AN AUDIT OF COMPLIANCE WITH THE BRITISH COMMITTEE FOR STANDARDS IN HAEMATOLOGY GUIDELINES ON THE INVESTIGATION OF ANTIPHOSPHOLIPID SYNDROME

A dissertation submitted to The University of Manchester for the degree of Master of Clinical Immunology in the Faculty of Medical and Human Sciences.

2016

9592081

SCHOOL OF MEDICAL SCIENCES
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ABSTRACT

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized mainly by thrombosis and pregnancy morbidity. The diagnosis of APS relies on the detection of antiphospholipid antibodies (aPL), namely Lupus anticoagulant (LA), anticardiolipin antibodies (aCL) and anti-Beta2-Glycoprotein-I antibodies (anti-B2GP1) which are laboratory tests with certain limitations. The treatment of APS consists of long-term anticoagulation therapy and varies according to the clinical and immunological profile of the patient. In order to address the need for clear diagnostic criteria of APS, the British Committee for Standards in Haematology (BCSH) published the most updated version of Guidelines on the investigation and management of APS.

This study undertook an audit aiming to evaluate whether or not the clinicians from Lancashire Teaching Hospitals and from primary care services across the region were requesting aPL tests according to the BCSH Guidelines. In the period March to August 2015, 2042 test requests for aPL testing were gathered. Of these, 648 were excluded because of duplicates, lack of aPL results and issues in the clinical information provided, leaving 1394 requests for this retrospective audit.

A set of three standards based on BCSH guidelines were created to assess compliance. The audit reveals that only 42 per cent of requests had clinical details that were recognized as fully compliant with the guidelines. Less than one percent of the tests included all three aPL tests (LA+aCL+anti-B2GP1) recognized in the laboratory criteria. Moreover, approximately one third of the requests had follow up testing within the time interval recommended in the BCSH Guidelines. Overall, the adherence to the BCSH Guidelines by the clinicians needs to be improved.

Consequently, this study recommends further investigation into the causes of noncompliance with aPL ordering practice, training options for health care staff and clearer definitions of pregnancy morbidity and thrombosis within the context of APS testing.
DECLARATION

No portion of the work referred to in the dissertation has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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DEDICATION
I dedicate all this year of study and MSc project to my husband, Fernando, my sons, Esteban and Joseph, and my daughter, Rosana. They have believed in me, encouraged me and supported me continuously. Finally, all my effort is dedicated to God who is the provider of all wisdom and intelligence.

ACKNOWLEDGEMENT
I would like to thank my supervisor Catherine Keymer for her corrections and her help in the development of this project. I also want to express my sincere recognition to Dr Anthony Rowbottom for suggesting this audit. Likewise, I would like to express my sincere gratitude to the Royal Preston Immunology Service for the data provided. My sincere gratitude also goes to the Bull Family, Paul, Nicola and Serenna, for their friendship, support and for hosting my family and me, and to all our friends who have helped us to face difficult times in England. Finally, I would like to express my gratefulness to my sponsor, the Ecuadorian Government, who granted me a scholarship to study at The University of Manchester with the program "Universidades de Excelencia, 2015".
1. INTRODUCTION: ANTIPHOSPHOLIPID SYNDROME

Antiphospholipid Syndrome (APS) is a systemic autoimmune disease defined by the presence of thrombosis (venous, arterial or microvascular) and/or pregnancy morbidity in an individual with persistent levels of a heterogeneous group of antibodies known as antiphospholipid antibodies (aPL).(1-3) Currently, Lupus anticoagulant (LA), anticardiolipin antibodies (aCL) and anti-Beta2-Glycoprotein-I antibodies (anti-B2GP1) are the aPL included in the classification criteria for APS. They can be detected by either coagulation assays or immunological assays. Other types of antibodies against plasma proteins have been identified, including anti-prothrombin (aPT), anti-phosphatidyl serine (aPS) and anti-phosphatidyl ethanolamine (aPE); but their clinical utility has not been fully demonstrated.(4, 5)

APS is characterised by a state of hypercoagulability which can affect any venous or arterial bed resulting in a wide variety of clinical manifestations. The most common manifestations are deep venous thrombosis, stroke, pulmonary embolism and obstetric morbidity (including both foetal and maternal complications).(6, 7) The definite diagnosis of APS requires a personalized medicine approach following strict guidelines which consider the presence of at least one clinical criterion with persistent positivity of aPL at least 12 weeks apart.(3) Life-long anticoagulation therapy is the treatment of choice. This approach aims to prevent, if not, reduce the risk of thromboembolic events and pregnancy complications.(1)

1.1. EPIDEMIOLOGY

According to several in the field of antiphospholipid syndrome research the prevalence of antiphospholipid antibodies in the general population may vary from 1-5 %. (8) These frequencies increase with age and the presence of a chronic disease. Testing positive for aPL does not necessarily mean a risk of developing APS because many patients could develop aPL as a result of vaccination, infections, certain drugs and malignancy.(9) Moreover, in a year, only about 1% of patients incidentally found to produce aPL are at risk of developing a thrombotic episode, compared to 40% of Systemic Lupus Erythematosus (SLE) patients who develop clinical features associated with APS after 20 years of follow-up.(10, 11) Although extensive research has been carried out on the frequency of aPL in APS patients, no single study indicating the incidence of the disease in the UK population could be found. Gómez-Puerta & Cervera (7) estimates that the incidence of the APS in the USA is around 5
new cases per 100,000 people per year and the prevalence around 40-50 cases per 100,000 people. Furthermore, in 2013 the Antiphospholipid Syndrome Alliance for Clinical Trials and International Networking (APS ACTION) performed a systematic literature review which estimates the aPL frequency in the most common clinical manifestation of APS. (12) Overall frequencies are: 6% for pregnancy morbidity, 13.5% for stroke, 11% for myocardial infarction, and 9.5% for deep vein thrombosis. However, it is noted that there are difficulties in determining the true prevalence of APS as figures for clinical manifestations of APS are highly variable due, at least in part, to the differences between the inclusion criteria for patients and to antiphospholipid antibody testing considerations.(13)

Antiphospholipid syndrome can occur in association with other systemic autoimmune diseases, mainly SLE. The prevalence of aPL in SLE patients vary between studies: 10-30% have lupus anticoagulant, 12-50% have positive aCL and 35-45% anti-B2GP1 (IgG, IgM and/or IgA isotypes).(9, 14) This high variability may be explained as a result of the disease activity which causes the intermittent production of aPL. Moreover, in some cases, the presence of aPL in SLE patients can be demonstrated only after a thrombotic episode.(8) However, it is not yet clear whether APS and SLE represent two elements of the same process, APS and SLE are two diseases coinciding in an individual, or whether SLE offers a setting for the development of APS.(15, 16) Additionally, Khamashta et al. (17) and Reynaud et al. (18) have reported that 30% of SLE patients will develop APS after a 20 year follow up with the highest risk of developing APS clinical manifestations being among the patients with positive LA. Foetal death during the first trimester of pregnancy has been associated with APS. 1 out of 100 women attempting to have children manifest recurrent miscarriage; of these women 10% to 15% are diagnosed with APS.(19, 20)

A small subset of patients with APS, less than 1%, develop a highly aggressive form of the disease. This condition is termed catastrophic APS (CAPS) which is characterized by the persistent presence of elevated titres of antiphospholipid antibodies and microthrombosis that leads to rapid multi-organ failure.(6, 7, 21)

1.2. PATHOGENESIS

The mechanisms behind the clinical manifestations of APS are not fully elucidated. However, the pathogenic role of aPL causing thrombosis and foetal death have been described as well as some clinical features caused by direct antibody damage have been reported.(1, 22)
1.2.1. Aetiology

APS is the result of a combination of environmental factors in an individual with genetic predisposition. HLA-DR4, HLA-DR7, and HLA-DRw53 have been associated with a higher risk of developing APS and thrombosis. Nevertheless, the environmental factors are most important since some patients with those HLA alleles do not develop APS. As a result, in a genetically susceptible individual the exposure to an environmental factor triggers the production of pathogenic autoantibodies associated with APS.

The presence of aPL has been found following infections. The findings in experimental models indicate that molecular mimicry between Beta-2-Glycoprotein-I (B2GP1) and infectious agents play an important role in the development of aPL. In the literature, several infectious diseases have been associated with APS or aPL positivity. Although in previous years the presence of this aPL was considered transient and not pathologic, there is some evidence that supports the presentation of APS symptoms in aPL positive patients with previous infections.

<table>
<thead>
<tr>
<th>Table 1. Infectious agents reported to be associated with aPL production (23-25)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Virus</strong></td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
</tr>
<tr>
<td><strong>Parasites</strong></td>
</tr>
</tbody>
</table>

Recently, Ruff et al. (26) have described the role of the microbiota in the development of aPL. According to their research, depletion of gut microbiome with broad spectrum antibiotics prevents thrombotic events and reduces anti-B2GP1 titres in APS-animal models. His findings suggest that commensal bacteria may cause conformational changes in the B2GP1 molecule or induce anti-B2GP1 antibodies from autoreactive CD4+ T cells. Furthermore, according to the revision of Giannakopoulos et al (20), oxidative stress is a common characteristic in patients with APS. This suggests that, under conditions of oxidative stress, formation of disulphide bonds in the molecule of B2GP1 make it immunogenic and capable of inducing aPL formation. Production of aPL has been associated with autoimmune conditions including not only SLE, but also rheumatoid arthritis, systemic sclerosis, Sjogren’s syndrome and temporal arteritis.
Likewise, presence of aPL has been reported in lymphoproliferative diseases, such as lymphoma and paraproteinemia.(11) In addition, other environmental factors have been linked with the aetiology of APS. The use of vaccines and certain drugs (including antiepileptic drugs, antihypertensive agents, antiarrhythmic drugs and antibiotics) have been reported to induce APS but the prevalence of aPL in these cases is unknown.(23)

### 1.2.2. Antiphospholipid antibodies (aPL)

The family of antiphospholipid antibodies includes various antibodies that recognize different antigenic targets(27):

1. Antibodies directed against phospholipids (PL) alone: anticardiolipin antibodies (aCL) and other autoantibodies against negatively charged phospholipids including phosphatidylinositol, phosphatidylserine, phosphatidic acid and phosphatidylethanolamine.
2. Antibodies directed against PL in complex with a (plasma-derived) phospholipid-binding ‘cofactor’ protein (B2GP1, prothrombin (PT)): anticardiolipin antibodies (aCL), antibodies PT/PE complexes.
4. Antibodies against coagulation cascade proteins, such as antibodies against protein C and protein S, Anexin V and Anexin II, and antibodies against plasma lipoproteins (anti-oxidized LDL antibodies).
5. Antibodies against other intravascular structures: anti-platelet antibodies, anti-erythrocyte antibodies, anti-endothelial cells antibodies.

Although a broad spectrum of aPL antibodies exist, only lupus anticoagulant, IgG/IgM aCL and IgG/IgM anti-B2GP1 antibodies are recognized as biomarkers of APS in the clinical practice.(3, 28) There is not enough evidence to support the clinical significance of the other autoantibodies in the context of APS. (25, 29)

Some studies have suggested that antibodies reacting against B2GP1 mediate the pathogenic mechanisms behind recurrent pregnancy loss and thrombotic events in APS.(23, 30-32) This hypothesis will be discussed in the following sections.
1.2.3. Beta-2 Glycoprotein 1 (B2GP1)

Human B2GP1 is a 50-kD phospholipid-binding protein and it belongs to the complement control family. It circulates at a plasma concentration of 200 µg/ml in healthy individuals and APS patients. The B2GP1 molecule structure possesses 326 amino acids organized in five repeating amino acid domains. (23, 31, 32) Four domains (domains I through IV) contain two disulphide bridges each and the fifth domain (domain V) contains an extra disulphide bridge linking cysteine Cys288 with Cys326 in a lysine-rich locus which forms the binding site for anionic phospholipid. (20, 23, 32)

B2GP1 is recognized as one of the main autoantigens for aPL. (1, 31) Some aCL bind cardiolipin in the presence of B2GP1 and some react with cardiolipin independently of B2GP1. The latter antibodies are associated with syphilis or other infectious diseases and their positivity in patients is transient, while the former are associated with APS clinical manifestations. (31-33) B2GP1 can exist in two conformations in plasma, a circular and a linear form. The circular form is stabilized by the interaction of domain I with domain V. When B2GP1 attaches to a negatively charged phospholipid through the domain V, the B2PG1 molecule opens its structure up to a fishhook configuration (linear structure). This conformational change exposes a cryptic epitope on domain I (Gly40-Arg43) allowing antiphospholipid antibody binding. (4, 20) (Figure 1) Although the function of B2GP1 is unknown in humans, it seems that the aCL present in APS patients induce a new function for this glycoprotein hampering haemostatic processes. (34, 35)

![Figure 1 Model of binding of B2GP1 to phospholipid in cell surfaces. B2GP1 circulates in plasma in a closed conformation. When B2GP1 attaches to a negatively charged phospholipid through the domain V, the B2PG1 molecule opens its structure up to a fishhook configuration. aPL antibodies bind to and stabilize B2GP1 in its linear conformation when it is able to interact with receptors on the surface of endothelial cells, monocytes and macrophages. (34)
Antibodies that bind the epitope on domain 1 have been correlated with a higher risk of thrombosis compared to other antibodies. Anti-domain 1 antibodies have Lupus anticoagulant activity and there is increased interest in these for diagnostic purposes.(20, 36)

1.2.4. The thrombotic mechanism
Thrombotic events occur occasionally in individuals with persistent serum levels of aPL.(30, 36) Currently, the “two hit” model of thrombosis associated with APS is widely accepted. This model proposes that alongside the presence of aPL another factor leading to thrombosis is necessary.(11, 35) The “first hit” is the induction of a procoagulant state. In order to induce this state, it has been proposed that aPL bind to endothelial cells, monocytes and platelets through B2GP1.(4, 19) Moreover, Giannakopolous et al. (20) have postulated that disturbance of the redox balance in the circulatory system of patients with APS primes the endothelium allowing formation of B2GP1 immunocomplexes on the cell surfaces.
While aPL-B2GP1 immunocomplexes have been shown to cause thrombosis in several animal models, the exact metabolic pathway and the target for those complexes in humans, remains elusive. Nevertheless, numerous possibilities have been suggested linking B2GP1 in the pathogenesis of the aPL.(7, 35) aPL antibodies cause a procoagulant state by several mechanisms which are indicated in table 2.
B2GP1 immunocomplexes have been described activating endothelial cells, monocytes and platelets through phosphorylation of p38 MAP kinase and subsequent NFκB activation.(30) As a result, monocytes and endothelial cells increase expression of proadhesive and procoagulant molecules, such as ICAM-1, VCAM-1, E-selectin and tissue factor, and the secretion of proinflammatory cytokines (IL-1b, IL-6, IL-8, TNFα). Platelets increase the synthesis of glycoprotein 2b-3a and thromboxane A2, as well as further activation of platelet aggregation by allowing the interaction between the Fc portion and the platelet surface FcγRII receptors.(20, 34, 36)(Figure 2) Additionally, activation of the complement system by aPL, has been observed, particularly between C5a and its receptor. (14)
Thereafter, all of the proinflammatory and prothrombotic markers in association with coagulation-regulatory proteins (protein C, prothrombin, plasmin) create a procoagulant environment in the vasculature in which a “second hit” is required for thrombus formation. (19, 30) For instance, infection, tobacco, inflammation, exogenous female hormone use and endothelial injury caused in surgical procedures or trauma are considered as trigger events. (2, 19, 20)

**Table 2 Proposed mechanisms of aPL-mediated thrombosis (1, 11, 34)**

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Target</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inhibition of protein C activity</strong></td>
<td>Protein C</td>
<td>Interference with the components of the coagulation cascade. Inhibition of antithrombin activity.</td>
</tr>
<tr>
<td></td>
<td>Protein S</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thrombomodulin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Activated protein C resistance</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endothelial protein C receptor</td>
<td></td>
</tr>
<tr>
<td><strong>Increased thrombin generation</strong></td>
<td>Prothrombin binding</td>
<td>Inhibition of tissue factor pathway inhibitor.</td>
</tr>
<tr>
<td></td>
<td>Heparin cofactor II</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protein Z</td>
<td>Induction of microparticles formation</td>
</tr>
<tr>
<td></td>
<td>Factor XI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Factor XII</td>
<td></td>
</tr>
<tr>
<td>Disruption of protective shield on endothelial cells</td>
<td>Annexin A5</td>
<td>Availability of cell surfaces for coagulation reactions</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Altered fibrinolysis</td>
<td>Tissue plasminogen factor</td>
<td>Decreased blood clot regulation</td>
</tr>
<tr>
<td></td>
<td>Anexin A2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B2GP1 cleavage</td>
<td></td>
</tr>
<tr>
<td>Unbalanced complement</td>
<td>C3, C5a</td>
<td>Complement activation and deposition</td>
</tr>
<tr>
<td></td>
<td>Membrane attack complex</td>
<td></td>
</tr>
<tr>
<td>Platelet adhesion</td>
<td>Von Willebrand factor</td>
<td>Fc-FcgRII receptors interaction</td>
</tr>
<tr>
<td>Platelet activation</td>
<td>Low-density lipoprotein receptor 8 (LRP8)</td>
<td>PF4-β2GP1 complexes activate GPIIb/IIIa receptor expression and thromboxaneA2 production by 38MAP kinase phosphorylation.</td>
</tr>
<tr>
<td></td>
<td>Glycoprotein Iba</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Platelet factor 4 (PF4)</td>
<td></td>
</tr>
<tr>
<td>Endothelial cell, monocyte and neutrophil activation</td>
<td>TLR4</td>
<td>Phosphorylation of p38MAP kinase and subsequent NFkB activation resulting in increasing expression of tissue factor, adhesion molecules (ICAM-1, VCAM-1, E-selectin), proinflammatory cytokines (IL-1b, IL-6, IL-8, TNFα) and prostaclycin.</td>
</tr>
<tr>
<td></td>
<td>TLR2</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>Increased atherosclerosis</td>
<td>Oxidized-LDL (low-density lipoprotein)</td>
<td>Inflammatory response caused by endothelial injury. aPL and anti-oxLDL are atherogenic and they can bind to oxLDL.</td>
</tr>
</tbody>
</table>

### 1.2.5. The obstetric mechanism

It is currently accepted that antiphospholipid antibodies are associated with prematurity and foetal death through foetal growth restriction and foetal distress. The pathways of how aPL exert their pathogenic properties, causing these complications, is not yet fully elucidated. Most of the evidence to date is based on the passive transfer of human antibodies within pregnant mice models. Nevertheless, the following mechanisms have been suggested:

1. Intraplacental thrombosis: A thrombotic mechanism mediating aPL-B2GP1 immunocomplexes binding to annexin V on the surface of the trophoblasts, breaking the anticoagulant shield of the placenta, causing damage and the production of IL-3 (involved in the process of embryo implantation).
2. Complement activation: aPL activate complement through the classical pathway generating C5a fragments which induces tissue factor from maternal neutrophils.
3. Inflammation: due to complement deposition, neutrophil infiltration, and TNFα secretion in decidual tissue.

4. Inhibition of syncytium-trophoblast differentiation: shown by the reduced secretion of human chorionic gonadotrophin (hCG). aPL decrease alpha 1 integrin and VE-cadherin and up-regulate alpha 5 integrin and E-cadherin affecting the implantation of the trophoblast into maternal tissues.

In the obstetric mechanism, as well as in the thrombotic mechanism, aPL have been associated with the induction of a placental hypercoagulable state. As a result, vasculopathy and clot formation may cause intrauterine growth restriction, signs of hypoxia (abnormal foetal heart rate), oligohydramnios, foetal distress, preterm delivery or miscarriages.(25, 37) However, it should be emphasized that the implantation of the embryo is a dynamic process and any alteration in the environment or in the state of the trophoblast may produce a failure in the implantation. A detailed investigation of the causes of obstetric complications is required before considering aPL as the cause, including anatomic, hormonal and chromosomal causes.(25, 37, 38)

1.3. CLINICAL FEATURES

The hallmarks of APS are thrombosis (venous, arterial and microvascular) and pregnancy morbidity. Any vascular bed may be affected, with potentially any organ and tissue affected, causing many different clinical manifestations. The symptoms depend on the affected thrombotic area.

1.3.1. Vascular manifestations

Lower limb deep vein thrombosis (DVT) and/or pulmonary embolism (PE) are the most common forms of APS affecting the venous beds.(3, 13) Other veins affected include superficial, portal, renal, mesenteric and intracranial veins.(2)

On the other hand, the most frequent site of arterial occlusion is the cerebral vasculature, resulting in Transient cerebral ischemic attacks (TIA) and stroke. Other potential sites include the brachial, subclavian, axillary, aorta, iliac, femoral, renal, mesenteric, retinal and peripheral arteries.(13, 22)

Pregnancy, surgery and inherited thrombophilias increase the risk of vascular thrombosis in APS patients. Also, young patients (<45 years) with cerebrovascular episodes should be investigated for APS.(16, 22)
1.3.2. Pregnancy manifestations

The most common feature of pregnancy morbidity in the context of APS is recurrent early miscarriages before the 10th week of gestation.(37) Further foetal death associations include prematurity due to eclampsia/severe pre-eclampsia, intrauterine growth restriction due to placental insufficiency and stillbirth. Less common features are placental abruption, late premature birth, in vitro fertilization failures and HELLP syndrome (hemolysis, liver enzyme elevation and thrombocytopenia).(11) Patients can present pregnancy complications alone or associated with vascular thrombosis.(37)

According to a multicentre prospective registry a two-fold increased risk of developing neurologic disabilities such as hyperactive behaviour, feeding disorders, language delay, and autism has been reported in babies born to mothers with APS.(37) However, the majority of mothers with APS give birth prematurely making a direct link between aPL and these disabilities unclear as they also link to preterm birth. Additionally, after a 5 year follow-up, systematic psychomotor and cognitive difficulties in children born to APS patients have been described.(37)

1.3.3. Cardiac Manifestations

Cardiac features include valve lesions in the form of vegetations and/or thickening.(15) The mitral valve is most commonly affected followed by the aortic valve. Additionally, myocardial infarction, intracardiac thrombus leading to pulmonary hypertension, cardiomyopathy and diastolic dysfunction have been reported.(2, 22) Intervention for acute myocardial infarction seems to produce an adverse cardiac outcome in patients with APS. Moreover, accelerated atherosclerosis associated with aPL against oxidized-LDL proteins has been described.(10, 22, 39)

1.3.4. Neurologic manifestations

Transient cerebral ischemic attack (TIA) and stroke are considered to be the most common thrombotic arterial manifestations. Although the mechanism behind some of the neurologic associations with APS remains poorly understood, some patients have been reported as presenting the following neurological symptoms(6, 10, 22, 36):

- Vertigo, weakness, transient paraesthesia, amaurosis fugax, transient global amnesia, idiopathic intracranial hypertension, Guillain Barre-like syndrome, neuropathy, dystonia and a Parkinson-like syndrome. Vision and hearing problems, including sensorineural hearing loss in the inner ear, transient blurred vision and transient
diplopia. These are linked to cerebral microvasculopathy caused by recurrent thrombosis.

- Cognitive dysfunction and multi infarct dementia causing poor memory, concentration difficulties and intractable headaches.
- Seizures, chorea and monophasic transverse myelitis. A direct pathologic effect of aPL on neurons has been proposed.
- Cerebrovascular accidents associated with the presence of emboli from valve lesions in the heart.
- Sagittal venous sinus thrombosis and acute ischemic encephalopathy are very rare.
- Migraine and epilepsy could be related to APS.

Some evidence suggests that SLE patients with persistent high titres of aPL have a higher risk of developing neurologic complications. (22) For clinicians it is important to exclude other causes of neurological manifestations before investigating for aPL. (11)

1.3.5. Pulmonary manifestations
Pulmonary thromboembolism falls in the spectrum of thrombotic vascular manifestations. Pulmonary hypertension caused by pulmonary embolism, as well as by tricuspid or pulmonary valve lesions, has been reported. (6, 22)

Acute respiratory distress syndrome (ARDS), in-situ pulmonary artery thrombosis, capillaritis, alveolar haemorrhage and fibrosing alveolitis constitute less common associations. These thrombotic events in the lung can cause cough, dyspnoea, fever with or without haemoptysis. (22, 40) Presence of alveolar infiltrates may cause hypoxia and anaemia in APS patients. Moreover, some of these associations have been reported in the setting of CAPS. (21, 22)

1.3.6. Osteo-articular manifestations
APS involvement in the musculoskeletal system has been reported in several studies. The pathophysiology is not well understood. The main clinical manifestations reported are arthralgia and arthritis, especially in APS secondary to SLE. Nevertheless, it is not clear whether the pathogenesis is linked to aPL or the non-erosive arthritis described in SLE. (6, 41, 42)

Moreover, some authors suggest that APS thrombotic events may lead to interruption of blood flow into bone cells as well as into hematopoietic bone marrow. As a result, avascular necrosis of bone (or osteonecrosis), bone marrow necrosis, osteoporosis and non-traumatic fractures
have been reported in APS patients. (6, 42) Additionally, diabetic muscle infarction and a few cases of complex regional pain syndrome type-1 (reflex sympathetic dystrophy) in association with aPL have been described.(41)

1.3.7. Dermatological manifestations
Although cutaneous features occur in nearly half of patients with APS, they are considered atypical manifestations.(22) Livedo reticularis, which is a persistent violaceous, red or blue, reticular or mottled pattern of the skin of the trunk, arms or legs, is the most common skin manifestation.(15) In the literature, livedo reticularis is associated with higher frequencies in APS associated with SLE. Moreover, livedo reticularis has been correlated with aCL IgG and arterial thrombosis causing cerebral and ocular ischemic events, arterial thrombosis, systemic hypertension, and heart valve abnormalities.(22)
Other rare and less common skin manifestations associated with aPL are: pseudo-vasculitic lesions, digital gangrene, skin ulcerations, superficial phlebitis, malignant atrophic papulos-like lesions, subungual splinter haemorrhages, skin necrosis, anetoderma (a circumscribed area of loss of dermal elastic tissue) and Sneddon syndrome (a severe condition characterized by livedo reticularis and multiple cerebrovascular accidents).(10, 13, 22)

1.3.8. Renal manifestations
Renal features presented in APS are referred as aPL-associated nephropathy. Thrombosis in the kidney due to aPL affects intraparenchymal medium-sized arteries, glomerular capillaries, and renal veins. Consequently, patients with APS may present renal symptoms including hypertension, renal infarction, hematuria, and thrombotic microangiopathy. The most common histologic characteristics associated with APS are fibrous intimal hyperplasia, focal cortical atrophy, and tubular thyroidization. SLE patients who develop APS have a worse renal prognosis compared to patients who present only APS.(10, 15)

1.3.9. Hematologic Manifestations
A mild thrombocytopenia from 100,000-150,000 platelets/µL, coombs positive haemolytic anaemia, osteonecrosis, and less commonly bone marrow necrosis (necrosis of the bone marrow with the preservation of bone) are associated with persistent titres of aPL.(30) It is not clear whether the thrombocytopenia presented in APS patients, also diagnosed with SLE, is caused by aPL or by antinuclear antibodies (ANA).(15) Furthermore, not only in patients with idiopathic thrombocytopenia but also in patients with the presence of aPL, antibodies against
platelet glycoproteins leading to thrombocytopenia but not to thrombosis have been identified. (15, 30)

1.3.10. Endocrine Manifestations
The most frequent endocrine glands affected are the adrenal glands. Primary adrenal failure or adrenal insufficiency have been reported in primary APS and as a part of catastrophic antiphospholipid syndrome. Additionally, ovarian vein thrombosis, testicular vein thrombosis and pituitary thrombosis may occur less frequently. (30)

1.3.11. Catastrophic antiphospholipid syndrome
Catastrophic antiphospholipid syndrome (CAPS) (or Asherson’s syndrome) can be the first manifestation or it can arise in patients diagnosed with APS with high aPL titres. It is a feature of this syndrome that micro thrombi cause extensive microvascular thrombosis leading to multi-organ failure with very high mortality. (10, 30) Three or more organs in various combinations can be affected most commonly including kidney, lungs, bowel, brain, heart and skin. Other organs affected are adrenal glands, pancreas, testes, spleen and liver. (10, 21) Common clinical associations include intra-abdominal involvement mainly with renal manifestations, intrathoracic involvement including pulmonary embolisms, cardiac failure and myocardial infarction and cerebrovascular accidents. (21) Additionally, encephalopathy, cardiac dysfunction and acute respiratory distress syndrome (ARDS) following the cytokine cascade released from the necrotic organs have been reported. (30)

It is widely accepted that the CAPS is triggered by the use of oral contraceptives, sudden withdrawal of anticoagulation, surgical procedures, and obstetric complications. (30) Less than 1% of patients with APS present this potentially life-threatening condition. (9) Of those, one half of patients presenting CAPS die due to cerebral involvement (primarily stroke), cardiac and pulmonary complications. Patients require attention in the intensive care unit and clinicians should be aware of this disease in order to take therapeutic decisions urgently. (11, 21)

1.4. CLASSIFICATION CRITERIA AND DIAGNOSIS
In October of 1998, the first classification criteria for the antiphospholipid syndrome (APS) were formulated. Investigators who were experts in the field achieved a consensus during a post-conference workshop following the Eighth International Symposium of APS in Sapporo, Japan. (43) These first classification criteria addressed the need to establish the most important
features of the syndrome in order to carry out clinical investigations and were based on a combination of laboratory and clinical criteria.\(^{(43)}\)

In 2006, the criteria were amended in a preconference workshop, preceding the Eleventh International Congress on antiphospholipid antibodies (aPL) in Sydney, Australia.\(^{(15)}\) Table 3 presents a summary of the main differences in the laboratory considerations between the Sapporo Criteria and the Sydney update.

*Table 3 Laboratory considerations in the Sapporo and the Sydney criteria for the classification of the APS \(^{(15, 43)}\)*

<table>
<thead>
<tr>
<th></th>
<th>Sapporo Criteria</th>
<th>Sydney Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA</td>
<td>Consider ISTH guidelines for testing recommendations: screening, mixing, and confirmation test. Persistency: two or more occasions at least 6 weeks apart.</td>
<td>Consider ISTH guidelines for testing recommendations: screening, mixing, and confirmation test. Persistency: two or more occasions at least 12 weeks apart.</td>
</tr>
<tr>
<td>aCL antibodies</td>
<td>-Standardized ELISA for (\beta)-2-glycoprotein I–dependent anticardiolipin antibodies. -IgG and/or IgM isotype in blood -Persistency: Medium or high titre, on 2 or more occasions, at least 6 weeks apart.</td>
<td>-Standardized ELISA -IgG and/or IgM isotype in serum or plasma -Persistency: Medium or high titre (i.e. &gt;40 GPL or MPL, or &gt;the 99(^{th}) percentile), on two or more occasions, at least 12 weeks apart.</td>
</tr>
<tr>
<td>Anti-B2GP1 antibodies</td>
<td>Not considered</td>
<td>-IgG and/or IgM isotype in serum or plasma -Persistency= titre &gt;the 99(^{th}) percentile, present on two or more occasions, at least 12 weeks apart. -Standardized ELISA</td>
</tr>
</tbody>
</table>

Despite the fact that the Sapporo criteria and the updated Sydney criteria were initially created for research purposes, clinicians tried to adapt them in order to investigate APS patients in the clinical setting. Some aspects not included in the Sapporo laboratory criteria were considered in the updated Sydney criteria improving the application of these criteria in the diagnosis of APS. These aspects included recommendations to\(^{(7, 15, 19, 44)}\):

- **Stratify APS patients in categories.**\(^{(45)}\) These categories are:
  
  - Classification Category I: more than one laboratory criteria (any combination)
  - Classification Category IIa: sole positivity of LA
  - Classification Category IIb: sole positivity of aCL
  - Classification Category IIc: sole positivity of anti-B2GP1
- Investigate inherited and acquired risk factors for thrombosis and pregnancy morbidity.(19)
- Consider individual patient circumstances in atypical manifestations not specific for APS including: cardiac valve disease, skin ulceration, thrombocytopenia, aPL nephropathy, and cognitive dysfunction, among others(30).
- Exclude testing for IgA isotype antibodies, antiphosphatidylserine antibodies (aPS), antiphosphatidylethanolamine (aPE) antibodies, antibodies against prothrombin alone (aPT-A), and antibodies to the phosphatidylserine–prothrombin (aPS/PT) complex, although they may contribute with useful information in seronegative APS patients.(3, 45)

Currently, a diagnosis of APS is considered when a patient presents with at least one clinical criterion (one episode of arterial, venous or small vessel thrombosis or pregnancy morbidity) and positivity in at least one of the tests investigating aPL, namely identifying the presence of aCL, LA or anti-B2GP1 on two or more occasion at least 12 weeks apart.(3)

1.4.1. BCSH Guidelines on the investigation and management of antiphospholipid syndrome

The BCSH Guidelines on the investigation and management of APS (2012) provide the current best practice approach for clinicians to diagnose APS(7, 19, 35). In February 2012, the British Committee for Standards in Haematology (BSCH) updated their previous guidelines (2000) on the investigation and management of APS to incorporate the 2006 Sydney classification criteria .(3) (Table 4 )

The updated guidelines consist of a comprehensive review of the relevant tests for antiphospholipid antibodies, important recommendations based on relevant publications and evidence that provides a simplified guide for the clinical investigation and treatment of APS.(3)
Table 4 Research criteria for defining the antiphospholipid syndrome. (3)

<table>
<thead>
<tr>
<th>Clinical criteria</th>
<th>Laboratory criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Vascular thrombosis</td>
<td>1. Lupus anticoagulant (LA) present in plasma, on two or more occasions at least 12 weeks apart</td>
</tr>
<tr>
<td>(a) One or more clinical episodes of arterial, venous or small vessel thrombosis</td>
<td>2. Anticardiolipin (aCL) antibody of immunoglobulin (Ig)G and/or IgM isotype in serum or plasma, present in medium or high titre (i.e. &gt;40GPL units or MPL units, or &gt; the 99th centile), on two or more occasions, at least 12 weeks apart</td>
</tr>
<tr>
<td>(b) One or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation</td>
<td>3. Anti-β2-glycoprotein 1 antibody of IgG and/or IgM isotype in serum or plasma (in titre &gt;the 99th centile), present on two or more occasions at least 12 weeks apart</td>
</tr>
<tr>
<td>(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</td>
<td>Antiphospholipid antibody syndrome (APS) is present if at least one of the clinical criteria and one of the laboratory criteria are met</td>
</tr>
</tbody>
</table>

The guidelines also include applying an individualized approach for some patients in the following situations:

- Patients that present with clinical features of APS that are not included in the classification criteria; such as heart valve disease, livedo reticularis, thrombocytopenia, nephropathy, neurological disorders, muscle-skeletal manifestations and catastrophic APS.(10, 22)
- Patients that present a thrombotic episode without a known acquired or inherited cause with negative antiphospholipid antibodies titres (seronegative APS).(19, 45)
- Asymptomatic patients that present with persistently low/medium titres of aPL, with or without clear risk factors of thrombosis.(28)
- Patients that need anticoagulant therapy before confirming the diagnosis of APS during the twelve-week interval for the investigation of aPL.(7, 19, 45)

The diagnosis of APS relies on the sensitivity and specificity of the laboratory tests namely Lupus anticoagulant, anticardiolipin antibodies test and anti-B2GP1 test.

1.4.2. Detection of antiphospholipid antibodies in the clinical laboratory.
There is a broad spectrum of antiphospholipid antibodies with different specificities (See section 1.2.2 Antiphospholipid antibodies). However, only Lupus anticoagulant (LA), anticardiolipin antibodies (aCL) and anti-B2-Glycoprotein-1 antibodies (anti-B2GP1) are considered in the diagnosis of APS and useful predictors of thrombosis. (16, 33, 46)
1.4.2.1. **Lupus anticoagulant**

Lupus anticoagulant is a group of liquid-phase tests that identify a prolongation of time in phospholipid-dependent clotting reactions. LA is caused by antiphospholipid antibodies blocking the phospholipid surfaces implicated in the clotting tests. Anti–B2GP1 antibodies, anti-prothrombin antibodies, and probably antibodies to other cofactors have been associated. (24, 27, 47)

In the 1980’s, the phenomenon was first detected in SLE patients (thereby giving the test its name) by a group of investigators at the Hammersmith Hospital in London.(48) Although LA detects a prolongation of coagulation time in the laboratory, it is highly associated with clinical manifestations of thrombosis in APS.(5, 17, 28, 49)

Three guidelines have been produced aiming to standardise LA testing. They are from the International Society on Thrombosis and Haemostasis (ISTH) in 2009(50), the British Committee for Standards in Haematology (BCSH) in 2012(3), and the Clinical and Laboratory Standards Institute (CLSI) in 2014(51). The guidelines contain recommendations about preparation of the samples; coagulation methods, internal quality control, use of normalised ratios, detection of LA in patients receiving anticoagulation therapy, interpretation and report of results. Standardization of LA performance has reduced interlaboratory variation and improved the reliability of the results in association with APS.(5, 27, 48)

After ruling out any undiagnosed coagulopathies and anticoagulant treatment, the detection of LA requires a three-step procedure which includes a screening step, a mixing step and a confirmatory step.(3, 47) The BCSH guidelines recommend that two test systems of different principles should be employed to ensure LA detection(3). Among APTT (activated partial thromboplastin time), dRVVT (dilute Russell viper venom time), SCT (silica clotting time), KCT (kaolin clotting time), dPT (dilute prothrombin time), and others; APTT and dRVVT are preferred because they are better standardized.(27)

1.4.2.2. **Anticardiolipin antibodies (aCL)**

There are two groups of anticardiolipin antibodies. The first consists of antibodies directed against phospholipids alone and the second consist of antibodies against phospholipids in complex with a 'cofactor' protein (B2GP1 or prothrombin).(27, 52) In the context of APS, the second group has the stronger association with thrombosis and pregnancy morbidity.(5)

Anticardiolipin antibodies are detected by solid phase assays, mainly Enzyme Linked Immunosorbent Assay (ELISA). This ELISA measures aCL that bind cardiolipin (a negatively
charged phospholipid) in the presence of bovine B2GP1 in diluted patient serum. Both antibodies that bind aCL alone and antibodies that bind cardiolipin-B2GP1 are detected. Additionally, aCL that bind with other negatively charged phospholipids, such as phosphatidyl serine and phosphatidyl ethanolamine, have been described but their role in APS is still poorly understood. Moreover, aCL that bind with phospholipids independently of B2GP1 have been detected following infections, malignancy and the use of certain medications. As a result, aCL testing is characterized by higher analytical sensitivity (including some false positive results) compared to Lupus anticoagulant testing.

The BCSH Guidelines recommend the use of 10% adult bovine serum or foetal calf serum as a blocking agent and sample diluent. The cut-off values should be established in each laboratory using the 99th centile or > 40 GLP units. The research criteria of the BCSH guidelines include both IgG and IgM isotypes of aCL. However, the evidence about the association between IgM aCL titres and thrombosis is controversial. Likewise, the role of IgA aCL is still unknown and there is not enough evidence to consider IgA aCL in the clinical practice. Additionally, during the 13th International Congress on Antiphospholipid Antibodies held in Galveston, Texas, USA in 2010; experts in the field created international consensus guidelines on aCL and anti-B2GP1 testing. These guidelines recommend the use of six calibrator standards in order to address the need to standardize practices for immunoassays.
addition, the guidelines describe semiquantitative measurement ranges to improve consistency in the interpretation of aCL and anti-B2GP1 antibodies results. Nevertheless, a considerable degree of inter-laboratory and inter-assay variation exists in the aCL testing despite the recommendations in the guidelines. This may limit the clinical utility of the aCL test in the diagnosis of APS.

1.4.2.3. Anti-Beta2-Glycoprotein-I antibodies
Anti-B2GP1 antibodies recognize B2GP1 protein in the presence of anionic phospholipids. (See section 1.2.3) The measurement of anti-B2GP1 is widely performed through a solid-phase assay. In recent years, a close association between anti-B2PG1 IgG antibodies and thromboembolic complications is recognized, since anti-B2GP1 IgM presents a higher association with infections, such as leprosy and syphilis. Furthermore, in the literature, anti-B2GP1 antibodies are associated with Lupus anticoagulant activity which represents a strong thrombotic risk factor with better correlations for the IgG isotype. These features play a key role in the diagnosis of APS. Additionally, the BCSH guidelines recommend that positive results should be considered when the antibody titre is above the 99th centile.

As mentioned earlier, anti-B2GP1 recommendations for testing have been included in the International guidelines created during the 13th International Congress on Antiphospholipid Antibodies, as well as in the BCSH guidelines. Relevant aspects include the use of whole human β2GP1 molecule with negatively charged (“high” binding or gamma-irradiated) microtiter plates and the use of a calibration curve in every assay along the internal quality control. However, anti-B2GP1 testing is still poorly standardized with high inter-laboratory variation.

1.4.2.4. Other antiphospholipid antibodies
Some studies have evaluated the clinical utility of antibodies against other anionic phospholipids; namely phosphatidylserine, phosphatidic acid, phosphatidylinositol, or phosphatidyglycerol and phosphatidylethanolamine. Additionally, some authors have developed new ELISA platforms in order to identify antiphospholipid antibodies that bind proteins other than B2GP1. These new assays include prothrombin, protein C, protein S, Vimentin, Anexin A5 and Anexin A2. Interestingly, antibodies against phosphatidylserine/prothrombin (aPS/PT) complex present a different specificity compared to
antibodies that bind to only the prothrombin protein. (36, 55) Some in vitro experiments suggest that these aPS/PT can cause thrombosis.(55) All of these antibodies are considered “non-criteria” aPL antibodies because their clinical utility is not recognized in the laboratory criteria of the BCSH Guidelines. The evidence showing an association between these “non-criteria” antibodies and clinical manifestations of APS is not consistent.(27, 35, 55) Neither standardized procedures nor calibrators in the immunoassays used for testing are available. (28, 55) However, testing for these antibodies are offered in some laboratories across Europe because there is some evidence that they may be used as additional serological markers of venous and arterial thrombosis in patients with negative results for LA, aCL and anti-B2GP1).

1.4.2.5. Antiphospholipid Score
The GAPSS (Global APS Score) and aPL-S (Antiphospholipid Score) seem to be potential tools in the diagnosis of APS. (56-58) These scores predict the risk of develop APS clinical manifestations based on the aPL and autoimmune profile, cardiovascular and environmental risk factors.(57, 58) The aPL profile consider LA, aCL, anti-B2GP1 and aPS/PT complexes. These new strategies could be useful when clinicians evaluate the risk of thrombosis and pregnancy loss in the APS patients as well as helping the selection of therapy options. (56-58)

1.5. MANAGEMENT
The management of APS patients involves long-term anticoagulation therapy in order to prevent, or at least reduce, the risk of recurrent thrombosis episodes. Also, supplements of vitamin D have shown reduce the risk of thrombosis.(59) However, one third of treated APS patients do have recurrent thrombosis.(1) Treatment also targets the prevention of miscarriage in pregnant APS patients through the use of heparin and aspirin(1, 3, 17, 24). The main anticoagulant approach in APS patients is heparin followed by long term anticoagulation with an oral vitamin K antagonist, such as warfarin.(1, 59) Treatment can be associated with immunosuppression, high dose steroids or plasma exchange when there is an underlying autoimmune disease or in catastrophic APS.(3, 60) Anticoagulant doses and the type of anticoagulant should be individualized according to the immunological and clinical profile of each patient. Single positivity for aPL requires a less intense or extended antithrombotic regimen. (27, 60) Nevertheless, the duration of the anticoagulation is still matter of debate. Clinicians should aim a balance between the risk of
thrombosis and the risk of bleeding (11, 19, 60) Avoidance of risk factors such as smoking, sedentary, obesity and exogenous female hormone, should be controlled carefully in all the patients (1, 11) There are some limitations related to the use of anticoagulants, for example therapy requires frequent INR monitoring, as well as measures taken to avoid negative drug interactions with the patients food intake and the management of bleeding due to reduced clotting ability. Moreover, some patients may be intolerant or resistant to the anticoagulant drugs. (1, 22, 59) Table 5 shows a summary with the management of APS patients.

Table 5: Summary of main therapy approaches in patients with APS (17, 19, 59)

<table>
<thead>
<tr>
<th>Secondary thromboprophylaxis: Recommended approaches in APS patients who have already had a thrombotic event.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with APS and previous venous thrombosis</td>
</tr>
<tr>
<td>Maintaining long-term anticoagulation with oral anticoagulants, INR 2.0 - 3.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primary thromboprophylaxis: Recommended approaches in asymptomatic aPL carriers without previous thrombosis.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients incidentally found to have aPL</td>
</tr>
<tr>
<td>Strict control of risk factors. No therapy</td>
</tr>
</tbody>
</table>

CAPS require combinations of anticoagulation (heparin/warfarin) and immunomodulatory therapies (plasmapheresis, hydroxychloroquine, intravenous human IgG, corticosteroids and rituximab).

There is an increased interest in the use of new immunomodulatory therapies. These new approaches are looking to prevent or cure the production of aPL rather than prevent clot formation. These novel treatments for APS are still in the research or clinical trial stages and their use in the clinical setting has not been approved yet. (1, 22) Table 6 shows a summary of these novel therapies and their antigenic targets.
Table 6 Novel immunomodulatory treatments in Antiphospholipid Syndrome (1, 30, 59)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Molecular target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxychloroquine</td>
<td>Restore Anexin A5, inhibition of Toll-like receptor (TLR) activation</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>Tissue Factor, IL6, IL1b, adhesion molecules</td>
</tr>
<tr>
<td>B-cell therapies: Rituximab</td>
<td>CD20</td>
</tr>
<tr>
<td>Belimumab/BAFF (B-cell-activating factor)</td>
<td>Blockage BAFF-R/BAFF</td>
</tr>
<tr>
<td>Complement inhibition</td>
<td>C3, C5, C6 fractions or C5a-R</td>
</tr>
<tr>
<td>Eculizumab</td>
<td>C5a</td>
</tr>
<tr>
<td>Defibrotide</td>
<td>Tissue Factor</td>
</tr>
<tr>
<td>Antiplatelet agents: Dilazep</td>
<td>Tissue Factor</td>
</tr>
<tr>
<td>Abciximab</td>
<td>GPIIb/IIIa-R</td>
</tr>
<tr>
<td>Co-stimulation inhibition: Abatacept (CTLA4-Ig)</td>
<td>CD80/CD86 (B7)-CD28 ligation</td>
</tr>
<tr>
<td>Intracellular pathways inhibition SB203580</td>
<td>p38MAP kinase</td>
</tr>
<tr>
<td>MG132 orSN50</td>
<td>NFκB</td>
</tr>
<tr>
<td>Cell-surface receptors</td>
<td>ApoER2, TLR4, AnxA2</td>
</tr>
<tr>
<td>Anti-cytokine therapies (Anti-TNFα)</td>
<td>TNFα</td>
</tr>
<tr>
<td>Statins (inhibitors of cholesterol)</td>
<td>Reduction of adhesion molecules and tissue factor expression</td>
</tr>
<tr>
<td>Vitamin D supplementation</td>
<td>Inhibition of B2GP1-mediated tissue factor expression</td>
</tr>
</tbody>
</table>

1.6. SUMMARY

Although the definite diagnosis and management of APS is challenging for the health care team, due to both the performance of the available tests and the large array of clinical manifestations a patient may present with, the BCSH Guidelines still provide the only and current best practice approach for the diagnosis of APS.(3, 7). A clinical audit of APS was therefore carried out to determine how well the clinicians adhered to the BCSH Guidelines when undertaking the antiphospholipid antibody investigations. The audit identified current practice and potential ways to improve the diagnosis of APS patients.
CHAPTER 2

2. AIMS AND OBJECTIVES

2.1. Aim
To assess whether the investigation of antiphospholipid antibodies (aPL) in Lancashire Teaching Hospitals and from primary care services across the region served by the Royal Preston Immunology service is being carried out according to the British Committee for Standards in Haematology (February 2012): Guidelines on the investigation and management of antiphospholipid syndrome.

2.2. Objectives

a. To collect patients’ test request forms and test results for antiphospholipid antibodies from the laboratory information system or from laboratory records in Preston Hospital in the period March-August 2015.

b. To set measurable audit standards based on the British Committee for Standards in Haematology (February 2012): Guidelines on the investigation and management of antiphospholipid syndrome.

c. To identify whether the patients are being selected for aPL testing, as well as follow up testing, in compliance with the BCSH (February 2012): Guidelines on the investigation and management of APS.
CHAPTER 3

3. METHODS

3.1. Audit design and data collection
A retrospective clinical audit was performed to assess antiphospholipid antibodies (aPL) test requests received by the Lancashire and Lakeland Regional Immunology Service at the Royal Preston Hospital from Lancashire Teaching Hospitals and from primary care services across the region served by the Royal Preston Immunology service. Based on the BCSH Guidelines on the investigation and management of Antiphospholipid Syndrome(3), testing for aPL include antcardiolipin antibodies (aCL) IgG/IgM, anti beta2 glycoprotein I antibodies (anti-B2GP1) IgG/IgM and Lupus Anticoagulant (LA).

The audit was approved by the University Research Ethics Committee and registered with the Clinical Audit department from Lancashire Teaching Hospitals NHS Foundation Trust. All the test reports for investigation of aCL were gathered from the Laboratory Information Management System (LIMS) Swisslabs over a 6-month period (from March to August of 2015). Data for anti-B2GP1 IgG/IgM and Lupus Anticoagulant testing, clinical details and demographics were then obtained for each patient. All the information was compiled in an anonymized database in Microsoft Excel. In order to anonymise the data, a random number was assigned to each patient by a third party who was not involved in the audit.

In the period March to August 2015, 2042 test requests for antcardiolipin antibodies were identified along with the date of the sample testing and clinical details for each patient. 648 out of 2042 test requests were excluded because of duplicates, lack of aPL results and issues in the clinical information provided (See Appendix I for a complete description), leaving 1394 test requests for assessment.

3.2. Audit standards
A set of three standards (see Table 5) were written based on the BCSH 2012: Guidelines on the investigation and management of Antiphospholipid Syndrome.
### Table 7 Audit Standards

<table>
<thead>
<tr>
<th>AUDIT CRITERIA</th>
<th>TARGET</th>
<th>EXCEPTIONS</th>
<th>SOURCE OF EVIDENCE</th>
<th>DEFINITIONS/INSTRUCTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The patient must meet one clinical criteria of APS</td>
<td>100 %</td>
<td>SLE patients and APS clinician requests</td>
<td>BCSH Guidelines on the investigation and management of antiphospholipid syndrome(3)</td>
<td>Clinical criteria of APS: Primarily venous thromboembolism and arterial ischaemic stroke, pregnancy morbidity. Partial compliance: Thrombocytopenia, heart valve disease, chorea, lived reticularis, nephropathy and neuropathy and others non-criteria clinical manifestations described.</td>
</tr>
<tr>
<td>2. The laboratory tests for antiphospholipid antibodies (aPL) should include Lupus anticoagulant (LA) and IgG anti-β2GP1/aCL</td>
<td>100 %</td>
<td>None</td>
<td>BSCH Guidelines on the investigation and management of antiphospholipid syndrome(3)</td>
<td>Antibodies to β2GP1 may be detected by anti-β2GP1 ELISA or aCL ELISA (detect antibodies to other phospholipid binding proteins as well as anti-β2GP1)</td>
</tr>
<tr>
<td>3. Time between test requests for antiphospholipid antibodies must be at least 12 weeks apart.</td>
<td>100 %</td>
<td>LA tests should not be performed if the patient is receiving therapeutic doses of unfractionated heparin, subsequent tests from initially negative results</td>
<td>BSCH Guidelines on the investigation and management of antiphospholipid syndrome(3)</td>
<td>The diagnosis of APS is confirmed when there is persistency in the presence of antibodies antiphospholipid after 12 weeks. aPL are LA, aCL of IgG and/or IgM and anti-β2GP1 IgG and/or IgM. aCL and anti-B2GP1 positivity cut-off value was considered above &gt;40 GPL-U/ml and MPL-U/ml</td>
</tr>
</tbody>
</table>

### 3.3. Data Categorisation

1394 aCL test requests were audited against the standards, and analysed using Microsoft Excel. Three spreadsheets were created, the first contains all the test request assessed, the second the test requests assessed without the identified repetitions for each patient and the third with the patients that had follow-up investigations. The data in the spreadsheets was analysed using...
filters and basic functions of Microsoft Excel. In order to assess the compliance with the standards, a table showing the frequencies of clinical manifestations according categories and the time between test requests were created. The findings were presented in their corresponding graphs in the result section.

3.3.1. Classification of clinical details

The test requests assessed were sorted into categories according to the available clinical information (Figure 3) using Microsoft Excel. Three main groups were defined. Group 1 includes test requests with clinical manifestations referred to in the guidelines, Group 2 consist of requests with manifestations reported in the literature in association to APS but not recognized as appropriately adhering to the published guidelines, and Group 3 contains the request with manifestations not related to APS.

![Figure 4 Flow diagram showing the classification strategy performed in the audit. The available LA and B2GP1 testing for each patient were added to the aCL requests gathered from the information management system.](image-url)

A detailed description of all the clinical information contained in each category, the exclusion criteria and the classification considerations can be found in Chapter 6: Appendix I.
3.4. Standard assessment

3.4.1. Standard 1: The patient must meet one clinical criteria of APS

The BCSH guidelines on the investigation and management of APS considers only thrombosis (primarily venous thromboembolism and arterial ischaemic stroke) and pregnancy morbidity in the clinical criteria. However, other clinical manifestations have been described in the context of APS, including livedo reticularis, thrombocytopenia, aPL nephropathy, heart thickening, heart valve disease, chorea, encephalopathy, transverse myelopathy, arthralgia, among others (3,6,2,44). For this audit, all the test requests with criteria clinical details (Group 1) were considered as fully compliant with the standard, while the requests with clinical details that are associated with APS but not explicitly stipulated in the BCSH guidelines were considered as a partial compliance of the published standards, and were assigned to a “non-criteria” group (Group 2).

Additionally, the number of patients (n=1291) in the audit cohort was estimated after subtracting the number of follow up investigation requests (n=103) from the number of test requests assessed (n=1394). Assessment of the Standard 1 considers the number of test requests while standard 2 and 3 considers the number of patients.

The estimated prevalence of anticardiolipin antibodies within the audit cohort was calculated considering the positive (medium/high titres > 40 units).

3.4.2. Standard 2: The laboratory tests for antiphospholipid antibodies (aPL) should include LA and IgG anti-β2GP1/aCL

Anticardiolipin antibodies IgG and IgM were the inclusion criteria considered in order to collect the test requests from the information system. 1291 patients with available lupus anticoagulant results, as well as anti-B2GP1 antibodies results, were identified and considered for the Standard 2 assessment.

3.4.3. Standard 3: Time between test requests for antiphospholipid antibodies must be at least 12 weeks apart

Initially, the data set contained quantitative and semi-quantitative results for antiphospholipid antibodies testing. In order to improve uniformity during the data analysis, all the quantitative data were converted to semi-quantitative data considering the cut-off titres recommended in the BCSH guidelines on the investigation and management of antiphospholipid syndrome. Table 8 shows the titres considered.
Table 8 Antiphospholipid antibody titres and results

<table>
<thead>
<tr>
<th>aPL test result</th>
<th>Anticardiolipin antibodies titres</th>
<th>Lupus anticoagulant</th>
<th>Anti-B2GP1 antibodies titres</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Negative</strong></td>
<td>&lt; 10</td>
<td>Not Demonstrated</td>
<td>&lt; 10</td>
</tr>
<tr>
<td><strong>Weak positive</strong></td>
<td>10 - 40</td>
<td>N/A</td>
<td>10-40</td>
</tr>
<tr>
<td><strong>Positive</strong></td>
<td>&gt; 40</td>
<td>Demonstrated</td>
<td>&gt; 40</td>
</tr>
</tbody>
</table>

From a cohort of 1394 test requests, the patients with repeated test requests for antiphospholipid antibodies were identified (N=87). 42 out of 87 patients with weak positive and positive results for anticardiolipin antibodies, Lupus anticoagulant and anti-B2GP1 were considered for assessing the Standard 3.
CHAPTER 4

4. RESULTS

4.1. Standard 1: The patient must meet one clinical criteria of APS before testing for antiphospholipid antibodies (aPL).

From a cohort of 1394 test requests, 596 (43%) were identified as fully compliant with the first audit standard; these requests were for patients with clinical details of venous or arterial thromboembolism, pregnancy morbidity, thrombosis and antiphospholipid syndrome. (description of clinical details in Appendix I) 583(42%) requests were made in patients with other clinical manifestations that were outside the specified conditions referred to in the guidelines (3) (the details of the reported conditions can be found in the Appendix I); these were considered as non-criteria (less common clinical manifestations of APS reported in the literature but not included in the guidelines). A further 215 (15%) requests were made with clinical details that were not related to APS.

Overall, 43% of the test requests were fully compliant with the standard and they meet one clinical criteria of APS, while 42% of the requests were considered as partially compliant and 15% do not meet the standard. (Figure 5).

![Figure 5 Standard 1 compliance.](image)

All the indications of aPL testing in accordance to the published Guidelines Criteria correspond to the group Criteria clinical manifestations, for instance Venous or arterial thromboembolism, pregnancy morbidity, thrombosis and antiphospholipid syndrome. The requests with clinical details not include in the Guidelines but considered within the scope of APS were included in the group Non-criteria clinical manifestations. All the requests investigating symptoms that are not related to APS do not meet the standard.
Further analysis of those requests that were fully compliant with the Guidelines (Group 1) identified that the highest frequency of aCL test requests were associated with the clinical details relating to pregnancy morbidity at 34% (201 test requests). (Figure 6)

**Figure 6 Group 1: Criteria Clinical Manifestations.**
Distributions of the frequencies for aCL testing in Group 1 based on the available clinical details. The highest frequency corresponds to Pregnancy morbidity (34%).

Of the 201 aCL test requests for IgG isotype, only one was positive, 4 were weak positive and the remaining negative. Likewise, in the data for IgM isotype only one test request was positive, 23 were weak positive and 177 negative. (Figure 7 and Figure 8).

**Figure 7 Pregnancy morbidity aCL IgM results.**
Only 1 out of 201 test requests for aCL IgM was positive, 23/201 were weak positive and 177 were negative.
When considering the distribution of clinical features for requests in Group 2 (Non-criteria clinical manifestations), the proportion of test requests with clinical details that were related to osteo-articular manifestations has the highest frequency of aPL testing with 23% (135 test requests). (Figure 9) This category was 2% higher than neurologic manifestations and other autoimmune diseases both of which corresponding to approximately 21% (neurologic manifestations, n= 124, and Other autoimmune diseases, n=121).

Similarly, of 135 test requests that were related to osteo-articular manifestations only one test request was positive for aCL IgG, as well as aCL IgM, 17 were weak positive for aCL IgM, 3 were weak positive for aCL IgG, and the remaining were negative. (Figure 9 and Figure 10)
Figure 10 Osteo-articular manifestations aCL IgM results. Only 1 out of 135 test requests for aCL IgM was positive, 17/135 were weak positive and 117 were negative.

Figure 11 Osteo-articular manifestations aCL IgG results. Only 1 out of 135 test requests for aCL IgG was positive, 3/135 were weak positive and 131 were negative.

Within the Group 3 (APS Non-related clinical manifestations) nearly half of the test requests (47%, N=100) presented with clinical manifestations other than cutaneous, haematological gastrointestinal and neurological manifestations (see Appendix I for details of the conditions). Of 100 test requests, two were positive for IgG aCL isotype and one was positive for aCL IgM. The reasons for performing aCL testing in those three cases were myalgia, hypothyroid shoulder pain and elevated inflammatory markers with high blood pressure, respectively. Additionally, six out of 100 test requests were weak positive for aCL IgM and eight were weak positive for aCL IgG.
4.2. Standard 2: The laboratory tests for antiphospholipid antibodies (aPL) should include LA and IgG anti-β2GP1/aCL

The inclusion criteria and audit design meant that all samples in the study had a request for anticardiolipin antibodies. Of the 1291 patients, 126/1291 were tested for anti-B2GP1 antibodies IgG and IgM while 56/1291 patients were tested for Lupus anticoagulant. However, of the 1291 patients who were tested for anticardiolipin antibodies, only 4 (0.31%) patients included both anti-B2GP1 IgG and Lupus anticoagulant. As a result, less than 1 percent (0.31%) of patients were compliant with the Standard 2. See Figure 5

Figure 12 Group 3: APS Non-related clinical manifestations.
Distributions of the frequencies for aCL testing in Group 3 based on the available clinical details. Nearly half of the test requests (47%) present clinical details other than cutaneous, haematological, gastrointestinal and neurological manifestations.

Figure 13 Standard 2 compliance.
From 1291 patients only four fulfilled the Standard 2. It means that less than 1% of patients were tested for antiphospholipid antibodies following the recommendations of the BCSH Guidelines.
4.3. Standard 3: Time between test requests for antiphospholipid antibodies must be at least 12 weeks apart

Only 87 out of 1291 patients could be identified as having repeat test requests for anticardiolipin antibodies, lupus anticoagulant and/or anti-B2GP1 antibodies over the study period. Of these 87 patients, 78 had one test request in addition to the initial investigation, and 9 patients had more than one test request subsequent to the initial investigation.

In order to assess whether the patients are being selected for follow up testing according to the recommendations of the BCSH guidelines of APS(3) and therefore in accordance with the audit standards, only the patients that had positive/weak positive results in the first investigations were considered for further analysis. Of the 87 patients, 41 patients had a positive and/or weak positive result in the initial investigation. Among these 41 patients, 34/41 patients had one test request in addition to the initial investigation, and 7/41 patients had more than one test request subsequent to the initial investigation.

Overall, 19/41 (46%) patients had aPL test repetitions performed in more than 12 weeks and 22/41 (54%) patients had the test repeated in less than 12 weeks. Therefore, 46% of patients fulfilled Standard 3. (Figure 15)

![Figure 14 Standard 3 compliance. Approximately one half of patients (46%) had the follow up testing at least 12 weeks apart. These frequencies correspond to patients who had positive results in the initial investigation and had repeated tests performed. Table 9 shows all the patients (n=42) that had positive results in the first investigation and have follow up testing. The most common clinical detail in these patients was SLE (N=8). All these](image-url)
patients had aCL test repetitions but only eight had LA repeated and nine anti-B2GP1 antibodies repeated (results shown in brackets between the respective aCL result in Table 9). As can be seen from Table 9, the results of patients 5, 19, 34 and 41 became negative in the follow up testing while the others remain positive. Additionally, patient 5’s negative results became weak positive in third test repeated. Regarding the patients who had more than one test request in the follow up testing. 7 out of 9 test requests had positive results in the first investigations with the remaining two having negative results. In line with the main inclusion criteria for the data collected, which was aCL testing and then the available LA test and anti-B2GP1 antibodies, of the two patients noted above, both had the aCL test repeated. However, only one patient had LA and anti-B2GP1 test repeated. Also, the result became weak positive for aCL IgG, remaining weak positive in the two next following repetitions. This patient’s clinical details correspond to “SLE”, while the other patient became weak positive for aCL IgG but in a third repetition became negative. This patient had LA and anti-B2GP1 testing with negative results and the clinical detail correspond to “APS clinicians requesting”. Moreover, patients number 5, 36 and 37 (see table 9) had three test repetitions with the last test performed in more than 12 weeks. Similarly, patient 31 had four repetitions, three of them performed in less than 12 weeks and the fourth repetition registered at least 12 weeks apart. The results remained the same in all these patients. Although, in these patients the first repetition was performed in less than 12 weeks, they were considered compliant with the Standard 3 because they register later repetitions after the 12-week period recommended in the guidelines. (Table 9)

When considering the 201 test requests with clinical details related to pregnancy morbidity (the most frequent clinical detail in Group 1. Section 4.1) The cases with positive results did not register any repetitions. Only one case with a weak positive result for IgG and four cases with IgM weak positive results were repeated with the same outcome. (See patients 24-28 in Table 9) Among the negative results, 13 test requests were repeated, with one becoming weak positive for IgM isotype. Likewise, of 135 patients with osteo-articular manifestations (the more frequent clinical detail in Group 2. Section 4.1), five patients had aPL test repeated within this group. Two initially negative results and three weak positive result for IgM isotype (See patients 14,15,16 in Table 9) with subsequent results remaining the same. In the two positive cases, there were no test repetitions identified for these patients.
Additionally, within the patients that reported “other clinical details” (the most common clinical association in Group 3), one case had repetitions with negative results in both the first and follow up testing. The clinical details given for this patient was raised creatinine kinase (CK).

Finally, seven patients present persistent positive aPL titres (medium/high titres above 40 units) after 12 weeks. (See patients 1,4,7,13,36,37 and 39 in Table 9)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical details</th>
<th>Results of repeat testing aPL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>APS clinicians requesting</td>
<td>aCL Pos IgG (aB2GP1 Neg)</td>
</tr>
<tr>
<td>2</td>
<td>APS clinicians requesting</td>
<td>Weak Pos IgG (aB2GP1 Neg)</td>
</tr>
<tr>
<td>3</td>
<td>APS clinicians requesting</td>
<td>Weak Pos IgG (aB2GP1 Weak Pos)</td>
</tr>
<tr>
<td>4</td>
<td>APS clinicians requesting</td>
<td>Pos IgG and Weak Pos IgM</td>
</tr>
<tr>
<td>5</td>
<td>APS clinicians requesting</td>
<td>Neg</td>
</tr>
<tr>
<td>6</td>
<td>Gastrointestinal manifestations non-criteria</td>
<td>Pos IgM</td>
</tr>
<tr>
<td>7</td>
<td>Neurologic manifestations</td>
<td>Pos IgM</td>
</tr>
<tr>
<td>8</td>
<td>Neurological manifestations non-related</td>
<td>Weak Pos IgG (LA Neg)</td>
</tr>
<tr>
<td>9</td>
<td>Neurological manifestations non-related</td>
<td>Weak Pos IgM</td>
</tr>
<tr>
<td>10</td>
<td>Neurological manifestations non-related</td>
<td>Weak Pos IgM</td>
</tr>
<tr>
<td>11</td>
<td>Neurological manifestations non-related</td>
<td>Weak Pos IgM (LA Neg)</td>
</tr>
<tr>
<td>12</td>
<td>Ophthalmologic manifestations</td>
<td>Pos IgG (LA Neg)</td>
</tr>
<tr>
<td>13</td>
<td>Ophthalmologic manifestations</td>
<td>Pos IgM (aB2GP1 Neg)</td>
</tr>
<tr>
<td>14</td>
<td>Osteo-articular manifestations</td>
<td>Weak Pos IgM</td>
</tr>
<tr>
<td>15</td>
<td>Osteo-articular manifestations</td>
<td>Weak Pos IgM (LA Neg)</td>
</tr>
<tr>
<td>16</td>
<td>Osteo-articular manifestations</td>
<td>Weak Pos IgM</td>
</tr>
<tr>
<td>17</td>
<td>Other autoimmune diseases</td>
<td>Weak Pos IgG</td>
</tr>
<tr>
<td>18</td>
<td>Other autoimmune diseases</td>
<td>Weak Pos IgG</td>
</tr>
</tbody>
</table>
46

<table>
<thead>
<tr>
<th></th>
<th>Other autoimmune diseases</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>Other autoimmune diseases</td>
<td>Weak Pos IgM</td>
</tr>
<tr>
<td>21</td>
<td>Peripheral thrombosis</td>
<td>Pos IgG</td>
</tr>
<tr>
<td>22</td>
<td>Peripheral thrombosis</td>
<td>Weak Pos IgG</td>
</tr>
<tr>
<td>23</td>
<td>Peripheral thrombosis</td>
<td>Weak Pos IgG (aΒ2GP1 Weak Pos IgG)</td>
</tr>
<tr>
<td>24</td>
<td>Pregnancy morbidity</td>
<td>Weak Pos IgG (LA Neg)</td>
</tr>
<tr>
<td>25</td>
<td>Pregnancy morbidity</td>
<td>Weak Pos IgM (aB2GP1 Neg)</td>
</tr>
<tr>
<td>26</td>
<td>Pregnancy morbidity</td>
<td>Weak Pos IgM</td>
</tr>
<tr>
<td>27</td>
<td>Pregnancy morbidity</td>
<td>Weak Pos IgM (LA Neg)</td>
</tr>
<tr>
<td>28</td>
<td>Pregnancy morbidity</td>
<td>Weak Pos IgG (a2GP1 Neg)</td>
</tr>
<tr>
<td>29</td>
<td>Renal manifestations</td>
<td>Weak Pos IgG</td>
</tr>
<tr>
<td>30</td>
<td>Renal manifestations</td>
<td>Pos IgG and Weak Pos IgM</td>
</tr>
<tr>
<td>31</td>
<td>Renal manifestations</td>
<td>Weak Pos IgM</td>
</tr>
<tr>
<td>32</td>
<td>SLE</td>
<td>Pos IgG</td>
</tr>
<tr>
<td>33</td>
<td>SLE</td>
<td>Weak Pos IgG (a2GP1 Weak Pos IgG)</td>
</tr>
<tr>
<td>34</td>
<td>SLE</td>
<td>Neg</td>
</tr>
<tr>
<td>35</td>
<td>SLE</td>
<td>Pos IgG and Weak Pos IgM</td>
</tr>
<tr>
<td>36</td>
<td>SLE</td>
<td>Pos IgG</td>
</tr>
<tr>
<td>37</td>
<td>SLE</td>
<td>Weak Pos IgG (LA Pos)</td>
</tr>
<tr>
<td>38</td>
<td>SLE</td>
<td>Pos IgG and Weak Pos IgM</td>
</tr>
<tr>
<td>39</td>
<td>Vasculitis</td>
<td>Pos IgM (a2GP1 Neg)</td>
</tr>
<tr>
<td>40</td>
<td>Vasculitis</td>
<td>Weak Pos IgG and IgM (LA Neg)</td>
</tr>
<tr>
<td>41</td>
<td>Vasculitis</td>
<td>Neg</td>
</tr>
</tbody>
</table>

aPL, antiphospholipid antibodies; aCL, anticardiolipin antibodies; aΒ2GP1, anti Beta2-Glycoprotein I antibodies; LA, Lupus anticoagulant; Pos, Positive; Neg, Negative.

Results are colour coded to indicate compliance, with green denoting results of tests conducted at least 12 weeks from the initial result and red denoting results of tests that were conducted within 12 weeks. All the patients were tested for aCL and the available LA and anti B2GP1 results are shown in brackets.
CHAPTER 5

5. DISCUSSIONS
In the following section, the outcome of the clinical audit will be explained considering the assessment of the audit standards.

5.1. Standard 1: The patient must meet one clinical criteria of APS.
The BCSH Guidelines for the investigation and management of APS considers only the most common and specific clinical manifestations of the syndrome which are thrombosis (deep venous thrombosis, pulmonary embolism, stroke and transient ischemic attack) and pregnancy morbidity (early and late miscarriage, foetal death, pre-term births due to eclampsia/severe pre-eclampsia), in the clinical criteria for diagnosis.\(^{(2, 3, 15, 22)}\) However, as pointed out by Favaloro and Wong \(^{(27)}\), as well as Ruiz-Irastorza \(^{(19)}\), the relevance of the clinical criteria may be limited because the criteria was initially coined in order to provide uniformity in the appropriate selection of APS patients into prospective clinical studies and not for diagnostic purposes.\(^{(43)}\)

Furthermore, currently in APS patients there is an increasing number of recognized clinical features affecting lungs, kidneys, liver, spleen, adrenal glands, the heart, brain, spinal cord, skin and eyes as reported by Cervera et al. \(^{(13)}\) and more recently by Katikaneni et al. \(^{(22)}\), which are not recognised under the current BCSH Guidelines\(^{(27)}\). Accordingly, in this audit less than half of the audited test requests \((43\%, n=596)\) met one clinical criteria of APS (Standard 1). Other clinical manifestations that did not satisfy the criteria but were described in the spectrum of APS were recognised as partially compliant. These test requests account for \(42\%(n=583)\) of the audit cohort. The remaining \(15\%\) of requests \((n=215)\) were made without appropriate clinical justification, although the information available regarding the clinical presentation was limited to the clinical details provided on the request forms received by the laboratory.

Regarding the audit, perhaps one of the most significant limitations was the lack of clear agreement in the terminology used to indicate the clinical manifestations in the test requests. The available information does not specify whether the patients were under investigation or a diagnosis has been confirmed. Also, the information does not reveal if the accounts of suspected thrombotic episodes are confirmed thrombosis or another underlying illness. Likewise, for the majority of obstetric patients, both data on the gestational age of the foetus and the number of miscarriages are unavailable. Both of which are important facts when
considering the definitions of clinical criteria of APS in line with the BCSH Guidelines.(3) (see section 1.2.4). Moreover, approximately one third (N= 648) of the test requests were excluded due to the uninformative nature of the clinical information. The lack of clarity within the clinical information was due either to missing details of patients’ clinical manifestations, or the information written in the requests could not been understood by the typists in laboratory or the information detailed was unable to be interpreted.

Interestingly, when considering the distribution of aPL requests based on the clinical features provided, pregnancy complication and osteo-articular manifestations represent the highest frequencies. However, only four cases related to these clinical details were positive for the antibodies from nearly one quarter (n=336) of the requests. Also, for pregnancy morbidity (n=201) 27 requests gave weak positive results (23 for IgG isotype and 4 for IgM isotype) and for osteo-articular manifestations (n=135), there were 17 IgG weak positive and 3 weak positive IgM results. The significance of these results is limited because of the lack of available patients’ clinical data.

The frequency of positive aCL in the patients who present pregnancy morbidity manifestations correspond to 1% (n=201) which is lower compared to the estimated frequency, 6 % (by any criteria test), reported by Andreoli et al.(12) in their critical review of the literature. Pregnancy morbidity is recognized as one of the hallmarks of APS.(19) However, according to recent reports, most of the studies directed to show the overall contribution of aPL in pregnancy adverse events have several limitations and their outcomes may be questioned.(17, 38) These studies are heterogeneous, lack specific definitions of foetal death, stillbirth and spontaneous abortion and do not follow the international clinical and laboratory criteria during the selection of the participants. Moreover, they do not address the possible contribution of other risk factors, present differences in the aPL test type performed, do not have confirmation after 12 weeks and were published more than ten years ago. (2, 9, 11, 12, 17, 22, 37, 38, 56, 61) Therefore, the estimated aPL frequencies in the literature may not be extrapolated to the audit cohort.

In contrast, recent research has suggested that antiphospholipid antibodies of IgG Isotype from thrombotic patients compared to obstetric patients may show different pathogenic mechanisms and have variable specificities and sensitivities.(4, 20, 25, 30) Furthermore, some evidence suggests that persistent low aPL titres (below <20 units) are important in patients with pure obstetric APS, with higher correlations for the IgG isotype.(11, 25, 38, 62) Nevertheless, the clinical significance of IgM isotype and low aPL titres in association to obstetrical morbidities should be evaluated cautiously. Additionally, other possible causes or aetiologies of pregnancy loss, such as foetal genetic abnormalities, maternal and paternal karyotypes, uterine defects,
hormonal problems, genetic hypercoagulable states, and chronic infections should be investigated and excluded first. (16, 25, 37, 38)

The prevalence of aCL IgG and IgM in the audit cohort is 2% for both (the prevalence was calculated considering the frequency of positive results, medium/high titres ≥ 40 units). The prevalence of LA and anti-B2GP1 could not be estimated because all of the patients do not have both tests. This prevalence is low compared to the estimated prevalence of aCL in venous thrombosis, 4-24%, and recurrent foetal loss, 10-19 %, reported by Biggioggero and Meroni (9) and the percentage of patients (38.7%) that reported arthralgia in the Euro-phospholipid cohort of 1000 APS subjects(6). The data reported here appear to support the assumption that the aPL clinicians ordering needs to be reviewed, since the audit cohort correspond to patients who were being investigated for potential APS and not healthy individuals. The prevalence in healthy subjects is about 1-5% (for LA, aCL and anti-B2GP1) with titres increasing with the age and the presence of chronic diseases.(2, 7, 10, 11) Further studies should be undertaken to investigate whether or not education and training options addressing the aPL ordering practice, as well as a better agreement regarding uniform definitions of foetal death and thrombosis, would improve APS investigation adherence to guidelines.(63) Additionally, a future study investigating the potential implementation of an informative management system, for clinicians in primary care services to improve the management of the information within the organization, could be very interesting.

5.2. Standard 2: The laboratory tests for antiphospholipid antibodies (aPL) should include LA and IgG anti-β2GP1

There is a broad spectrum in the family of antiphospholipid antibodies(aPL). They are extremely heterogeneous with frequencies varying in different populations and from one patient to another.(10, 46) Among the aPL family of antibodies only LA (detected by liquid phase assays), aCL and anti-B2GP1 antibodies (both detected by solid phase assays) are considered in the laboratory criteria in the investigation of APS. The BCSH guidelines recognise the importance of performing both liquid and solid assays along with the confirmation of the aPL persistent positivity at least after 3 months in order to confirm the diagnosis of the syndrome.(3)

Testing for LA, aCL and anti-B2GP1 antibodies do not necessarily identify the same antibodies. LA detect a group of antibodies with B2GP1 dependant activity, anti–B2GP1 antibodies, anti-prothrombin antibodies, and probably antibodies to other cofactors.(10, 27, 47) aCL have been related to antibodies directed against cardiolipin alone and cardiolipin in
association with phospholipid binding proteins, principally B2GP1-aCL antibody complexes are clinically relevant in the context of APS. (10, 24, 46) Anti-B2GP1 antibodies recognise different domains of B2GP1 but only the antibodies with Domain I specificity have been significantly associated with LA and vascular thrombosis. (36, 52, 64) Moreover, some aCL and anti-β2GP1 antibodies have no lupus anticoagulant activity. (46, 65)

Several studies have reported that LA is a stronger predictor of thrombotic complications and pregnancy morbidity than aCL or anti-β2GP1 antibodies. (3-5, 17, 20, 24, 27, 38, 44, 49) As a result, lupus anticoagulant antibodies are more specific for the antiphospholipid syndrome, whereas anticardiolipin antibodies are more sensitive. This sensitivity increases with the titre and is higher for IgG isotype rather than IgM isotype. Moreover, Gris and Bouvier (4) and Pericleous and Rahman (64) have demonstrated that anti-B2GP1 antibodies towards Domain I of the B2GP1 molecule show LA activity and they are highly specific markers for a risk of thromboembolic complications. Furthermore, the so-called triple positivity (LA+aCL+anti-B2GP1), regardless of the titre, is the most predictive profile for clinical manifestations of thrombosis and/or pregnancy loss. (3-5, 10, 17, 20, 24, 27, 38, 44, 49, 66) Despite the fact that IgG and IgM isotypes of aCL and anti-B2GP1 are included in the research criteria of the BCSH guidelines, testing for IgM aCL and IgM anti-B2GP1 are not recommended as a “routine first-line testing” since these isotypes are not significantly associated with thrombosis. (3, 17, 27) Similarly, there is not enough evidence that adding IgA aCL and IgA anti-B2GP1 in routine aPL testing improves the diagnosis of APS. (3, 53, 54) Nevertheless, the role of isolated IgM aCL or IgA aCL in the APS should be confirmed in further studies. (3, 5, 53, 54)

Assessment of compliance for the Standard 2 reveals that less than 1 % of the test requests show adherence with the laboratory criteria from the published guidelines which is the worst performing aspect in the investigation of APS covered in the audit. Since only four patients had the three aPL tests requested and the recommendations in the BCSH Guidelines(3) clearly state “When assessing clinical significance account should be taken of whether the patient has LA, aCL/anti-B2GP1, or both and of the isotype and titre in the solid phase tests”. However, it seems possible that these results were caused by the data collection criteria applied. The initial inclusion criterion considered in order to collect the test requests were requests for aCL testing and then the available LA and anti-B2GP1 for each patient. This approach could lead to misidentifying other requests that test for LA besides aCL/anti-B2GP1 within the audit period (March-August 2015). These issues could be addressed in future work.

Additionally, the Standard 2 compliance suggests that a weak link may exist between the laboratory department and the clinicians. The clinicians might not be aware of the differences
and the clinical significance of the aPL assays. Moreover, Favaloro and Wong (27) have described a potential bias in the perception of the clinical utility of the aPL test panels by the clinicians. Since liquid and solid phase aPL assays are performed in different departments (haematology vs immunology laboratory respectively) and require different instrumentation and samples. This is an important issue for future studies.

5.3. Standard 3: Time between test requests for antiphospholipid antibodies must be at least 12 weeks apart

Assessment of this standard has found that approximately one half of the patients (46%) had confirmatory aPL test repeated after at least 12 weeks. As previously stated, the BCSH Guidelines on investigation of APS recommends that positive aPL test results should be repeated at least 12 weeks apart. (3) This period of time aims to decrease the risk of misclassification due transient aPL which are clinically irrelevant antibodies in the context of APS. These can be detected in other clinical scenarios, including ongoing infectious diseases, malignancy, use of some drugs and within healthy individuals.(11, 20, 22, 23) Moreover, routine testing of aPL in individuals without clinical features of APS it is not recommended because it may cause unnecessary further investigations.(27, 62, 63)

The audit aimed to assess whether or not confirmation of positivity in aPL test were made at least 12 weeks apart. Consequently, only patients with positive or weak positive results that have follow up investigations were considered in the Standard 3 assessment. However, 42 patients that tested positive for aPL in medium/high titres did not have a repeat test within the 6-month period audited. These results include 1 positive result for IgM anti-B2GP1, 4 for LA, 18 for IgG aCL and 19 for IgG aCL. There are two likely causes. First, it is possible that some repetitions were requested on dates outside of the audit cohort and some results correspond to follow up testing from previous requests. Secondly, it is possible that the patients lost the local follow up because they were discharged from hospital to the community service.(63) This is an important issue for future research.

On the other hand, 45 patients with aPL negative results were identified with test repeated. There are two possible explanations. First of all, the clinicians want to confirm the diagnosis of APS in patients who present symptoms related to thrombosis or pregnancy morbidity but other possible causes and risk factors of thrombosis have been excluded. Nevertheless, since the seronegative form of APS is characterized by negative titres of aPL, the value of this might be questioned. Secondly, it might be that the clinicians are aware of the aPL method limitations regarding the analytical sensitivity which means the lowest concentration detectable in a
biological sample. However, none of these patients were tested for the three criteria aPL. As a result, a better agreement in the panel of antiphospholipid antibodies ordered by the clinicians may improve the clinical significance of the aPL testing. As explained in the previous section, the diagnosis of APS could not be excluded based on the sole negativity of one laboratory criteria. When LA, IgG/IgM aCL and IgG/IgM anti-B2GP1 are negative in a patient with high level of suspicion for APS. A possible approach to consider is testing for “non-criteria aPL”. These aPL include IgA aCL, IgA anti-B2GP1, anti-prothrombin, antibodies against phosphatidylserine, phosphatidylethanolamine, phosphatidic acid and anti domain I antibodies. Moreover, anti-phosphatidylserine-phrothrombin complexes antibodies (aPS/PT) have been described as an independent risk factor for thrombosis and they can be used as a confirmatory test for APS. However, additional well designed studies considering the international classification criteria and the recommendations in testing standardization in order to entirely evaluate and confirm the reported clinical associations of non-criteria aPL with thrombosis and pregnancy complications are required.

Another interesting finding was the time between repetitions in the nine patients that had more than one test request subsequent to the initial investigation. (see Table 9). Patients 5, 36 and 37 had three test repetitions and patient 31 had four repetitions within 12 weeks with the same outcome. These request were considered in adherence with the published guidelines despite the fact that only the last repetition was performed at least 12 weeks apart. Unnecessary test repetitions may be considered as an arguable practice. Besides the fact that only one out of nine patients were tested for the three criteria aPL. To develop a full picture of why the clinicians were requesting more than 1 repetition within 12 weeks and not considering the three criteria aPL tests, additional studies are needed to consider how ordering practice could be improved. Moreover, two patients were identified with negative results for aCL with these becoming weak positive on repeated testing. One of these remained weak positive in further test repetitions and the other became negative. Transient aPL positivity and the intermittent production of aPL in SLE patients may cause inadequate ordering practice. Similarly, certain factors may cause false positive and false negative aPL results. For instance, acute phase proteins may cause false negative LA results whereas coagulation factor inhibitors may cause a false LA identification. Moreover, IgM rheumatoid factor may produce a false positive signal in the detection of aCL and anti-B2GP1 of the IgM isotype. Clinicians and laboratory staff should be aware of these limitations and provide appropriate interpretative comments in the aPL results.
SLE was the most common feature in the patients who had tests repeated, a higher frequency of thrombotic events in aPL positive SLE patients has been reported in the literature.\(\text{(9, 15, 17, 61)}\) Furthermore, antiphospholipid syndrome can occur in association with other systemic autoimmune diseases, mainly SLE, and 30% of SLE patients who develop APS symptoms after a 20 year follow-up.\(\text{(6, 16, 17, 21)}\) Hence, testing for aPL in SLE patients may help to predict and prevent the risk of thrombotic episodes and/or pregnancy loss. However, more research is needed to better understand the exact association of APS and SLE.

### 5.4. Conclusions

The findings of this clinical audit highlight that the adherence to the BCSH guidelines in the investigations of APS\((3)\) by clinicians in Lancashire Teaching Hospitals and primary care services across the region needs to be improved. This audit highlighted a number of potential explanations for this result, including the limitations of the audit, the different presentations of the disease and the testing limitations. Therefore, this result may not be a full reflection of current clinical practice.

However, further research into whether education and training for clinicians that highlights the importance of testing for all three APS antibodies as well as the importance of follow up testing in the right time period would improve their adherence to the guidelines, is suggested.\(\text{(5, 27, 63)}\) Research into whether better standardization and harmonization of the methodological considerations in aPL testing is also suggested, as aPL antibodies are significant biomarkers for APS.\(\text{(3, 17, 27, 46)}\) Moreover, narrowed APS criteria would allow better selection of aPL test panels and the appropriate interpretation of results from a personalized medicine approach in APS.\(\text{(4, 10, 44)}\)

Additionally, future studies could be undertaken to explore what measures could be put in place to improve the ordering practice of clinicians, both for initial testing and follow up testing. Possible suggestions could be examining whether a more collaborative relationship between clinicians and the laboratory could improve performance.\(\text{(27)}\)
CHAPTER 6

6. APPENDIX

6.1. APPENDIX I Exclusion criteria and considerations for the classification of test requests

Classification of test requests was based on the available clinical details. The test requests with multiple clinical details were grouped considering the most important manifestations related to the antiphospholipid syndrome.

EXCLUSIONS

Absence of clinical details: (N=350) Test requests that contain the reference “None” in the clinical details.

Duplicates: (N=6) Test request that have the same clinical information: same age, gender, date and hour of sampling, clinical details, test results and random number.

Illegible clinical details: (N=82) Test requests that contain the reference “Illegible” or “Not readable” in the clinical details.

Lack of antiphospholipid antibodies (aPL) results: (N=27) Absence of anticardiolipin antibodies (aCL) results in test request it was observed due to sampling error which included insufficient sample received, incorrect sample received, no sample received, immunology error and input in error. There were four patients with results for aCL but absence of Lupus anticoagulant (LA) results which could not be processed due to sampling errors. It is important highlight the fact that in these patients the clinicians require LA but the results are not available. In contrast, the requests investigating aCL without lupus anticoagulant testing were included in the audit because the main inclusion criteria in the collection data was aCL testing and the available LA test.


GROUP 1: CRITERIA CLINICAL MANIFESTATIONS

This group contains the test requests with the most specific clinical manifestations in the presentation of APS. The BCSH current guidelines (3) recognise these clinical details as part of the clinical criteria.

For the audit purposes, the test request within the categories, “Antiphospholipid syndrome clinicians requesting”, “SLE (Systemic Lupus Erythematosus)” and “Thrombosis” were included in the “Group 1: Criteria clinical manifestations”. It was considered that clinicians are aware of the antiphospholipid syndrome and they are investigating patient’s symptoms with this references in the context of APS.

Antiphospholipid syndrome clinicians requesting: This category does not have clear clinical manifestations however the details written are related to antiphospholipid antibodies or antiphospholipid syndrome terminology. Any requests related to antiphospholipid syndrome and its acronyms (APLA, APLS, APS), Hughes syndrome and positive or weak positive aPL results (anticardiolipin antibodies, anti beta2glicoprotein antibodies and lupus anticoagulant) were included.

Neurologic Manifestations: Requests with terms referred to ischemic episodes affecting blood supply in the brain. Mainly stroke, transient ischemic attacks (TIA), cerebrovascular accident (CVA), middle Cerebral Artery Occlusion (MCA) and occipital infarct.

Peripheral thrombosis: Clinical details related to thrombotic events in veins or arteries from upper and lower limbs. The terms included are deep vein thrombosis (DVT), venous sinus thrombosis, venous thromboembolism (VTE), ischemic leg and arterial occlusion.
**Pregnancy morbidity:** There is no agreement in the terminology used to term the pregnancy complications in the clinical details. Also, some patient’s information does include neither the gestational age nor the number of foetal demise events. As a result, all the test requests including any reference of foetal death at or beyond the 10th week of gestation were included within this group. For instance, recurrent miscarriage, stillbirth, foetal death in utero (FDIU), intrauterine foetal demise (IUD) spontaneous delivery and intrauterine growth restriction (IUGR). Additionally, requests with clinical details related to prematurity caused by maternal morbidity (preeclampsia, eclampsia, placental failure and abruptio placentae) were included.

**Pulmonary Manifestations:** Thrombus formation in the lungs, mainly pulmonary embolism was classified.

**SLE (Systemic Lupus Erythematosus):** APS has been widely studied in patients with SLE. Thrombotic events and pregnancy morbidity are possible clinical presentations in SLE patients with aPL positivity. The clinical details in the test requests with any reference of SLE were included in this group.

**Thrombosis:** One of the hallmarks of APS is thrombophilia. Indications for testing included are thrombophilia screen, investigations for family history of thrombophilia, recurrent thrombosis, raised APTT, low protein c and risk factors for thrombosis.

**GROUP 2: NON-CRITERIA CLINICAL MANIFESTATIONS**

This group includes test requests with less common clinical manifestations of APS reported in the literature. There are several authors describing these clinical features in the context of APS. The BCSH does not recognize them as part of the classification criteria. However, the BCSH suggests investigating aPL in these cases when other causes or risk factors of thrombosis have been excluded.

**Cardiac Manifestations:** Pericarditis, bradycardia, tachycardia and cardiomyopathy.

**Cutaneous Manifestations:** Livedo reticularis, mouth ulcers, thrombophlebitis and vasculitic rash.

**Haematological Manifestations:** Low platelets, thrombocytopenia and haemolityc anemia.

**Intraabdominal Manifestations:** Ischemic colitis and bowel ischaemia.

**Neurologic Manifestations:** Currently there are some studies linking aPL with some neurological disorders due to thrombus formation affecting the blood supply in the nervous system or by a direct pathologic effect. (11) References considering any neuropathy were included in this category: chorea, seizures, headache, migraine, collapses, weakness,
numbness, dementia, amnesia, syncope, sensitive changes, mononeuritis multiplex, spinal infarct, multiple embolic infarctions, sensorineural hearing loss, myelopathy, demyelination, neuroinflammatory and cognitive decline.

**Ophthalmologic Manifestations:** Papillitis, optic neuritis, uveitis, papilloedema, bilateral ureitis, diplopia, loss of vision, myoclonus, retinal vein occlusion and amaurosis fugax.

**Osteo-articular Manifestations:** Joint and muscle pains, stiffness, arthralgia, myalgia, polymyalgia, arthritis, polyarthritis and inflammatory arthritis.

**Other related autoimmune diseases:** Sjogren Syndrome, connective tissue disease (CTD), spondylitis, mixed connective tissue disease (MCTD), Raynauds phenomenon, reumathoid arthritis (RA), multiple sclerosis (MS), sarcoidosis and systemic sclerosis.

**Pulmonary Manifestations:** Shortness of breath, breathlessness, pleural effusions, interstitial lung disease and pulmonary oedema.

**Renal Manifestations:** Chronic kidney disease, chronic renal failure, renal transplant due to clotting, acute kidney injury, renal failure and haematuria.

**Vasculitis:** Vasculitis screen, vasculitis, vasculopathy, vascular problems and arteritis.

**GROUP 3: APS NON-RELATED CLINICAL MANIFESTATIONS**

This group contains test requests with clinical manifestations not considered within the spectrum of antiphospholipid syndrome. There is no enough evidence to consider the investigation of antiphospholipid antibodies in these clinical symptoms.

**Cutaneous Manifestations:** Alopecia, urticaria, angioedema, bruising, arm swelling, facial rash, sarcoidosis, dermatitis and erythema nodosum.

**Gastrointestinal Manifestations:** Deranged liver function tests, left upper quadrant pain, proteinuria, hepatosplenomegaly, primary biliary cirrhosis, cholecystitis, diarrhoea, colitis and abdominal pain.

**Haematological Manifestations:** Anaemia, raised ESR, lymphocytosis, leucocytosis, leucopenia, neutropenia, thrombocytosis, pancytopenia, idiopathic thrombocytopenic purpura (ITP), promyelocitic leukaemia, chronic lymphoid leukaemia, Monoclonal gammopathy of undetermined significance, raised packed cell volume (PCV) and myeloma.

**Neurologic Manifestations:** Tiredness, Postural tachycardia syndrome (POTS), fatigue, chronic fatigue syndrome (CFS), dizziness, diabetic stroke, tired all the time (TATT), headache with neutropenia secondary to chemotherapy and lethargy.
Others: Pregnancy, monitoring anticoagulant (heparin warfarin), preconception, raised inflammatory markers, myopathy, asthma, widespread pain, Graves disease, coeliac disease, high blood pressure, hypothyroidism, myalgia, unwell, back pain, widespread pain, low vitamin B12, polychondrytis, prostate cancer, raised ferritin, hemochromatosis, nutritional deficiencies, diabetes, fertility screen, swelling, linear IgG disease, sinusitis, weight loss, lymphadenopathy, breast pain, recurrent chest infections, haemoptysis, palpitations, aches and pains, dry eyes and skin, febrile episodes, thrombocytopenia due to surgery, pulmonary fibrosis, post-partum, lactation problems, amenorrhoea, risk of infection, termination of pregnancy due to foetal abnormalities, penile herpes, cryoglobulinaemia, HIV, sepsis, glomerulonephritis, hypertension, dry mouth, nose bleeding, malignancy, palpitations, overweight, raised troponin, polyserositis, erythromelalgia and Crohn’s disease.
REFERENCES