et al.*, 2014)
- Psychosis	(Cardinal *et al.*, 2009; Paz-Silva *et al.*, 2014)
- Chorea and other movement disorders	(Avcin *et al.*, 2008; da Silva and de Carvalho, 2014)
- Cognitive Impairment	(Jacobson *et al.*, 1999; Tektonidou *et al.*, 2006)

- Bipolar disorder	(Raza *et al.*, 2008; Avari and Young, 2012)
- Transverse myelitis	(D'Cruz *et al.*, 2004; Rodrigues and de Carvalho, 2011)
• **Cardiac**	
- Myocardial infarction	(Cervera *et al.*, 2015)
- Cardiac valve abnormalities	(Cervera *et al.*, 2011)
• **Pulmonary**	
- Pulmonary emboli and infarction	(Cervera *et al.*, 2015)
- Pulmonary hypertension and intra-alveolar haemorrhage	(Stojanovich *et al.*, 2012)
• **Dermatological**	
- Livedo reticularis	(Toubi and Shoenfeld, 2007)
• **Renal**	
- 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 β2-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 (PGI2), thromboxane A2 (TXA2)] (Carreras and Vermylen, 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-Pedrera *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-α) (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). β2-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-β2-GPI antibodies can bind with human trophoblasts and the endothelium via the domain 5 phospholipid binding site of β2-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. Asymptotic APS subjects with very high antibody titres, triple positive subjects (LA, aCL and anti-β2-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.

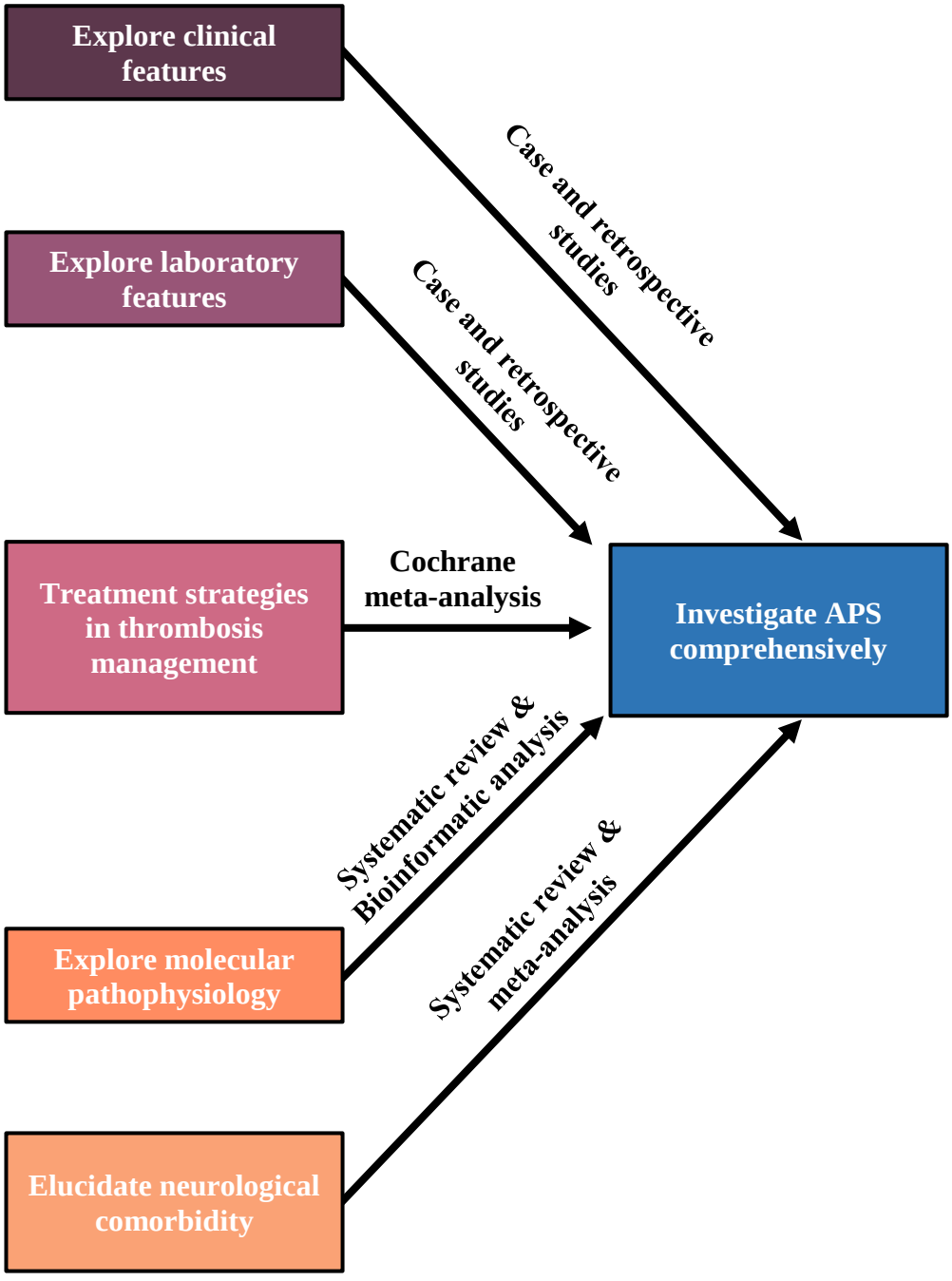

Figure 1.1: Theoretical Framework of the study

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$_{260/280}$ ($A_{260/280}$) and $A_{260/230}$ ratios, which should ideally be ≥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 ≥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 ≥1.8; RIN ≥7; total RNA ≥1 µg and concentration ≥65 ng/µl) 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 ± 5). The supernatant was discarded and another 2 ml of 1x PBS was re-suspended. From there, 10 µL cell suspensions was mixed with 10 µL 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® 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 µl) supplemented with β-mercaptoethanol (12 µl) 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 µl of 100% ethanol was added into the sample and mixed by pipetting (15-20 times). Then, 700 µl 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 µl wash buffer 1 and 600 µl wash buffer 2 and centrifuged at both 13000 and 14000 rpm for 60, 75 and 90 seconds. Again, the column was washed with 250 µl 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 µl 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 µl RNA sample) by using the Epoch Microplate Spectrophotometer (BioTek, Vermont, United States). Nuclease free water (2 µl) 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 µg of RNA, 1 µl 10x reaction buffer with magnesium chloride ($MgCl_2$) and 1 µl DNase I (RNase-free) was used. To scale up the solution to 10 µl, 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/µl. 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 µl by adding nuclease free water followed by adding 300 µl lysis buffer without β-mercaptoethanol and mixed thoroughly by pipetting. Then, 180 µl 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 µl wash buffer 1 and 600 µl wash buffer 2 and centrifuged at both 13000 and 14000 rpm for 60, 75 and 90 seconds. Again, the column was washed with 250 µl 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 µl RNA sample) by using the Epoch Microplate Spectrophotometer (BioTek, Vermont, United States). Nuclease free water (2 µl) 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 µl) was mixed with 1 µl 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 ± 0.41 µg) with high concentration (74.28 ± 8.17 ng/µL), 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)
	$A_{260/280}$	$A_{260/230}$	
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.

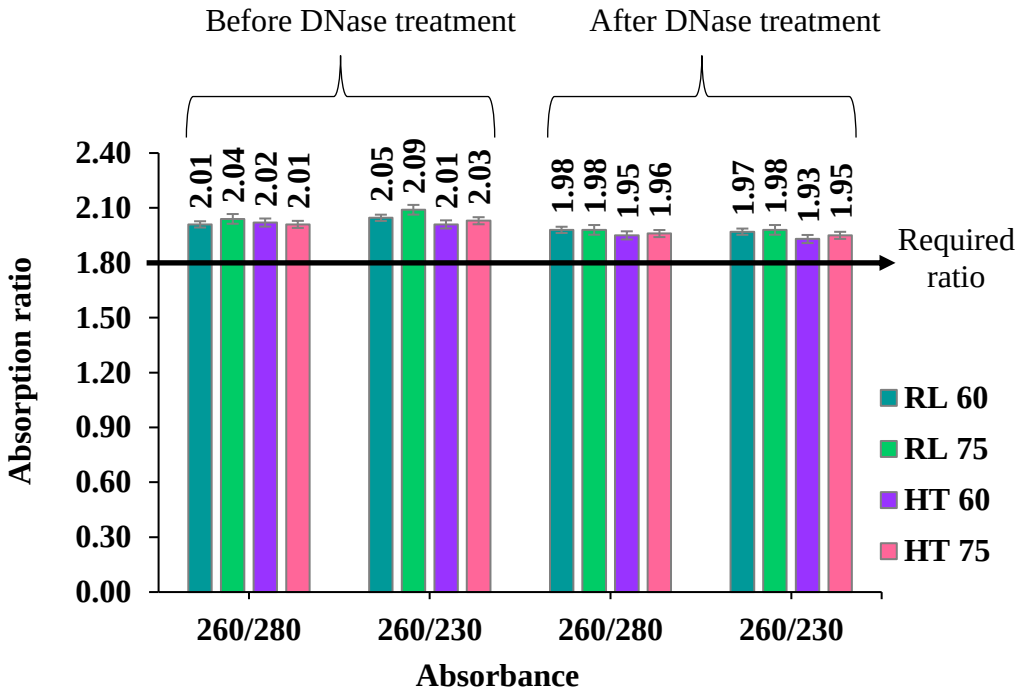

Figure 2.1: RNA quality in human lymphoma cell lines

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/µl)	Ideal condition
	$A_{260/280}$	$A_{260/230}$		
Before DNase treatment				
60	1.86 ± 0.03	1.82 ± 0.03	72.33 ± 5.86	X
75	**1.95 ± 0.04**	**1.87 ± 0.03**	**104.33 ± 4.16**	✓
90	1.69 ± 0.04	1.75 ± 0.03	87.33 ± 6.03	X
After DNase treatment				
60	1.76 ± 0.02	1.74 ± 0.03	54.67 ± 3.51	X
75	**1.89 ± 0.04**	**1.84 ± 0.02**	**78.67 ± 2.52**	✓
90	1.65 ± 0.02	1.70 ± 0.01	63.33 ± 1.53	X