THE CLINICAL AND LABORATORY SIGNIFICANCE OF ANTIPHOSPHOLIPID SYNDROME PATIENTS IN HOSPITAL USM

by
DHAMIRAH NAZIHAH BT MOHD NASIRUDDIN

Thesis submitted in fulfilment of the requirements for the degree of Masters of Pathology (Haematology)

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

β	beta

LIST OF ABBREVIATIONS

anti-β2GPI	Anti-β_2-glycoprotein I antibodies
aCL	Anticardiolipin antibodies
aPL	Antiphospholipid antibodies
APS	Antiphospholipid syndrome
aPTT	Activated partial thromboplastin time
aPTT-LA	Lupus Anticoagulant-sensitive activated partial thromboplastin time
BCSH	British Committee for Standards in Haematology
CAPS	Catastrophic Antiphospholipid syndrome
CLSI	The Clinical and Laboratory Standards Institute
dPT	dilute prothrombin time
dRVVT	dilute Russell viper venom time
ELISA	enzyme-linked immunosorbent assay
EUROAPS	European Registry of Obstetric Antiphospholipid Syndrome
HPE	Hexagonal phase phosphatidylethanolamine
IPS	Institut Pengajian Siswazah
INR	International Normalized Ratio
ISTH	International Society on Hemostasis and Thrombosis
KCT	Kaolin clotting time
LA	Lupus Anticoagulant
LAR- aPTT	LA-responsive aPTT
OAPS	Obstetric APS
PL	Phospholipids
PPP	Platelet poor plasma
SCT	Silica clotting time
SLE	Systemic Lupus Erythematosus
TF	Tissue factor
USM	Universiti Sains Malaysia

LIST OF APPENDICES

TANDA-TANDA KLINIKAL DAN MAKMAL PESAKIT SINDROM ANTIPHOSPHOLIPID DI HOSPITAL USM

ABSTRAK

Sindrom antiphospholipid (APS) didiagnosis pada pesakit dengan trombosis vaskular atau morbiditi kehamilan yang mana ujian makmalnya menunjukkan antibodi antiphospholipid yang berterusan (aPL). Untuk mengesahkan keberadaannya, antibodi antiphospholipid ini, yang merupakan antikoagulan lupus (LA), antibodi antikardiolipin (aCL) dan antibodi anti-β2-glikoprotein I (anti-β2GPI), perlu diulang selepas 12 minggu. Objektif kajian ini adalah untuk menganalisis ciri klinikal dan ciri makmal pesakit dengan gejala APS yang dihantar untuk ujian LA dan aCL di Hospital USM.

Kajian retrospektif terhadap 391 sampel yang dihantar untuk ujian LA dalam tempoh 6 tahun di makmal Hematologi Hospital USM dianalisis. Enam puluh sembilan sampel menunjukkan positif bagi LA pada sampel pertama. Walau bagaimanapun, hanya tiga puluh lima yang dihantar untuk ujian ulangan. Hasil ujian aCL mereka juga dianalisis untuk setiap pesakit yang dihantar untuk ujian LA. Oleh itu, bahagian pesakit APS yang disahkan mengikut kriteria Sydney di Hospital USM adalah 19.5% (17/81).

Keputusannya bersama dengan ciri klinikal dan makmal setiap pesakit dianalisis. Hasil regresi logistik sederhana menunjukkan pesakit APS yang disahkan (LA atau aCL positif) lebih kerap berlaku pada masa tromboplastin separa aktif yang sensitif LA (APTT-LA) indeks yang berpanjangan, DRVVT positif (nisbah LA) dan Indeks Rosner (RI) yang positif. Analisis multivariate menunjukkan bahawa nisbah DRVVT yang berpanjangan (nisbah LA> 1.2) didapati sebagai satu-satunya faktor risiko bebas yang bertanggungjawab untuk APS yang disahkan dalam kajian ini.

Jumlah sampel yang dihantar untuk ujian kedua lebih rendah daripada jumlah yang dijangkakan menunjukkan kurangnya kesedaran di kalangan pengamal perubatan mengenai amalan memperoleh diagnosis APS pada sampel kedua. Oleh itu, lebih banyak usaha perlu dilakukan untuk memastikan penyiasatan APS yang tepat.

THE CLINICAL AND LABORATORY SIGNIFICANCE OF ANTIPHOSPHOLIPID SYNDROME PATIENTS IN HOSPITAL USM

ABSTRACT

Antiphospholipid syndrome (APS) is diagnosed in patients with vascular thrombosis or pregnancy morbidity whose laboratory assays demonstrate persistent antiphospholipid antibodies (aPL). To confirm their existence, these antiphospholipid antibodies, which are lupus anticoagulant (LA), anticardiolipin antibodies (aCL) and anti-β2-glycoprotein I antibodies (anti-β2GPI), need to be repeated after 12 weeks. The objective of this study is to analyse the clinical and laboratory characteristics of patients with confirmed APS sent for LA and aCL tests in Hospital USM. A retrospective study on 391 samples sent for LA tests within 6 years in Haematology laboratory of Hospital USM were analysed. Sixty-nine out of three hundred ninety-one samples showed positivity for LA in the first samples. However only thirty-five repeated sample were available. The results of their aCL tests were also analysed for each patient who were sent for LA testing. Therefore, proportion of confirmed APS patients according to Sydney criteria are 19.5% (17/81).

Their result along with clinical and laboratory characteristics of each patient were analysed. Confirmed APS patients (LA or aCL positive) is more common in patients aged between 20-40 years old, female gender with female to male ratio 4.7:1, Malay race, vascular thrombosis, patients with autoimmune diseases such as systemic lupus erythematosus (SLE), normal platelet count, normal prothrombin time (PT), prolonged activated partial thromboplastin time (APTT), normal international normalized ratio (INR), corrected mixing study, prolonged LA-sensitive activated partial thromboplastin time (APTT-LA), positive Rosner's index, positive DRVVT (LA ratio), positive LA and negative aCL. Multivariate analysis shows prolonged

DRVVT ratio (LA ratio >1.2) and positive Rosner's index were the only independent risk factors responsible for confirmed APS in this study.

In conclusion, the number of samples sent for second testing are lesser than the expected amount shows the lack of awareness among healthcare practitioners on the practice of attaining diagnosis of APS on second sample. Therefore, more efforts are needed to be strained to ensure proper investigation of APS.

CHAPTER 1

CHAPTER 1

INTRODUCTION

The antiphospholipid syndrome (APS) is defined by venous or arterial thrombosis and/or pregnancy morbidity with persistent presence of antiphospholipid antibodies (aPL). Since thrombosis and pregnancy morbidity are not exclusive to APS, the diagnosis is based on accurate and robust detection of aPL. In semi-quantitative solid-phase assays, two of the requirements for antibodies, anticardiolipin antibodies (aCL) and anti-β2-glycoprotein I antibodies (anti-β2GPI), are identified, while lupus anticoagulants (LA) are identified in a medley of phospholipid-dependent coagulation assays by inference based on antibody behaviour (Moore *et al.*, 2017). To prevent unnecessary long - term secondary thromboprophylaxis, correct diagnosis of the condition is crucial. The classification criteria for the antiphospholipid syndrome involves clinical and laboratory criteria. Laboratory criteria include three antiphospholipid antibodies subtypes: lupus anticoagulants (LA), anticardiolipin antibodies (aCL) and anti-β_2-glycoprotein I (anti-β2GPI) antibodies. Only lupus anticoagulants are unquestionably linked to thrombosis, which is why the serological criteria for APS were under discussion. Although progress has been made in the detection of lupus anticoagulants, all assays used to detect antiphospholipid antibodies need better standardization. The combination of both anticardiolipin and anti-β2GPI antibodies with thrombosis is an inconsistent cause of alarm (Urbanus and de Groot, 2011).

A recent study in Thailand described the characteristics of patients with APS in an Asian population (Jatuworapruk *et al.*, 2018). They concluded that APS patients in central Thailand demonstrated high prevalence of stroke, late foetal loss, LA positivity, and multiorgan thrombosis at first presentation, leading to poor outcomes.

However, their data on the clinical and laboratory parameters were concluded based on the outcome of definite or probable APS while we know that thrombosis and pregnancy complications are multifactorial. Thus, consideration of non-aPL thrombotic risk factors is critical in evaluating patients who are positive for aPL. Therefore, this study was aimed to know the correlation between clinical characteristics and laboratory parameters of APS patients in Kelantan population.

Another study was done in obstetric population in Sabah, Malaysia which audited indications and outcomes of APS screening in the pregnant population in their centre (Valayatham, 2012). They found the need to increase awareness among obstetricians on the indications for screening for APS and the prevalence of APS with obstetric manifestations in the study population is lower than rates published in other literatures. The target population in their study is limited to only obstetric population while in our study, patients from all units were involved.

A study done on laboratory and clinical significance of rare antiphospholipid antibodies in preeclampsia patients, and they found that the prevalence of these rare antiphospholipid antibodies is low 2.4%. They are also associated with shortened activated partial thromboplastin time (APTT) in severe preeclampsia. The association of rare antiphospholipid antibodies with APS is uncertain as it is IgM type (Abdullah, 2015).

The research questions for our study are (a) what is the proportion of confirmed APS patients in Hospital USM? (b) what are the clinical characteristics and laboratory parameters of confirmed APS patients? (c) is there any significant association of patients' clinical characteristics and laboratory parameters with APS positive cases in HUSM?

CHAPTER 2

CHAPTER 2

LITERATURE REVIEW

2.1 Antiphospholipid Syndrome (APS)

2.1.1 Definition of APS

Antiphospholipid syndrome (APS) is an autoimmune prothrombotic disorder related to the presence of antiphospholipid antibodies (aPL). This condition is also known as Hughes' syndrome in honor of the physician Graham R. V. Hughes, who first narrated it in 1983 (Negrini *et al.*, 2017).

2.1.2 Clinical features of APS

APS clinical manifestations include recurrence of arterial and venous thrombosis as well as pregnancy-related complications, but a wide spectrum of events could also be observed (Negrini *et al.*, 2017).

The presence of aPL can be associated with different clinical situations: asymptomatic "aPL carrier" patients, "classical" APS with recurrent vascular events (venous and/or arterial thrombosis); APS limited to pregnancy morbidity (obstetric APS); or aPL-positive patients presenting with non-thrombotic or obstetric clinical manifestations. In addition, a minority of APS patients can develop a vociferous and life-threatening form of disease, defined as catastrophic APS (CAPS), characterized by a rapid occurrence of multiple occlusive events leading to multiorgan failure. Furthermore, in 2003, Hughes and Khamashta suggested the definition "seronegative APS" to describe patients with clinical manifestations of APS and persistently negative aPL (Negrini *et al.*, 2017).

APS is defined "primary" if it is not associated with other underlying diseases or "secondary" if it occurs in relation with other conditions, such as autoimmune

disorders, particularly systemic lupus erythematosus (SLE). aPL positivity has also been described in association with infections, drugs, and malignancies; however, in these cases, aPL titers are usually transient and low, hence not increasing the risk of thrombosis or of an adverse pregnancy outcome.

Clinical features of primary or secondary APS can be identical; however, the clinical manifestation of secondary forms could be complicated by the expression of the underlying disease (Negrini *et al.*, 2017).

Besides thrombosis and pregnancy morbidity, there have been many claims of other clinical associations with aPL. Thrombocytopenia, heart valve disease (which is most commonly occult), chorea, livedo reticularis/ racemosa and nephropathy are likely associations, although like the thrombotic and pregnancy manifestations, none is specific to APS (Miyakis *et al.*, 2006). Transverse myelopathy occurs in SLE and may be more frequent in those with aPL (Cervera *et al.*, 2002). A purported association with infertility has not been substantiated (Buckingham and Chamley, 2009) and an association with migraine is controversial with one recent study finding a relationship (Cavestro *et al.*, 2011) but others did not find a relationship (Montalban *et al.*, 1992; Tietjen *et al.*, 1998).

Another controversial concept is that APS may manifest as a disorder closely mimicking multiple sclerosis and responsive to anticoagulant therapy (Hughes, 2003). However, aPL may be present in some cases of otherwise typical multiple sclerosis (Heinzlef *et al.*, 2002) perhaps representing an epiphenomenon in a disorder with an immune pathogenesis. Even more contentious is the possibility that a seronegative form of APS may exist (Hughes and Khamashta, 2003). The main manifestations of APS, thrombosis and pregnancy failure, are usual and in most cases do not have an

autoimmune basis; as such it would be difficult to sustain the diagnosis of 'seronegative APS' (Keeling *et al.*, 2012).

2.1.3 Prevalence of APS

aPLs can be diagnosed incidentally in normal individuals as low-titre aCL occurs in less than 10% of normal blood donors and moderate-high titre aCL and/or positive LA test occurs in less than 1%. The prevalence of positive aPL tests increases with age; because the differential diagnosis of vascular occlusion is broader than it is in young adults, particular care. is necessary in diagnosing APS in older patients. Thirty to 40% of SLE patients and approximately one-fifth of rheumatoid arthritis patients have positive tests for aPL (Erkan and Lockshin, 2013).

Ten per cent of healthy blood donors are positive for antibodies to anticardiolipin and one per cent positive for lupus anticoagulant. However, less than one per cent of these samples are still positive after one year (Garcia and Erkan, 2018). About 20% to 30% of patients with SLE have recurrent antiphospholipid-antibody profiles of moderate - high risk associated with an increased risk of clinical sequelae (Garcia and Erkan, 2018).

Incidence of APS is around 5 new cases per 100, 000 persons per year with a prevalence of around 40-50 cases per 100, 000 persons. (Durcan and Petri, 2017).

The prevalence of antiphospholipid-antibody positivity among patients without autoimmune disease is 6% among women with pregnancy complications, 10% among patients with venous thrombosis, 11% among patients with myocardial infarction and 17% among patients with stroke younger than 50 years of age. However, these prevalence estimates were derived mostly from studies that included patients who underwent antiphospholipid antibody testing only once, those in whom test results were borderline positive, or both. Large studies that use rigorous definitions of clinical events

and strict criteria for antiphospholipid-antibody positivity are needed (Garcia and Erkan, 2018).

2.1.4 Pathophysiology of APS

2.1.4(a) Introduction

A' two-hit' theory has been suggested: the first hit is a prothrombotic / inflammatory condition caused by antiphospholipid antibodies (aPL), and the second is exposure to an immediate precipitating event such as surgery, immobilization, exogenous oestrogen or pregnancy. Interestingly, pregnancy does not merely serve as a precipitating prothrombotic state, since comparison of conception products from aPL-positive and aPL-negative women with recurrent early miscarriage reveals a clear defect in the Obstetric APS (OAPS) invasion of decidual endovascular trophoblasts and shows that placental infarction is not unique to APS (Clark and Giles, 2018).

Experimental evidence increasingly implies non-thrombotic role in OAPS pathogenesis by aPL-mediated activation of the complement, inflammation, and impairment of placental development and function. However, the European Registry of Obstetric Antiphospholipid Syndrome (EUROAPS) clinical data on 247 patients with OAPS shows that progression to thrombosis and SLE is small compared to patients with thrombotic APS; this gives evidence to the theory that OAPS is a specific subset of APS (Clark and Giles, 2018).

2.1.4(b) Thrombosis

The association between aPL and both venous and arterial thrombosis is widely accepted, and the development of clots is the key event underlying the vascular manifestations. Most of the pathogenic mechanisms potentially responsible for thrombus formation have only been shown using in vitro models. Nonetheless, in vivo thrombosis models –induced by mechanical, chemical, or photochemical trauma

in mice and hasmters–have reported that aPL can increase the formation of thrombus in the venous and arterial trees. In addition, it was shown that passive infusion of human aPL IgG together with a small amount of lipopolysaccharide (LPS) triggered clotting in mesenteric microcirculation of rats. In arterial endothelia, altered expression of endothelial adhesion molecules and upregulation of nitric oxide and tissue factor (TF) expression have been reported after infusion of aPL with or without dimeric β2GPI, supporting a key role for aPL in causing vascular anomalies. Platelets were found to be involved in arterial photochemical-induced thrombus formation, and upregulated expression of TF was reported on passively injected mice monocytes with aPL. Nevertheless, more detailed studies are needed to further identify the functions of the various steps of the coagulation cascade, and the types of cells involved in contributing to the formation of clots (Meroni *et al.*, 2011).

Considering increasing subpopulations of aPL drive thrombotic mechanisms, it should be noted that in all corresponding in vivo models aPL reacting with human β2GPI and reacting with mouse, rat and hamster molecules has been shown to be pathogenic. In particular, thrombotic in vivo effects were developed using affinity-purified anti-β2GPI IgG and inhibited by specific anti-β2GPI activity absorption. In view of these experimental findings, the antibody subpopulation responsible for the thrombotic manifestations of APS should be known as the β2GPI-dependent aPL. In line with this assessment, β2GPI-dependent LA is reported to correlate with thrombosis better than LA in general (Meroni *et al.*, 2011).

However, in addition to β2GPI, aPL is also known to react with other phospholipid-binding proteins, especially prothrombin. The way anti-prothrombin antibodies exert their procoagulant effect is still a matter of research, but in vitro studies have suggested that endothelial cell function might be disrupted by reacting with the

target molecule expressed on the cell surface. However, there is a lack of in vivo studies using purified antiprothrombin polyclonal antibodies, and the pathogenic activity of these autoantibodies is mainly supported by experiments with monoclonal preparations and their epidemiologic association with thrombosis. The most convincing link between antiprothrombin antibodies and thrombosis has been reported for antibodies detected by the phosphatidylserine-prothrombin assay, suggesting that the pathogenic antibodies may recognize a conformational epitope or epitopes expressed by prothrombin when in complex with anionic phospholipids in the presence of calcium ions (Meroni *et al.*, 2011).

Contrary to what is known as β2GPI, there is a lack of information on the cross - reactivity of antiprothrombin antibodies with prothrombin from different species, making it difficult to assess in vivo pathogenicity of this type of aPL. Nevertheless, the suggestion that anti-β2GPI antibodies are the only pathogenic aPL for thrombosis is in apparent contrast with the widely accepted view that LA positivity is strongly associated with thrombotic risk; the finding that LA activity in patients with APS is more frequently associated with antiprothrombin thrombosis antibodies (Meroni *et al.*, 2011).

The procoagulant mechanisms mediated by aPL are related to their ability to react with phospholipid-binding proteins expressed on the cell membranes of different cell types. The antibody is thought to form a complex with the corresponding antigen, leading to cell membrane perturbation and eventually to signaling to the nucleus (Figure 2-1). Depending on their biological functions, perturbed cells will mediate different responses, which might contribute to the variety of APS clinical manifestations.

It is not yet clear whether aPL react substantially with phospholipid-binding proteins (in particular β2GPI and prothrombin) in the fluid phase. In this regard, all aPL are characterized by low avidity, suggesting that complex formation in the fluid phase

requires stoichiometric antigen-antibody ratios that are not commonly present in patients. This point further supports the hypothesis that the main pathogenic mechanisms mediated by aPL are those related to their reactivity with the target molecules expressed on cell membranes. In particular, β2GPI is expressed on cell membranes at high antigenic density, and so is more easily recognized by low avidity autoantibodies than more sparsely expressed molecules would be (Meroni *et al.*, 2011). The question of whether aPL can affect the biological function of target proteins in the fluid phase is another matter for investigation. For example, autoantibodies directed against enzymes may increase the enzymatic activity of the target molecules.

There is some evidence that antiprothrombin antibodies might induce a 'gain of function' of prothrombin, leading for example, to increased fibrin production. Assessing whether anti- β2GPI can alter the function of their target protein is, however, more difficult, as we still do not know what the true physiological role of β2GPI is in the coagulation cascade. A mild natural anticoagulant activity and a powerful effect in protecting against cell death induced by endothelial oxidative stress have been described for β2GPI, but whether anti-β2GPI antibodies increase these putative physiological activities remains to be investigated (Meroni *et al.*, 2011).

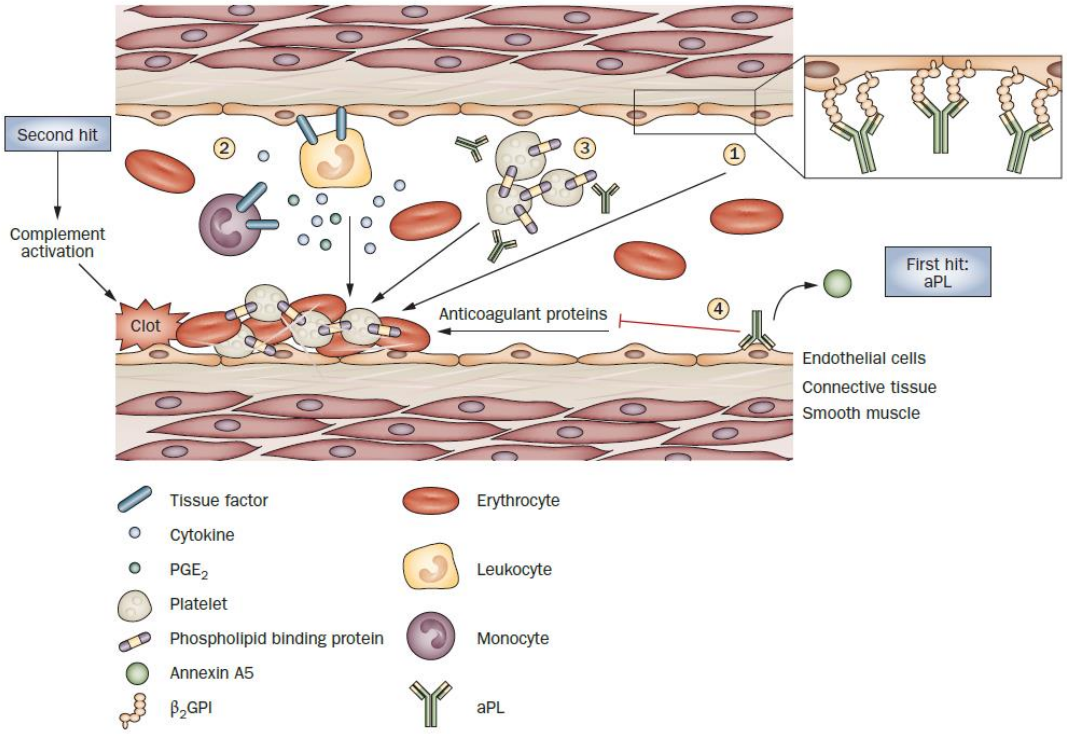

Figure 2.1 Pathogenic clotting mechanisms mediated by aPL

Adopted from (Meroni *et al.*, 2011).

aPL actions favour clot formation through several routes.

1. aPL interact with endothelial cells, primarily through binding of β2GPI on the cell surface, and induce a procoagulant and proinflammatory endothelial phenotype.

2. aPL upregulate tissue factor expression on endothelial cells and blood monocytes, and promote endothelial leukocyte adhesion, cytokine secretion and PGE_2 synthesis.

3. aPL recognize phospholipid-binding proteins expressed on platelets- aPL binding potentiates platelet aggregation induced by another agonist.

4. aPL interfere with plasma components of the coagulation cascade, by inhibiting anticoagulant activity, by affecting fibrinolysis, and by displacing the binding of the natural anticoagulant annexin A5 to anionic structures. These mechanisms all contribute to a procoagulant state that is necessary but not sufficient for clotting. Clot formation seems to require two steps: the presence of aPL provides the ‘first hit’, which produces

clotting when accompanied by another procoagulant condition, a ‘second hit’. Complement activation seems to be necessary for clot formation in vivo.

Abbreviations: aPL, antiphospholipid antibodies; β2GPI, β2 glycoprotein I; PGE_2, prostaglandin E_2.

2.1.4(c) Fetal loss

The presence of aPL represents the most frequent acquired risk factor for a treatable cause for recurrent pregnancy loss, and for pregnancy complications (early and severe preeclampsia). Such an association is supported by several epidemiological studies and by experimental models showing that passive transfer of aPL IgG induces fetal loss and growth retardation in pregnant naïve mice.

In line with the thrombophilic effect of aPL, intraplacental thrombosis, with impairment of maternal-fetal blood exchange, was initially suggested to be the main pathogenic mechanism of fetal loss. Placental thrombosis and infarction were reported, and in vitro studies showed that aPL might induce a procoagulant state at the placental level through several mechanisms, including the ability of the aPL antibodies (specifically, anti- β2GPI antibodies) to disrupt the anticoagulant annexin A5 shield on trophoblast and endothelial cell monolayers. Supporting the in vitro findings, the distribution of annexin A5 covering the intervillous surfaces was found to be considerably sparser in the placentas of aPL-positive women, in comparison with those lacking the autoantibodies. Nevertheless, these observations were not confirmed by other studies, which have failed to show intravascular or intervillous blood clots. Indeed, histopathological findings suggestive of thrombosis cannot be detected in most samples from miscarried fetuses and placentas from women with APS (Meroni *et al.*, 2011).

Fine-tuning of the maternal immune response takes place during embryo implantation, to allow normal progression of pregnancy. There is evidence for a dynamic balance between proinflammatory and anti-inflammatory mediators in normal pregnancy, with fluctuations between which signals predominate occurring during gestation. Acute inflammatory events are widely accepted to be generally responsible for a negative pregnancy outcome, and proinflammatory mediators (such as complement, tumor necrosis factor [TNF], and CC chemokines) have been shown to have a role in animal models of aPL-induced fetal loss (Meroni *et al.*, 2011).

Repeated intraperitoneal injections of large amounts of human IgG with aPL activity (10mg/mouse per injection) to pregnant naïve mice after embryo implantation induces considerable placental inflammatory damage that results in fetal resorption and growth retardation at day 15 of pregnancy. Immunohistochemical and histological examination of decidua showed deposition of human IgG and mouse complement, neutrophil infiltration and local TNF secretion, in association with a transient but significant increase in blood TNF levels. Several lines of evidence support involvement of the complement system in inducing aPL-mediated fetal loss in this mouse model, as suggested by the protection that deficiency in complement component confers on the animals, or that follows from in vivo inhibition of complement. The cleavage product C5a of the complement component C5 is the key effector in this model, and acts through the upregulated expression of TF on neutrophils infiltrating placental tissues. The hypothesis that complements is involved in the fetal loss induced by aPL is further supported by the demonstration that the protective effect of heparin in the mouse model is linked to the anticomplement, rather than to the anticoagulant, activity. However, the amount of IgG injected during these studies was much larger than the levels of antibodies that spontaneously occur in aPL patients, and the β2GPI specificity of the

IgG preparation was not investigated; these points need to be further addressed (Meroni *et al.*, 2011).

In another experimental model of fetal loss, mice deficient in chemokine-binding protein D6 (also known as chemokine-binding protein 2), a placental receptor that recognizes majority of inflammatory CC chemokines and targets them for degradation, are more susceptible to fetal loss when passively infused with a small amount of human aPL IgG than wild-type mice or mice-infused with normal IgG. Altogether, these findings suggest that a local acute inflammatory response might have a role in experimental aPL-mediated fetal loss (Meroni *et al.*, 2011).

From a clinical point of view, the contribution of acute inflammation to aPL-associated recurrent fetal loss would support a beneficial effect of corticosteroids in treating or preventing such complications. Although low corticosteroid doses (<20 mg/day) are occasionally used, particularly in women unresponsive to the standard therapy (low-dose aspirin and heparin), there is no sound evidence to support the routine use of corticosteroids. Furthermore, immunohistological analysis of abortive material or full-term placentae from women with APS has not provided conclusive information about the pathogenic contributions of acute local inflammatory events and complement deposition (Meroni *et al.*, 2011).

Finally, an inflammatory process does not seem to participate in another model of fetal resorption and growth retardation, elicited by intravenous injection of a small amount of human aPL IgG (10-50 µg/ mouse) into mice before implantation, as indicated by a lack of histological evidence of inflammation in the placentae (Meroni *et al.*, 2011).

Besides thrombosis, evidence indicates that alternative aPL-mediated pathogenic mechanisms impede placentation, involving direct targeting of maternal

decidua and the invading trophoblast (Figure 2-2). On the fetal side, aPL (in particular β2GPI-dependent antibodies) bind to human trophoblasts and affect several cell functions in vitro (Figure 2-3), inducing cell injury and apoptosis, inhibition of proliferation and syncitia formation, decreased production of human chorionic gonadtrophin, defective secretion of growth factors and impaired invasiveness. All of these aPL-mediated effects might participate in causing defective placentation (Meroni *et al.*, 2011).

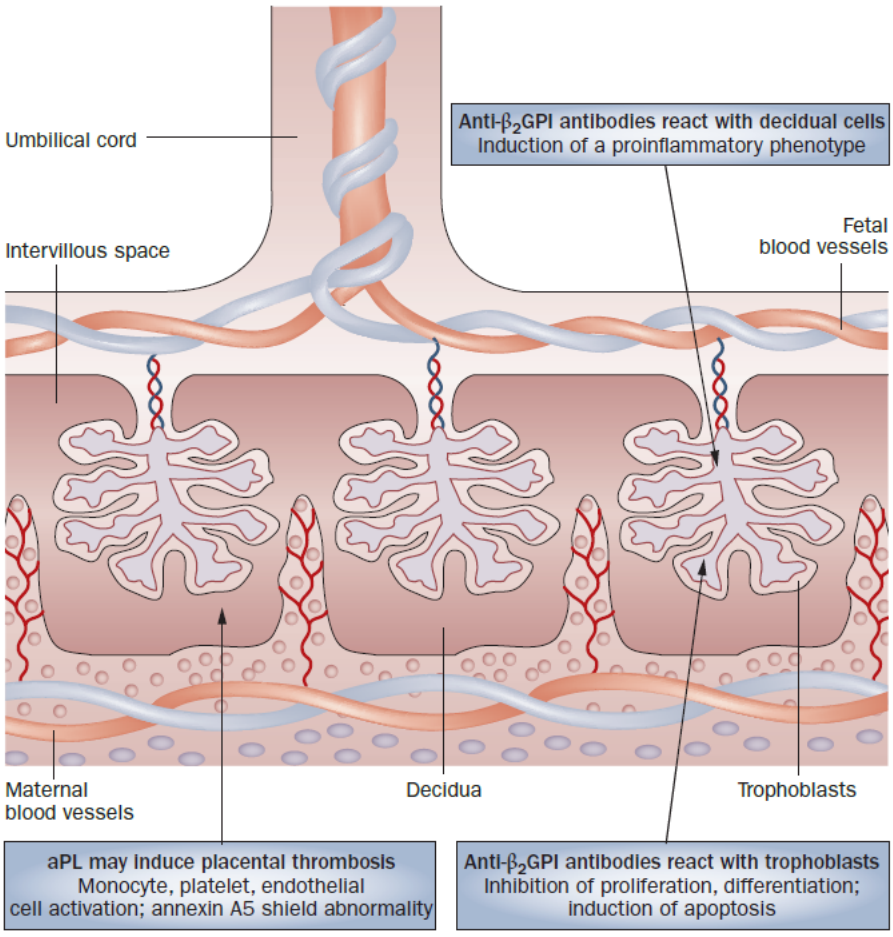

Figure 2.2 Main effects of aPL on placenta.

Adopted from (Meroni *et al.*, 2011).

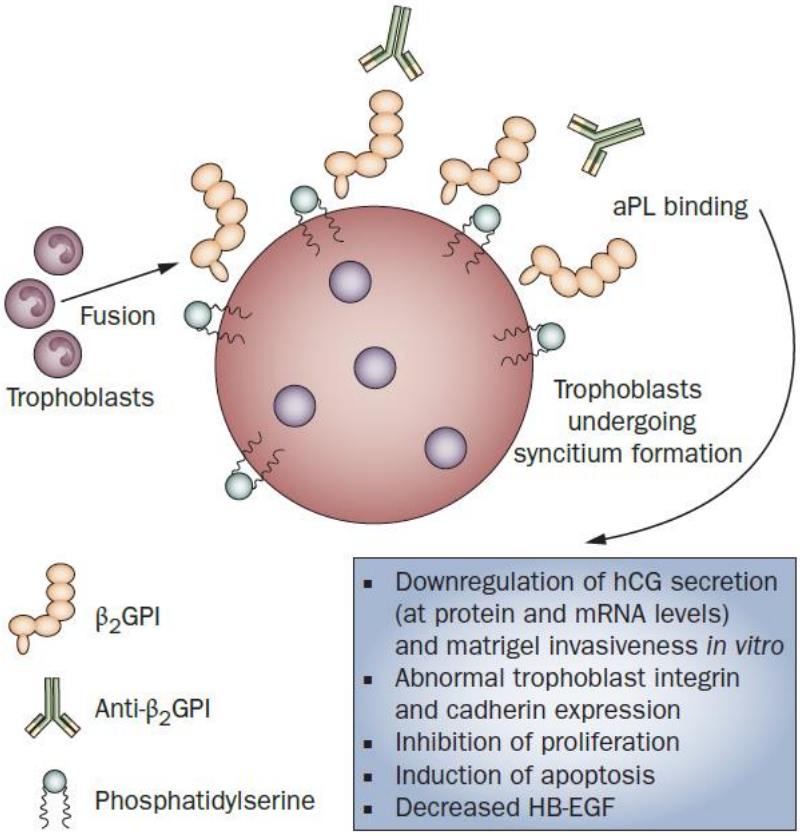

Figure 2.3 aPL effects on trophoblasts.

Adopted from (Meroni *et al.*, 2011)

Data have shown that aPL also cause abnormalities at the maternal side of the placenta. In fact, impaired endometrial differentiation, and reduced expression of complement decay-accelerating factor (also known as CD55) were found on endometrial biopsies. These alterations before conception might compromise implantation and predispose to complement-mediated pregnancy failure. In addition, β2GPI-dependent aPL are able to react with human stromal decidual cells in vitro, inducing a proinflammatory phenotype.

As a whole, these findings suggest that APS-associated pregnancy complications can be mediated by several distinct pathogenic events that are not necessarily related to the procoagulant or proinflammatory effects of aPL. On the other hand, data from in vivo animal models are biased by the fact that findings are restricted

to the period of pregnancy when the investigation was performed or are dependent on the timing of passive infusion of the putative pathogenic autoantibodies. For example, examination of animals immediately after the administration of large amount of autoantibodies midgestation might show an inflammatory signature that can go undetected if smaller amounts of the same autoantibodies are infused soon after mating. For the same reason, histological examination of full-term human placentae might show a clear picture of events that take place at the beginning of pregnancy, showing only the resulting damage (Meroni *et al.*, 2011).

2.1.5 Laboratory Diagnosis of APS

2.1.5(a) Diagnostic criteria

Initially, classification criteria for APS were established in 1999 ('Sapporo criteria') and then updated during the Sydney International Antiphospholipid Antibodies Congress in 2006 and are now referred to as the updated Sapporo or Sydney criteria. However, as (Favaloro and Wong, 2014) and also (Harris and Pierangeli, 2008) have previously pointed out, and as their name ('classification criteria') suggests, these 'criteria' were established primarily as tools for classification (as opposed to diagnostic). They were therefore designed and refined to increase the likelihood that patients who meet these conditions genuinely have APS as a disease to be able to join prospective clinical trials appropriately. Any patients who currently have APS would also not meet these classification requirements (Favaloro and Wong, 2014).

Consensus standards for the diagnosis of APS were accepted to ensure consistency in the research (Revised classification criteria for the APS, modified from (Miyakis *et al.*, 2006)).

Clinical criteria

1. Vascular thrombosis

One or more clinical episodes of arterial, venous, or small vessel thrombosis, in any tissue or organ. Thrombosis must be confirmed by objective validated criteria.

2. Pregnancy morbidity
    a) One or more unexplained deaths of a morphologically normal fetus or beyond the 10th week of gestation, with normal fetal morphology documented by ultrasound or by direct examination of the fetus.
    b) One or more pre-term births of a morphologically normal neonate before 34th week of gestation because of: (i) eclampsia or severe pre-eclampsia or (ii) recognized features of placental insufficiency, or
    c) Three or more unexplained consecutive spontaneous miscarriages before the 10th week of gestation, with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded.

Laboratory criteria

1. Lupus anticoagulant (LA) present in plasma, on two or more occasions at least 12 weeks apart, detected according to the guidelines of the International Society on Thrombosis (ISTH).
2. Anticardiolipin antibody (aCL) of IgG and/or IgM isotype in serum or plasma, present in medium or high titre (i.e. >40GPL units or MPL units, or >the 99th centile), on two or more occasions, at least 12 weeks apart, measured by a standardized ELISA.
3. Anti-beta-2 glycoprotein I (anti-β2GPI) antibody of IgG and/or IgM isotype in serum or plasma (in titre >the 99th centile), present on two or more occasions, at least 12 weeks apart, measured by a standardized ELISA.

APS is present if at least one of the clinical criteria and one the laboratory criteria are met (Sen *et al.*, 2013).

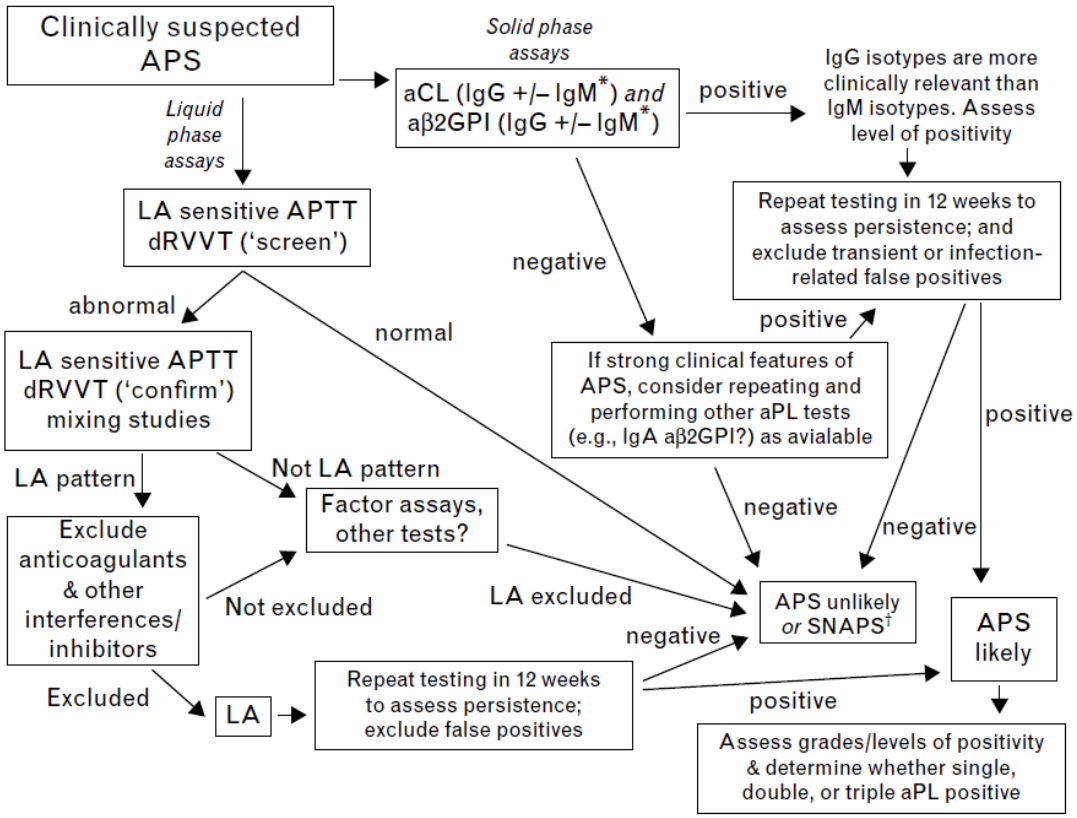

Figure 2.4 Algorithm of laboratory testing with solid and liquid phase assays for evaluation of possible APS.
* Routine first-line testing for IgM aCL and IgM anti-β2GPI for diagnostic purposes is no longer recommended based on the current evidence base (please refer to the section on 'Assay Selection' in the text for further details).
* Differentiation between 'APS unlikely' and 'SNAPS' is heavily dependent on the clinical scenario and pre-test probability of the subject having APS.
anti-β2GPI, anti-beta 2 glycoprotein I antibody; aCL, anticardiolipin antibody; aPL, antiphospholipid antibody; APS, antiphospholipid syndrome; APTT, activated partial thromboplastin time; dRVVT, dilute Russell viper venom time; LA, lupus anticoagulant; SNAPS, seronegative antiphospholipid syndrome. Adopted from (Favaloro and Wong, 2014).

One possible algorithm to incorporate laboratory testing with both solid and liquid phase assays for the evaluation of possible APS. The major points illustrated are: (i) both solid phase and liquid phase testing should be undertaken (i.e., neither should be undertaken without the other); (ii) for liquid phase testing, (a) at least two different tests

should be performed and found to be negative before excluding LA (APTT and dRVVT in this example), (b) mixing with normal plasma should show evidence of inhibitor, (c) an abnormal screening test result should show evidence of correction with excess phospholipid (PL) ('confirmation'), and (d) other potential confounding abnormalities (e.g., factor deficiencies, anticoagulant therapy) should be excluded; (iii) for solid phase testing, performance of both IgG aCL and IgG anti-β2GPI assays are recommended in subjects suspected of having APS to help determine their aPL profile, in particular the presence of triple aPL positivity (namely positive IgG aCL and IgG anti-β2GPI along with positive LA); and (iv) for both solid phase and liquid phase testing repeat testing (after 12 weeks) is recommended following initial positive tests to confirm persistence, and also following initial negative tests in high pre-test probability scenarios to exclude falsely negative results at the time of thrombosis (Favaloro and Wong, 2014).

2.1.5(b) Antiphospholipid antibodies profile and thrombotic risk

Unlike the original Sapporo Criteria, the Sydney Criteria strongly recommended that patients with APS be classified into those with positivity for one aPL (LA only, aCL only, or anti-β2GPI only) and those with more than one laboratory criteria present in any combination. This was based on the concept that, compared to multiple (particularly triple) positivity, positive for one aPL carries a lower risk of thrombosis or pregnancy complications. Consequently, assessing patients using the LA, aCL or anti-β2GPI aPL profile, rather than an individual test, may be useful in assessing thrombotic risk. These laboratory findings may be explained by an understanding of the antigenic aim of pathologic aPLs. In 1990 autoantibodies directed against β2GPI in patients with APS were identified as an important subgroup of antibodies. It was then elucidated that thrombosis-associated aCLs tend to be β2GPI-recognizing autoantibodies, which have a high affinity for cardiolipin. Two types of aCL appear to exist: anti-β2GPI-dependent

aCLs, which are associated with complications of thrombosis and pregnancy, and anti-β2GPI-independent aCL antibodies which appear to be associated with infection. The latter are generally transient antibodies and are not associated with thrombosis. There are many aCL kits which include a β2GPI source. Similarly, the autoantibodies against β2GPI were later identified as having LA activity. LA that binds β2GPI and is associated with thrombosis and LA that binds prothrombin may be classified into LA, but these are not currently available outside of a research setting. Distinguishing anti-β2GPI-dependent and anti-β2GPI-independent aCLs and LA can have important clinical implications and allow clinicians to distinguish between pathological antibodies associated with thrombosis and nonpathological antibodies (Lim, 2013).

Therefore, laboratory tests evaluating LA, aCLs, and anti-β2GPI antibodies tend to detect overlapping, but various, autoantibodies populations, which may account for the varied clinical manifestations of APS. LA-assays seem to detect the pathological aPLs better than aCLs or anti-β2GPI assays. Apart from distinguishing whether aPLs bind β2GPI, different subpopulations of anti-β2GPI antibodies appear to recognize different epitopes on β2GPI. Antibodies that bind β2GPI domain I seem to interact with complications of thrombosis and pregnancy relative to antibodies that bind other domains. In laboratory assays, autoantibodies that bind domain I tend to result in triple positivity, identifying patients at higher risk for thrombotic complications. Other variables that predict thrombosis tend to be persistence of aPLs and a high titre of aPL that culminated in Sydney Criteria being included (Lim, 2013).

2.1.1(b)(i) Risk of thrombosis with positive testing for one aPL

A systematic review found no link between thrombosis and aCL, and this was later verified in prospective studies. Even when assessed at moderate to high titre (aCL >40 IgG or IgM phospholipid units), the presence of aCL alone was not

associated with thrombosis, although pregnancy morbidity was observed. Similarly, patients with anti-β2GPI only positive tend to have no correlation with thrombosis or the loss of pregnancy. By comparison, LA tends to be more strongly associated with thrombosis and complications of pregnancy, although contradictory evidence indicate that isolated LA may not be associated with elevated thrombotic risk. The ELISA assays for aCLs and anti-β2GPI have been plagued by poor standardisation and unreliable results. By comparison, LA assays have greater standardisation and strong association with the clinical manifestation of APS (Lim, 2013).

2.1.1(b)(ii) Risk of thrombosis with positive testing for 2 or more aPLs

Analysis of patient samples taken at Warfarin enrolment in the Antiphospholipid Syndrome (WAPS) study showed that patients with positive LA testing and positive anti-β2GPI were at elevated risk of total thrombosis (odd ratio [OR] = 2.5, 95 % CI, 1.0-6.0). In retrospective and prospective studies patients with three positive aPL tests were shown to be at increased risk of thrombosis or morbidity of pregnancy, with thrombosis ORs ranging from ~5 to 33. In a retrospective study of 160 patients with triple-positive aPL testing, a cumulative incidence of thrombosis of 12.2%, 26.1% and 44.2% was found after 1, 5 and 10 years of follow-up, respectively. In a prospective study of 194 patients with persistent LA and/or aCL, the highest incidence of thrombosis was observed in patients with persistent LA who were also positive for anti-β2GPI and anti-prothrombin antibodies estimated at 8.4% per patient-year. Further study is needed to determine whether aPL profiles will affect APS classification and influence clinical decision making. APL profiles and their effect on thrombotic risk have not been specifically studied in CAPS patients or in CAPS registries (Lim, 2013).

Other aspects that affect thrombotic risk include the existence of other thrombotic risk factors (e.g., hereditary thrombophilia, pregnancy, immobilisation, surgery) and the existence of systemic lupus erythematosus (SLE), in addition to the patients' aPL profile. Patients with SLE have an elevated risk of thrombosis relative to the general population and those who has isolated but persistently positive aPL tend to be at greater risk (Lim, 2013).

2.1.5(c) Pre-analytical, analytical and post-analytical considerations in APL testing

1) Pre-analytical considerations	a) Sample type and sample processing	• Serum is the preferred sample for solid phase assays • Plasma represents the required sample for LA testing • Citrate anticoagulated plasma may lead to a 10% dilution factor • Errors during sample collection → Laboratory staff have difficulty in differentiating plasma and serum as they are visually identical → laboratory should reject inappropriate samples and arrange collection of the appropriate sample type • Sample needs to be platelet poor ($<10x10^9$), particularly if it is to be frozen prior to testing → can cause false negative result if not platelet poor → achieve platelet poor by a process of double centrifugation • Specimen transport conducted according to current recommendations • Specimens should be capped when not in use to avoid changes in pH → may yield false positive LA results
	b) Anticoagulation therapy	• Testing of LA should ideally not be undertaken on patients who are being treated with anticoagulant therapy • Vitamin K agonists (VKAs), heparin and direct oral anticoagulants (e.g., dabigatran,

		rivaroxaban, apixaban, edoxaban) will complicate test performance and interpretation → lead to false positive result in some cases → misdiagnose APS. • In vivo anticoagulants may affect the in vitro assays used in the laboratory • It is possible to test for LA whilst on VKA therapy using mixing assays • LA testing is not recommended while patients are on heparin or the new direct oral anticoagulants since these essentially act like coagulation inhibitors → can lead to false positive results. • Anticoagulation thrapy is not expected to affect solid phase assays such as aCL and anti-β2GPI → these aPL assays can still be performed as normal.
	c) Assay selection – liquid phase assays	• dRVVT as the primary test in LA testing • APTT as the second test • No more than 2 tests should be performed → this may increase the risk of false positive LA diagnosis • LA can be identified if either or both of the tests is positive • Clinician may order a single test or a large number of tests → false positive LA detection
	d) Assay selection – solid phase assays	• Current classification criteria require that IgG and IgM isotype testing for both aCL and anti-β2GPI • IgM isotypes of aCL and anti-β2GPI were not significantly associated with thrombosis or obstetric manifestations • IgG isotypes of aCL and anti-β2GPI have higher clinical utility than the IgM isotypes • Testing for IgA anti-β2GPI (rather than IgA aCL) may be helpful when other isotypes are negative in the setting of a high pre-test probability for APS