The antiphospholipid syndrome

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THE ANTIPHOSPHOLIPID SYNDROME

The clinical importance of detecting
antiphospholipid antibodies by immunoassays

Walid Chayouâ

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voor papa & mama

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1

General introduction and outline

INTRODUCTION

History of the antiphospholipid syndrome

The antiphospholipid syndrome (APS) is an autoimmune disease characterized by the persistent presence of antiphospholipid (aPL) antibodies and thrombosis or pregnancy morbidity (1). The first classification criteria for definite APS were published in 1999 (2). However, the APS story started in 1906 with the development of a diagnostic test for syphilis (Figure 1) (3). The Wassermann (reagin) test is a serological assay based on complement fixation (3). The 'Wassermann reagent' contained a liver extract of newborns who had died from congenital syphilis (3–5). In the following years, research showed that tissue extracts of animals never exposed to the causative organism of syphilis (*Treponema pallidum*), for example an ethanolic extract of beef heart could also be used (4,5). In 1941 a breakthrough occurred as Pangborn identified the antigenic component of the reagin test extracted from beef heart, cardiolipin (6). This breakthrough resulted in the development and standardization of new tests, including the Venereal Disease Research Laboratory (VDRL) test (4,5,7,8). The VDRL test was a screening tool for syphilis based upon microflocculation (7,8). Heated serum is mixed with reagin containing cardiolipin, lecithin and cholesterol (7,8). In case of positivity, flocculation or clumping can be observed using a microscope (7,8). Population screening using the VDRL test showed a group of patients that were biologically false positive. The lack of specificity to the antigen was already seen with the Wassermann test, which was confirmed by the *T. pallidum* immobilization test (4,5,9). In 1947, Lubinski listed diseases and conditions with biological false positive syphilis reactions (10). The list included diseases and conditions like malaria, lupus erythematosus and pregnancy (10). In 1952, Mohr and Moore found a high incidence of biological false positives due to infections (e.g. leprosy and malaria) (11). However, non-infectious diseases like systemic lupus erythematosus (SLE) and rheumatoid arthritis had also a high approximate incidence of biological false positives of 20% and 5%, respectively.

In the same year Conley and Hartman described two patients with lupus erythematosus who both had a biological false positive serological test for syphilis and a prolonged prothrombin time (12). This was one of the first descriptions of lupus anticoagulant (LAC), a phospholipid dependent prolongation of the clotting time. In 1954, Beaumont et al. published a report of a patient with LAC and seven previous abortions (13). More than two decades later, Firkin et al. suggested a possible relationship between LAC and recurrent abortion, which was later confirmed by Bowie et al. (14,15). In the 1950's it became apparent that some patients with SLE also had a circulating anticoagulant (12,16–18). In the 1960's the association between LAC and thrombosis

Figure 1. Timeline of key events and discoveries in APS.

was established (19). Bowie et al. demonstrated that the prolonged clotting time in patients could be corrected using soybean phosphatide (inosithin, a platelet substitute) and that this correction was related to thrombosis (19). In 1983, Harris et al. described a radioimmunoassay for the detection of anti-cardiolipin (aCL) antibodies (20). This milestone, resulted in the correlation between aCL antibodies and thrombosis within SLE patients. aCL antibodies were also found to correlate with LAC (20). Two years later, an enzyme-linked immunosorbent assay (ELISA) for the detection of aCL antibodies was described (21). In the 1980's, the nomenclature 'antiphospholipid antibody syndrome' (APS) was starting to be widely used (4). However, it was not until 1987 that the first article using this terminology was published by Bingley and Hoffbrand (22). In 1988, Fleck et al. established a correlation between anti-prothrombin antibodies (aPT) and LAC (23). LAC was hypothesized to have immunological specificity for several anionic phospholipids including phosphatidylserine, cardiolipin, phosphatidylinositol and phosphatidic acid (24). However, Fleck et al. demonstrated that aPT immunoglobulin (Ig)G antibodies possessed LAC activity (23). In 1990, it became apparent that β 2glycoprotein I (β 2GPI) was the main target for antibody binding instead of cardiolipin itself. These publications already suggest that the term antiphospholipid antibody is a misnomer, as the antibodies are not directed against phospholipids per se, but rather to phospholipid binding proteins like β 2GPI and prothrombin. In 1998, Iverson et al. suggested that antibodies directed against the first domain of β 2GPI are clinically important (25). Seven years later, de Laat et al. confirmed the importance of anti-domain I β 2GPI antibodies and suggested that these antibodies might be pathogenic as they cause LAC activity and strongly correlate with thrombosis (26). In 1999, an international consensus was reached for the classification of definite APS and updated in 2006 (1,2). First-line treatment for a first or recurrent APS-related venous thrombotic event is treatment with vitamin K-antagonists (VKA) (27). According to international guidelines, treatment with VKAs is recommended in APS patients with a first arterial thrombosis (28). In a recent consensus paper published in The Netherlands Journal of Medicine, either treatment with clopidogrel (anti-platelet therapy) or VKAs is recommended in APS patients with a first arterial thrombosis (27). Still, both papers discourage the use of direct oral anticoagulants (DOACs) like rivaroxaban (direct Xa inhibitor) (27–29). DOACs have shown to be non-inferior to VKAs for treatment and secondary prevention of venous thromboembolic events and prevention of stroke and systemic embolism in patients with non-valvular atrial fibrillation (27,30). The use of DOACs over VKAs has many advantageous, including no need for frequent laboratory monitoring, which is very much desired in patients with APS who are mostly young women of fertile age (31). It was until 2018 that an article was published evaluating the use of DOACs in triple positive (combined positivity for LAC, aCL and a β 2GPI antibodies) APS patients (32).

Pengo et al. showed that rivaroxaban therapy was associated with an increased rate of arterial events, compared to warfarin treatment (32). However, an explanation for the observed increased rate of arterial events in patients treated with DOACs is unknown and should be further explored. Overall, the efficacy of DOAC use in APS patients should be further studied.

Pathophysiology of the antiphospholipid syndrome

Multiple studies have reported an association between aPL antibodies and thrombosis or pregnancy morbidity (1). However, the underlying mechanisms are poorly understood (33,34). Patients with the persistent presence of aPL antibodies do not constantly develop thrombosis, suggesting that other factors play a role in the development of thrombotic APS (35). A “two hit” theory is suggested, in which aPL antibodies induce a thrombophilic state (first hit), and the second hit is another thrombophilic stimulus (36,37). In agreement, antibodies from APS patients only induce thrombosis in mice upon induction of vessel wall injury (38,39). However, the “two hit” theory seems not to apply for obstetric complications as antibodies with aPL activity induce fetal loss in naive pregnant mice (36).

B cells produce a β 2GPI antibodies which can form β 2GPI immune complexes on cellular surfaces (33). Binding of β 2GPI immune complexes to endothelial cells requires priming with pro-inflammatory factors (40). β 2GPI immune complexes have also shown to bind to monocytes and platelets, inducing their proinflammatory and procoagulant state (41,42). Activation of inflammatory and endothelial cells by β 2GPI immune complexes results in the activation of the complement pathway, upregulation of adhesion molecules (E-selectin), upregulation of TF, increased level of vascular endothelial growth factor and the release of neutrophil extracellular traps (33,34). Prothrombotic changes by aPL antibodies may be caused by increased expression of the platelet fibrinogen receptor glycoprotein IIb/IIIa, suppressing the activity of tissue factor pathway inhibitor, protein C activity and fibrinolysis (33,34). aPL antibodies have also an effect on the placenta, non-thrombotic mechanisms include activation of the complement system, increased trophoblast apoptosis, decreased human chorionic gonadotropin, proliferation and syncytia formation (33,34). Activation of coagulation, inflammatory and endothelial cells and interference with trophoblasts and decidual cells can lead to clinical manifestations of APS like thrombosis and pregnancy complications (33,34). However, aPL antibodies are also correlated with non-criteria manifestations like inflammation and vasculopathy (33,34). Despite all the efforts made, our understanding of the molecular mechanisms causing the development of aPL antibodies and APS related clinical manifestations is poorly understood.

Antiphospholipid antibodies

For the classification of APS, a patient has to meet at least one clinical and one laboratory criterion (Table 1). Laboratory criteria include positivity for LAC, aCL IgG/M or a β 2GPI IgG/M antibodies on two occasions at least 12 weeks apart (1,43). The prevalence of APS is estimated to be around 50 per 100,000 of the population (44). Frequency of aPL is estimated at 9.5% in deep vein thrombosis, 11% in myocardial infarction, 13.5% in stroke and 6% in pregnancy morbidity (45). As clinical features of APS occur frequently and independent of APS, reliable aPL detecting assays are of utmost importance.

TABLE 1. Classification criteria of APS.

APS is present if a patient meets at least one of the following clinical criteria and one of the laboratory criteria (1)	
Clinical criteria	Laboratory criteria
<p>1. Vascular thrombosis One or more clinical episodes of confirmed arterial, venous, or small vessel thrombosis, in any tissue or organ.</p> <p>2. Pregnancy morbidity (a) One or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation, or (b) One or more premature births of a morphologically normal neonate before the 34th week of gestation, or (c) Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation.</p>	<p>1. Lupus anticoagulant present in plasma, on two or more occasions at least 12 weeks apart, detected according to the guidelines of the ISTH-SSC (46).</p> <p>2. Anticardiolipin antibody of IgG and/or IgM isotype present in serum or plasma on two or more occasions, at least 12 weeks apart (47).</p> <p>3. Anti-β2 glycoprotein-I antibody of IgG and/or IgM isotype present in serum or plasma on two or more occasions, at least 12 weeks apart (47).</p>

Lupus anticoagulant

LAC is detected by a phospholipid dependent prolongation of the clotting time (1). The detection of LAC is a three-step procedure including a screening, mixing and confirmatory test. Citrated blood is collected and platelet free plasma is made by double centrifugation (46). LAC should be detected based on two different principles, preferably using a dilute Russell's viper venom time (dRVVT) and a LAC sensitive activated partial thromboplastin time (aPTT) (46). Patients referred for LAC testing are tested for prolongation of the clotting time (screening). Next, patient plasma is diluted with pooled normal plasma (1:1) to exclude clotting factor deficiencies (mixing). Lastly, LAC is confirmed using an excess of hexagonal phase II phospholipids (46). LAC is the only functional assay within the aPL-panel and considered the strongest predictor of

thrombosis and pregnancy morbidity (48,49). LAC has been shown to be dependent on aPT or a β 2GPI antibodies (23,50–52). However, it has also been demonstrated that LAC can occur in the absence of these antibodies (53). Isolated LAC positivity is considered to be less correlated with thrombosis than triple positivity (54). Still, the risk of a first thrombotic event in isolated LAC positives is higher than controls without aPL antibodies of the same age (54,55). Many efforts have been made in standardization of aPL antibody testing. In 2009, the International Society on Thrombosis and Haemostasis-Scientific and Standardization Committee (ISTH-SSC) subcommittee on lupus anticoagulant/anti-phospholipid antibodies published detailed guidelines for LAC detection (46). Laboratory testing for LAC remains complicated with many pitfalls in the implemented procedure as well as in the interpretation (56). LAC detection has many drawbacks, including sensitivity to anticoagulant therapy, difficulties in the interpretation of mixing studies, the use of normal pooled plasma and lack of consensus on the calculation of cut-off values (46,57–62).

Anti-cardiolipin antibodies

Traditionally, aCL antibodies are measured by an ELISA. Typically, the ELISA plate is coated with cardiolipin and incubated overnight (63). Plates are blocked with the use of adult bovine serum (ABS) or fetal bovine serum (FBS). The use of phosphate buffered saline (PBS), bovine serum albumin (BSA) or casein to block non-specific binding are not recommended as they do not contain β 2GPI. ABS and FBS do contain β 2GPI, which is crucial for binding of pathogenic 'aCL' antibodies (63,64). After blocking the plate, samples are diluted and incubated. A secondary conjugated antibody (anti-IgG, anti-IgM or anti-IgA) is incubated for the detection of aCL antibodies (63). Quality control is important for the detection of aCL antibodies and inter-assay variation should not exceed 20% for ELISAs detecting aCL antibodies (47). Nowadays, automated immunoassays have become available for the detection of aCL and a β 2GPI antibodies using different solid phases (magnetic particles, microbeads, coated polystyrene cups or membranes) with various detection systems like chemiluminescence, flow cytometry and multiplex systems (65–67). These automated systems have shown to have less inter-assay variation and inter-laboratory variation compared to traditional ELISAs (47,65,68). Automated systems are also less labor intensive, more rapid and can detect several aPL antibodies simultaneously in one sample (65). Cut-off values are recommended calculated from at least 120 plasmas or sera of which the 99th percentile is calculated (47). However, it is not always possible to collect samples from 120 healthy donors. Alternatively, manufacturer's recommended cut-off values can be validated on

a limited number of at least 20 healthy donors and transferred (47). Similar to LAC and a β 2GPI, aCL positivity should be confirmed after at least 12 weeks to rule out transient positivity induced by infections or drugs (1).

Anti- β 2glycoprotein I antibodies

a β 2GPI antibodies are traditionally detected by ELISA, but equally as for aCL automated systems are available (65–67). For the detection of a β 2GPI antibodies, (high-binding) ELISA plates are coated with β 2GPI overnight (69). Wells are then blocked with a blocking agent and samples are diluted and incubated (69). A secondary conjugated antibody (anti-IgG, anti-IgM or anti-IgA) is then incubated for the detecting of a β 2GPI antibodies (69). The absence of cardiolipin is theorized to increase the detection of clinically relevant antibodies (70). However, not all a β 2GPI antibodies are correlated with thrombosis (26).

Cofactors for anti-phospholipid antibodies

β 2GPI and prothrombin are considered the most important cofactors for aPL antibodies.

β 2glycoprotein I

β 2GPI is an abundant plasma protein (approximately 200 μ g/ml) which is produced in the liver and placenta (26,71–73). β 2GPI is an anionic 50-kDa protein, is evolutionary conserved and has shown to regulate haemostasis as well as the complement system (38,39,72,74–77). Also, β 2GPI is considered the main antigenic target for aPL antibodies (78). Still, β 2GPI-deficient humans and mice do not express a clear phenotype of thrombosis or bleeding, indicating it is not essential for life (71,72,79,80). Notably, breeding of β 2GPI null mice results in reduced percentage of offspring, suggesting that the lack of β 2GPI is a selective disadvantage for survival (72,75,80). β 2GPI is a highly glycosylated phospholipid-binding protein, consisting of four complement control protein (CCP) domains of 60 amino acids each. Domain V is different as it consists of 82 amino acids and a phospholipid-binding site (26,72,81–86). The CCP domain functions as a module for protein-protein interactions in many different proteins (72). Domain V of β 2GPI contains a large positively charged patch that determines the affinity for anionic phospholipids. β 2GPI can be found in two distinct conformations: a ‘circular’ (native) conformation in plasma and an ‘activated’ (open) conformation (87). Upon binding of β 2GPI to an anionic phospholipid surface, circular β 2GPI unfolds to an open conformation, exposing cryptic epitopes. One of these cryptic epitopes is Gly40-Arg43, located in the first domain of β 2GPI. Antibodies against the cryptic epitope Gly40-Arg43 are associated with LAC and strongly correlate with thrombosis (26,88).

Prothrombin

Prothrombin, also known as coagulation factor (F)II, is a 72-kDa, vitamin K-dependent zymogen produced in the liver. Prothrombin has a plasma concentration of approximately 100 µg/mL. Prothrombin is converted to thrombin upon vessel wall injury. Coagulation factor Xa cleaves prothrombin at Arg271-Thr272 and Arg320-Ile321 generating the enzyme thrombin and fragment 1.2 (89,90). Thrombin is a central coagulation factor that cleaves fibrinogen to fibrin to produce a firm clot.

Genetic depletion of prothrombin in adult mice is not compatible with survival (91,92). To date, no living patient has been reported with undetectable plasma prothrombin, suggesting that prothrombin is essential for life. Like β 2GPI, prothrombin can be found in an open and closed conformation. aPT antibodies are a heterogeneous group of antibodies of which it has become more apparent that the conformation of prothrombin and the target epitope is of clinical importance, similar to β 2GPI (93–96).

Non-Criteria anti-phospholipid antibodies

The first international consensus statement on the classification criteria for definite APS included the detection of LAC, aCL IgG and aCL IgM (2). In 2006, classification criteria were revised and the detection of a β 2GPI IgG and a β 2GPI IgM was added to the aPL panel (1). Inclusion of other aPL antibodies like aCL IgA, a β 2GPI IgA, aPT and anti-phosphatidylserine/prothrombin (aPS/PT) antibodies needed further investigation (1).

Domain I antibodies

a β 2GPI antibodies may be directed to any epitope or part of the molecule. However, antibodies directed to the first domain of β 2GPI have shown to be pathogenic by both *in vivo* and *in vitro* studies. *In vitro*, anti-domain I antibodies have shown to display LAC activity, increase resistance to the anticoagulant properties of annexin A5 and activated protein C (APC) and neutralize the inhibitory function of β 2GPI on von Willebrand factor induced platelet aggregation (26,97–102). *In vivo*, domain I antibodies have shown to be pathogenic by inducing thrombosis and/or fetal loss in mice and rats (103–105). Antibodies against domain V failed to exert a thrombogenic effect in rats (105). However, APS patients could have both anti-domain I and anti-domain V antibodies. Antibodies against domain V bind circulating β 2GPI, blocking interaction of β 2GPI with anionic phospholipid surfaces and therefore hindering the conformational change of β 2GPI to an open conformation and exposing cryptic epitopes. It is hypothesized that patients with anti-domain I β 2GPI IgG antibodies and anti-domain V antibodies have a lower thrombotic risk if the anti-domain V level exceeds the level of anti-domain I (105,106). Anti-domain V antibodies might therefore be protective as these antagonize the

procoagulant activity of domain I antibodies. Calculating a ratio of anti-domain I to anti-domain 4/5 might be useful for risk stratification (106). Although domain I antibodies are not part of the current classification aPL-panel, commercial assays for the detection of anti-domain I antibodies have become available (107). Unfortunately, a commercial chemiluminescence immunoassay (CIA) detecting anti-domain I antibodies failed to expose the cryptic epitope Gly40-Arg43, hampering the use of anti-domain I antibodies in APS patients (107). These results highlight the importance of standardization of solid phase assays detecting aPL antibodies (59,107).

Anti-cardiolipin and anti- β 2glycoprotein I IgA antibodies

aCL and a β 2GPI IgA antibodies are not included in the current classification criteria for APS (1). Nevertheless, multiple studies have demonstrated an association between IgA aPL antibodies and thrombosis or pregnancy morbidity (108–118). The ISTH-SSC committee concluded in 2008 that aCL IgA does not add diagnostic power to the current aPL-panel. Data supporting the inclusion of a β 2GPI IgA antibody testing was found to be inadequate (1). Another point of discussion raised by the committee, is the lack of uniform guidelines on how to perform the assay, unavailability of control material and lack of consensus on units of measurement (1). Since 2006, new data on the association between IgA aPL antibodies and clinical manifestations of APS became available (108,111–116,118). A systematic review was published concerning the clinical relevance of aCL and a β 2GPI IgA antibodies and concluded that there is not enough evidence to recommend aCL and/or a β 2GPI IgA antibody testing as diagnostic accuracy is not improved (119). However, new assays for the detection of aCL and a β 2GPI IgA antibodies have become commercially available, but their value in the current aPL panel remains to be investigated (117).

Anti-prothrombin antibodies and anti-phosphatidylserine/prothrombin antibodies

For the detection of aPT antibodies, prothrombin is directly coated on irradiated ELISA plates (120). Detection of anti-phosphatidylserine/prothrombin (aPS/PT) antibodies is performed by coating phosphatidylserine/prothrombin complex on non-irradiated ELISA plates (120,121). Although a correlation has been shown between aPS/PT and aPT antibodies, it is hypothesized that these aPL antibodies belong to a different population of antibodies (120,122,123). In a systematic review, aPT antibodies correlated less with thrombosis than aPS/PT antibodies. Already in 1988, an association was established between aPT antibodies and LAC, which is considered the strongest predictor of thrombosis and pregnancy morbidity in APS, making aPS/PT antibodies an interesting group of aPL antibodies to further investigate (23,48,49). aPS/PT antibodies

are frequently found in patients with APS and suggested to be included in the current aPL-criteria (1,121,124). They may have a potential role in risk stratification and are strongly correlated with isolated LAC in APS patients, as well as in asymptomatic carriers (94,95,125–127).

Antiphospholipid profile

Although every aPL antibody in the current panel (LAC, aCL IgG, aCL IgM, a β 2GPI IgG, a β 2GPI IgM) for the classification of APS has the same diagnostic value, it is more apparent to evaluate the complete aPL-profile (1,128,129). Evaluation of an aPL profile can be important for risk stratification of thrombosis or pregnancy morbidity and “reliability” of diagnosis. Single positivity or isolated positivity, is positivity for one of the five consensus aPL antibodies but negative results for the other four aPL antibodies. Double positivity is referred as aCL and a β 2GPI positivity, but negative for LAC. Triple positivity is referred as combined positivity for LAC, aCL and a β 2GPI from the same isotype. LAC is considered the main predictor of thrombosis and pregnancy morbidity in APS (49,130). However, single LAC positivity is not considered a high risk profile for thrombosis or pregnancy morbidity in comparison to triple positivity (131–133). A strong correlation with thrombosis is found when a β 2GPI antibodies are present with LAC (134). Single LAC positivity in patients receiving anticoagulation must be interpreted carefully as anticoagulation therapy can induce a false positive LAC result (62). Antibodies against other cardiolipin binding proteins (complement factor 4 or Factor H) and β 2GPI-independent aCL antibodies are also detected (135,136). aCL and a β 2GPI assays contain the same antigen (β 2GPI), an overlap is therefore expected. A positive aCL result with the absence of a β 2GPI antibodies and LAC should therefore be interpreted with care as no association is found for isolated aCL positivity (131). Also, isolated a β 2GPI antibodies are not correlated with thrombosis and hypothesized to be of no importance as these antibodies do not induce LAC activity (131,136). For example, a β 2GPI antibodies might occur in patients with leprosy in which thrombosis is uncommon (137). However, these a β 2GPI antibodies are not directed against the first epitope of β 2GPI (137). It became apparent that positivity for multiple aPL is highly associated with clinical manifestations of APS (138–140). Triple positivity is more strongly associated with thrombosis than single and double positivity (129,141). Not only for recurrent thrombosis, but also for a first thrombotic event in asymptomatic carriers, triple positivity is a risk factor (141,142). Double positive patients are at lower risk to develop APS related clinical manifestations than triple positive patients, and single positive patients are even less likely to develop thrombosis (43,67,136).

Anti-cardiolipin and anti- β 2glycoprotein I isotype

The isotype of aCL and a β 2GPI antibodies should be taken into consideration for diagnosis of APS and correct interpretation of the assays (136). A laboratory test result with positivity for aCL IgG and a β 2GPI IgM, but not for aCL IgM and a β 2GPI IgG aPL should be classified as “inappropriate” (136). Concordance of isotype in aCL and a β 2GPI detection is essential, but not required according to the updated classification criteria (1,136). The isotype of aCL and a β 2GPI is not only a valuable tool for the interpretation of the aPL assays, but may also help in evaluating the risk for thrombosis or pregnancy morbidity. A meta-analysis found more significant correlations with thrombosis for the IgG isotype compared to the IgM isotype (143). aCL IgM antibodies have shown to be important in obstetric APS (130,144). However, the relevance of aCL and a β 2GPI IgM antibodies in thrombosis is debated (143).

OUTLINE

Classification of APS is dependent on the detection of aPL antibodies by laboratory tests. This includes the detection of LAC, aCL IgG/M antibodies and a β 2GPI IgG/M antibodies. Multiple studies have concluded that aPL antibody detecting assays show a large inter-assay variability. It is hypothesized that the inter-assay variation for aCL and a β 2GPI detected automated systems is lower compared to traditional ELISAs. **Chapter2** assesses the agreement between four commercially available and fully automated assays detecting aCL and a β 2GPI IgG/M antibodies in patients with APS and controls. In addition, the association of aPL antibodies with clinical manifestations is investigated. Patients with triple positive aPL antibodies have a high risk for a first thrombotic event and recurrence. Subsequently, clinicians may adapt their treatment strategy. Altogether, reliable detection is of utmost importance in triple positive patients. Triple positivity is often associated with high aCL and a β 2GPI titers and therefore hypothesized to be less affected by variations between solid phase assays. In **chapter3**, agreement between four aCL and a β 2GPI assays in the detection of triple positive APS patients is investigated. In the current classification criteria, positivity for aCL or a β 2GPI, independent of the isotype (IgG/M) is sufficient to fulfill the laboratory criteria of APS. However, the relevance of IgM antibodies in the classification of thrombotic APS is debated. We therefore investigated in **chapter 4** the relevance of aCL and a β 2GPI IgM antibodies in thrombosis and pregnancy morbidity. **Chapter 5** studies the added value of aCL IgA and a β 2GPI IgA antibodies in APS. aCL IgA and a β 2GPI IgA antibodies have shown to correlate with thrombosis and induce thrombosis in mice. However, IgA is still not included in the current aPL-panel for the classification of APS. Antibodies against domain I of β 2GPI are considered pathogenic and correlate well with LAC. The

significance of anti-domain I antibodies in APS is reviewed in **chapter 6**. In **chapter 7**, we investigate whether anti-domain I antibodies can be used as a tool to identify high-risk APS patients. The role of aPT antibodies in platelet activation is investigated in **chapter 8**. Finally, **chapter 9** summarizes key findings of this thesis and discusses these findings in relation to the literature.

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Detection of anti-cardiolipin and anti- β 2glycoprotein I antibodies differs between platforms without influence on association with clinical symptoms

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ABSTRACT

Background: The antiphospholipid syndrome (APS) is characterized by thrombosis and/or pregnancy morbidity with persistent presence of antiphospholipid antibodies (aPL). Laboratory criteria include aPL detection by coagulation tests for lupus anticoagulant (LAC) or solid phase assays measuring anti- β 2glycoprotein I (a β 2GPI) or anti-cardiolipin (aCL) immunoglobulin (Ig) G/IgM antibodies. External quality control programs illustrate that commercially available aPL assays produce variable results.

Objective: We aimed to investigate the agreement and diagnostic accuracy of solid phase assays.

Materials and Methods: In this multi-centre study, 1,168 patient samples were tested on one site for aCL and a β 2GPI IgG/IgM antibodies by four solid phase test systems. Samples included APS patients, controls and monoclonal antibodies (MoAB) against different epitopes of β 2GPI. LAC was determined by the local centre.

Results: aCL IgM assays resulted in the most discrepancies (60%), while aCL IgG and a β 2GPI IgM assays resulted in lower discrepancies (36%), suggesting better agreement. Discrepant samples displayed lower median aPL titers. Dependent on the solid phase test system, odds ratios ORs for thrombosis and pregnancy morbidity ranged from 1.98 to 2.56 and 3.42 to 4.78, respectively. Three platforms showed lower sensitivity for MoAB directed against the glycine (Gly) 40-arginine (Arg) 43 epitope of domain I of β 2GPI.

Conclusion: Poor agreement was observed between different commercially available aCL and a β 2GPI IgG/IgM assays, hampering uniformity in the identification of aPL-positive patients. Clinical association was globally concordant between solid phase test systems considering results of the four aPL together. An assay sensitive in detecting the MoAB against Gly40-Arg43 of domain I of β 2GPI reached the highest OR for thrombosis.

INTRODUCTION

The anti-phospholipid syndrome (APS) is characterized by thrombosis and/or pregnancy morbidity with the persistent presence of anti-phospholipid antibodies (aPL) (1). Laboratory criteria include aPL detection by phospholipid-dependent prolongation of coagulation tests referred to as lupus anticoagulant (LAC) or by solid phase assays measuring anti- β 2glycoprotein I (a β 2GPI) and anti-cardiolipin (aCL) immunoglobulin (Ig) G/IgM antibodies (1). For the classification of APS at least one clinical and one persistent positive laboratory criterion is required (1). Given the high frequency of clinical manifestations (thrombosis and pregnancy morbidity) associated with APS in the general population, laboratory tests are of utmost importance for the classification of patients with APS. Although LAC positivity is considered the strongest predictor of clinical manifestations of APS, aCL and a β 2GPI IgG/IgM antibodies have the same value in the current classification criteria (1–5)

Anti- β 2GPI antibodies are detected using β 2GPI as antigen, while both cardiolipin and β 2GPI are used as antigens in aCL immunoassays. The use of antigens from human sources is preferred above antigens from animal origin (e.g. bovine) to avoid false-positives (6). In contrast to a β 2GPI immunoassays, antigens used in aCL immunoassays are not exclusively from human origin (Supplemental Table 1). Traditionally, aCL and a β 2GPI antibodies are detected with an enzyme-linked immunosorbent assay (ELISA). Nowadays, more advanced and automated systems have become available for the detection of aCL and a β 2GPI aPL (7–10). Automated systems make use of an alternative solid phase (e.g. magnetic beads) and use alternative detection methods, such as chemiluminescence or (enzyme linked) fluorescence. A large variety of assays are used in clinical laboratories as there is no consensus on a ‘gold standard’ for the detection of aCL and a β 2GPI aPL (6). Reports from external quality control programs illustrate that aPL assays produce variable results (11,12). Detection of a β 2GPI antibodies is challenging as some antibodies may be directed against a cryptic epitope that is only exposed after conformational shape change (13,14). Exposure of this cryptic epitope, spanning glycine (Gly) 40-arginine (Arg) 43 in the first domain of β 2GPI, has been shown to vary across commercial a β 2GPI IgG assays (7,15). In addition, variability between assays might be due to the heterogeneous origin of aPL, differences in local working conditions, differences in assay design and a lack of standardization (6). As classification of APS heavily depends on the detection of aPL, variation within these tests will affect the treatment strategy.

Therefore, we aimed to investigate the agreement and diagnostic accuracy of commonly used commercially available solid phase assays measuring IgG and IgM aCL and a β 2GPI

aPL. Samples and normal pooled plasma supplemented with monoclonal antibodies (MoAB) against different domains of β 2GPI were tested with four assays at one location by a single technician to exclude inter-laboratory and inter-operator variation.

MATERIALS AND METHODS

Patient cohort

We obtained 1,168 samples from 8 European centers. Classification of APS was based on the Sydney criteria (1). Patients were classified by the corresponding centre resulting in 259 thrombotic APS patients (APS thrombosis), 204 patients with a history of thrombosis and negative for laboratory criteria of APS (non-APS thrombosis), 122 obstetric APS patients (APS obstetric), 33 patients with pregnancy complications and negative for laboratory criteria of APS (non-APS obstetric), 196 patients with an autoimmune disease other than APS (autoimmune diseases), 100 individuals with a normal pregnancy (normal pregnancy), 194 controls that were referred for aPL testing for other reasons than the clinical criteria of APS, like subfertility and prolonged activated partial thromboplastin time (controls) and 60 women that were diagnosed with APS without information on the specification of the clinical manifestations (unspecified APS). Centers with the indicated number of samples included Ghent (469), London (196), Nîmes (164), Nancy (114), Kraków (101), Milan (52), Geneva (50) and Apeldoorn (22). The study was approved by the central and the local ethical committees.

Assays

aCL IgG, aCL IgM, a β 2GPI IgG and a β 2GPI IgM aPL were measured in the Ghent University Hospital (Ghent, Belgium) by four commercially available immunoassays: BioPlex2200 (Bio-Rad, Bio-Rad Laboratories, Hercules, USA), ImmunoCapEliA (Thermo Fisher Scientific/Phadia, Uppsala, Sweden), ACL AcuStar (Werfen/Instrumentation Laboratories, Bedford, USA) and QUANTA Lite ELISA (Inova Diagnostics, San Diego, USA) (Supplemental Table 1). Assays were selected based on frequently used assays in the External quality Control of diagnostic Assays and Tests program and the willingness of manufacturers for collaboration. Due to shortage of patient sample, three and two patients were excluded for aCL IgG and a β 2GPI IgG detection by BioPlex2200, respectively. Manufacturers' recommended cut-off values were used upon confirmation in 20 healthy volunteers, in accordance with the Scientific and Standardization committee (SSC) guidelines of the International Society on Thrombosis and Haemostasis (16). Assays were performed according to the manufacturer's instructions. All four methods were performed in parallel in runs of 40 samples. According to the

guidelines, single measurement was used on the automated systems (BioPlex2200, ImmunoCapEliA and ACL AcuStar) as the intra- and inter-run imprecision coefficient of variation was <10% and duplicate measurement for the ELISA (QUANTA Lite ELISA) (16). aPL titers were expressed in arbitrary units (GPL, MPL, U/ml, SGU and SMU). All samples were measured by the same technician and values below the calculated limit of detection (LOD) were replaced by the calculated LOD.

Monoclonal antibodies

Two human-derived MoAB P2-6 and P1-117 were used to test the specificity and sensitivity of the four commercially available aCL and a β 2GPI IgG assays. P2-6 recognizes β 2GPI independently of its conformation and P1-117 recognizes β 2GPI in its open conformation binding to the Gly40-Arg43 epitope of the domain I (15,17). Serial dilutions of antibodies (0-250 μ g/ml) in normal pooled plasma were tested in duplicate for all platforms included in the study. Platforms were used according to the manufacturer's recommendations and these spiked samples were handled as patient samples and tested in the same conditions. The threshold for positivity corresponding to a positive titer of aCL or a β 2GPI was determined.

Statistical analysis

Solid phase assays were compared pairwise as no 'gold standard' for aPL detection exists. Agreement between assays in positivity was assessed by 2 x 2 contingency tables in all measured samples. Comparison of discrepancies between aCL IgG, aCL IgM and a β 2GPI IgG and a β 2GPI IgM positivity was calculated: positivity discrepancy (%) = (only positive for method A + only positive for method B) / (all positives) x 100. Median aPL titers within one platform were calculated with titers above the cut-off value. Correlation between solid phase assays was performed by a Spearman's rank correlation coefficient. Diagnostic accuracy was assessed by calculating odds ratios, sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV) and the receiver operator curve (ROC). The area under the ROC curve between solid phase assays was compared using DeLong et al.'s method (18). Significance of differences was determined with the Mann-Whitney U test, as appropriate using the statistical package for social sciences (SPSS 23.0; SPSS, Chicago, Illinois, United States) and MedCalc Statistical Software version 17.7.2 (MedCalc Software bvba, Ostend, Belgium).

RESULTS

We measured aCL IgG/IgM and a β 2GPI IgG/IgM aPL in 1,168 individuals with a mean age of 43 years ranging from 16 to 87 years old (Table 1) with four commercially available assays (Supplemental Table 1).

TABLE 1. Demographic and clinical characteristics of the study population

	Patients (n)	Female	Age [year, mean (range)]	Pregnancy morbidity	Thrombosis				
					Venous	Arterial	Venous and Arterial	Small vessel	Not specified
APS thrombosis	259	164 (63%)	50 (17-87)	22	160	55	26	4	14
Non-APS thrombosis	204	116 (57%)	46 (19-85)	0	149	47	5	0	3
APS obstetric	122	122 (100%)	35 (19-61)	122	4	2	0	0	4
Non-APS obstetric	33	33 (100%)	33 (20-52)	33	NA	NA	NA	NA	NA
Autoimmune diseases	196	158 (81%)	46 (16-83)	NA	NA	NA	NA	NA	NA
Normal pregnancy	100	100 (100%)	31 (27-36)	0	NA	NA	NA	NA	NA
Controls	194	170 (88%)	39 (18-82)	NA	NA	NA	NA	NA	NA
Unspecified APS	60	60 (100%)	48 (24-70)	NS	NS	NS	NS	NS	NS
Total population	1168	923 (79%)	43 (16-87)	177	313	104	31	4	21

Abbreviations: APS, anti-phospholipid syndrome; NA= not applicable; NS = not specified.

Laboratory criteria for the classification of APS require at least one positive aPL. We therefore compared positivity for at least one aPL between platforms using 2 x 2 contingency tables (Table 2). Discrepancies varied between 79 (ACL AcuStar vs. BioPlex2200) and 164 (ACL AcuStar vs. ImmunoCapEliA) samples. aCL IgM and a β 2GPI IgG positivity resulted in the most discrepancies, varying from 69 to 162 and 44 to 153 samples, respectively (Table 3). On the other hand, aCL IgG and a β 2GPI IgM positivity

resulted in less discrepancies between assays, varying from 49 to 98 and 34 to 58 samples, respectively. Comparison of discrepancies between aCL IgG/IgM and a β 2GPI IgG/IgM positivity was calculated by the percentage of discrepancies from all positives, resulting in a maximum discrepancy of 36, 60, 53 and 36% for aCL IgG, aCL IgM, a β 2GPI IgG and a β 2GPI IgM positivity, respectively. In accordance, aCL IgM titers were less correlated between the solid phase assays compared to aCL IgG and a β 2GPI IgM with Spearman's rank correlation coefficients.

TABLE 2. Number of samples positive for aCL IgG, aCL IgM, a β 2GPI IgG or a β 2GPI IgM by BioPlex2200, ImmunoCapEliA, ACL AcuStar and QUANTA Lite ELISA are compared pairwise

		BioPlex 2200		ImmunoCap EliA		ACL AcuStar	
		-	+	-	+	-	+
BioPlex 2200	-						
	+						
ImmunoCap EliA	-	755	37				
	+	108	268				
ACL AcuStar	-	795	11	717	89		
	+	68	294	75	287		
QUANTA Lite ELISA	-	792	64	751	105	753	103
	+	71	241	41	271	53	259

Abbreviations: aCL, anti-cardiolipin; a β 2GPI anti- β 2 glycoprotein I; Ig, immunoglobulin.

In accordance, aCL IgM titers were less correlated between the solid phase assays compared to aCL IgG and a β 2GPI IgM with Spearman's rank correlation coefficients of ≥ 0.514 (95% confidence interval [CI], 0.471-0.555), ≥ 0.635 (95% CI, 0.599-0.668) and ≥ 0.738 (95% CI, 0.711-0.763) respectively (Table 4).

aPL positive samples not in agreement across the platforms were characterized by lower median aPL titers than positives in agreement (Figure 1). However, for the majority of discrepancies observed, respective individuals suffered from clinical manifestations of APS (thrombosis and/or pregnancy morbidity) (Figure 1). Clinical implications of the observed (dis)agreements were assessed by calculating the sensitivity, specificity, NPV, PPV and OR for thrombosis, pregnancy morbidity or both clinical criteria (Table 5). Positivity was defined when at least one aPL was positive. Clinically affected and non-clinically affected patients were set as outcome variable rather than APS/non-APS in order to be independent of aPL presence previously detected by the medical centers

TABLE 3. Discrepancies in aPL positivity detection between platforms

		BioPlex® 2200		ImmunoCap® EliA		ACL AcuStar®	
		-	+	-	+	-	+
A	BioPlex® 2200	-					
		+					
	ImmunoCap® EliA	-	892	59			
		+	25	190			
	ACL AcuStar®	-	905	37	913	30	
		+	12	212	40	185	
	QUANTA Lite ELISA®	-	897	78	913	46	919
		+	20	171	22	169	24
							58
							167
B	BioPlex® 2200	-					
		+					
	ImmunoCap® EliA	-	895	69			
		+	18	184			
	ACL AcuStar®	-	874	5	867	14	
		+	39	248	99	188	
	QUANTA Lite ELISA®	-	909	115	964	62	877
		+	4	138	2	140	4
							149
							138
C	BioPlex® 2200	-					
		+					
	ImmunoCap® EliA	-	930	28			
		+	119	91			
	ACL AcuStar®	-	989	9	897	101	
		+	60	110	61	109	
	QUANTA Lite ELISA®	-	971	27	906	92	946
		+	78	92	52	118	52
							52
							118
D	BioPlex® 2200	-					
		+					
	ImmunoCap® EliA	-	1007	23			
		+	29	109			
	ACL AcuStar®	-	1028	26	1024	30	
		+	8	106	6	108	
	QUANTA Lite ELISA®	-	997	19	996	20	1006
		+	39	113	34	118	48
							10
							104

Abbreviations: aCL, anti-cardiolipin; aβ2GPI anti- β2 glycoprotein I; aPL, anti-phospholipid antibodies; Ig, immunoglobulin. Note: Number of samples positive for aCL IgG, aβ2GPI IgG, aCL IgM and aβ2GPI IgM by BioPlex2200, ImmunoCapEliA, ACL AcuStar and QUANTA Lite ELISA are compared pairwise.

FIGURE 1. Anti-phospholipid antibodies (aPL) titers of samples in (dis)agreement. Titers of samples positive for all assays and assay discrepancies are shown as detected with (A) BioPlex2200, (B) ImmunoCapEliA, (C) ACL AcuStar and (D) QUANTA Lite ELISA. Patients with thrombosis and/or pregnancy morbidity are indicated in black and without in red. Titers are expressed as the median value of positive aPL titers with interquartile ranges. *** $p < 0.001$. AU, arbitrary units.

that collected the samples, minimizing selection bias. Independent from the assay used, a statistically significant association with thrombosis and/or pregnancy morbidity was found. ORs for thrombosis varied between 1.98 (95% CI, 1.46-2.69) and 2.56 (95% CI, 1.82-3.59) detected by ImmunoCapEliA and BioPlex2200, respectively. ORs for pregnancy morbidity ranged between 3.42 (95% CI, 2.32-5.05) and 4.78 (95% CI, 3.14-7.27) detected by ImmunoCapEliA and QUANTA Lite ELISA, respectively. Although not the most sensitive aPL detection platform for thrombosis and/or pregnancy morbidity, the QUANTA Lite ELISA resulted in the highest specificity (87.76% [95% CI, 84.52-90.52%])

and OR (4.24 [95% CI, 3.10-5.79]). PPVs for thrombosis and/or pregnancy morbidity ranged from 74.73% (95% CI, 70.74-78.35%) to 80.77% (95% CI, 76.47-84.44%) and NPVs ranged from 49.71% (95% CI, 48.07-51.35%) to 50.23% (95% CI, 48.57-51.90%) as shown in Table 5. The area under the ROC curve of the solid phase assays for thrombosis and/or pregnancy morbidity was low as expected and was not significantly different among the tested solid phase assays.

BioPlex2200 aCL and a β 2GPI IgG showed the highest and equal sensitivity for both MoAB P2-6, that recognizes β 2GPI independently of its conformation, and P1-117, which only recognizes β 2GPI upon exposure of the Gly40-Arg43 epitope in domain I (Table 6). ACL AcuStar aCL and a β 2GPI IgG sensitivity was slightly lower for P1-117, in the same extent for aCL and a β 2GPI. QUANTA Lite ELISA and ImmunoCapEliA showed much lower sensitivity for P2-6 and P1-117, with large difference in sensitivity for P2-6 and P1-117. Both aCL assays did not detect P1-117 at all. OR for thrombosis and/or pregnancy morbidity for aCL and a β 2GPI (Table 5) ranged from 4.24 to 6.12 and 3.49 to 6.56, respectively. The platform (BioPlex2200) with the highest sensitivity in detecting the P1-117 MoAB also reached the highest OR for thrombosis, but not for pregnancy morbidity.

TABLE 4. Correlation between aCL IgG, aCL IgM, a β 2GPI IgG and a β 2GPI IgM titers of solid phase assays

	aCL IgG (95% CI)	aCL IgM (95% CI)	a β 2GPI IgG (95% CI)	a β 2GPI IgM (95% CI)
BioPlex2200 vs ImmunoCapEliA	0.712 (0.682-0.739)	0.514 (0.471-0.555)	0.784 (0.716-0.806)	0.739 (0.712-0.764)
BioPlex2200 vs ACL AcuStar	0.767 (0.742-0.789)	0.803 (0.781-0.822)	0.900 (0.889-0.911)	0.813 (0.792-0.831)
BioPlex2200 vs QUANTA Lite ELISA	0.744 (0.717-0.768)	0.586 (0.547-0.622)	0.676 (0.644-0.706)	0.701 (0.671-0.729)
ImmunoCapEliA vs ACL AcuStar	0.635 (0.599-0.668)	0.521 (0.478-0.562)	0.758 (0.732-0.781)	0.775 (0.752-0.797)
ImmunoCapEliA vs QUANTA Lite ELISA	0.716 (0.687-0.743)	0.562 (0.521-0.600)	0.686 (0.655-0.716)	0.764 (0.739-0.787)
ACL AcuSta vs QUANTA Lite ELISA	0.673 (0.640-0.703)	0.580 (0.541-0.617)	0.632 (0.596-0.665)	0.738 (0.711-0.763)

Abbreviations: aCL, anti-cardiolipin; a β 2GPI, anti- β 2 glycoprotein I; CI, Confidence interval; Ig, immunoglobulin.

Note: Spearman Rank Correlation rho coefficients with their respective 95% confidence intervals are shown.

TABLE 5. Diagnostic accuracy of aPL detection by BioPlex2200, ImmunoCapEliA, ACL AcuStar and QUANTA Lite ELISA. Samples were defined as positive if at least one aPL was positive, excluding LAC.

	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	PPV (%, 95% CI)	NPV (%, 95% CI)	Odds ratio (95% CI)	AUC^a (95% CI)
Thrombosis N=853^b						
BioPlex® 2200	31.32 (27.12-35.76)	84.87 (80.92-88.28)	71.08 (65.21-76.32)	51.00 (49.14-52.86)	2.56 (1.82-3.59)	0.58 (0.55-0.61)
ImmunoCap ®EliA	36.29 (31.90-40.85)	77.69 (73.23-81.73)	65.88 (60.75-70.66)	50.67 (48.5-52.84)	1.98 (1.46-2.69)	0.57 (0.54-0.60)
ACL AcuStar®	35.85 (31.48-40.41)	79.74 (75.41-83.62)	67.76 (62.50-72.59)	51.15 (49.04-53.26)	2.20 (1.61-3.00)	0.58 (0.54-0.61)
QUANTA Lite ELISA®	31.10 (26.91-35.54)	84.62 (80.65-88.05)	70.59 (64.71-75.86)	50.85 (48.99-52.71)	2.48 (1.77-3.48)	0.58 (0.55-0.61)
Pregnancy morbidity N=645^c						
BioPlex® 2200	38.06 (30.39-46.20)	87.55 (84.30-90.34)	49.17 (41.52-56.85)	81.71 (79.73-83.55)	4.32 (2.84-6.58)	0.63 (0.59-0.67)
ImmunoCap ®EliA	45.16 (37.17-53.35)	80.61 (76.83-84.02)	42.42 (36.45-48.63)	82.29 (80.01-84.36)	3.42 (2.32-5.05)	0.63 (0.59-0.67)
ACL AcuStar®	42.58 (34.68-50.77)	82.45 (78.79-85.71)	43.42 (37.06-50.01)	81.95 (79.76-83.95)	3.48 (2.35-5.17)	0.63 (0.59-0.66)
QUANTA Lite ELISA®	40.00 (32.22-48.17)	87.76 (84.52-90.52)	50.82 (43.22-58.38)	82.22 (80.19-84.08)	4.78 (3.14-7.27)	0.64 (0.60-0.68)
Clinical criteria N=1,168						
BioPlex® 2200	35.99 (32.37-39.73)	87.55 (84.30-90.34)	80.00 (75.60-83.78)	49.71 (48.07-51.35)	3.95 (2.90-5.39)	0.62 (0.59-0.65)
ImmunoCap ®EliA	41.45 (37.71-45.26)	80.61 (76.83-84.02)	74.73 (70.74-78.35)	49.87 (47.96-51.79)	2.94 (2.24-3.86)	0.61 (0.59-0.65)
ACL AcuStar®	40.71 (36.98-44.51)	82.45 (78.79-85.71)	76.24 (72.19-79.87)	50.12 (48.26-51.99)	3.23 (2.44-4.26)	0.62 (0.59-0.64)
QUANTA Lite ELISA®	37.17 (33.52-40.93)	87.76 (84.52-90.52)	80.77 (76.47-84.44)	50.23 (48.57-51.90)	4.24 (3.10-5.79)	0.63 (0.60-0.65)

Abbreviations: aPL, anti-phospholipid antibodies; APS, anti-phospholipid syndrome; AUC, area under the curve; CI, confidence interval; LAC, lupus anticoagulant; NPV, negative predictive value; PPV, positive predictive value.

Note: Samples were defined as positive if at least one aPL was positive, excluding LAC.

^aAUC from the receiver operator curve (ROC) were not significantly different between solid phase assays.

^bAPS thrombosis + non-APS thrombosis + AID + controls.

^cAPS obstetric + non-APS obstetric + AID + normal pregnancy + controls.

DISCUSSION

APS classification strongly depends on the laboratory criteria. Besides the clinical criteria, thrombosis or pregnancy morbidity, often due to other causes than aPL, APS is defined by the persistent presence of aPL (1). aPLs are detected by LAC assays or by semi-quantitative solid phase assays measuring aCL IgG, aCL IgM, a β 2GPI IgG or a β 2GPI IgM antibodies (1). Many studies on head-to-head comparisons of solid phase assays with different study designs, already have shown that solid phase assays differ in performance and agreement. In our study we analyzed a large cohort of APS and non-APS patients simultaneously with four different platforms, allowing comparison of different methods on the same patient population. LAC was determined by the local center but excluded from the comparative analysis to investigate the variation solely introduced by solid phase assays. aPLs have a low prevalence (1-5%) in the general population, and APS is even more infrequent (40-50/100,000 persons) (19,20). As a consequence, studies comparing aPL detection methods are often based on a small patient group and/or lack diagnostic accuracy. We investigated the (dis)agreement and diagnostic accuracy of four commercially available solid phase assays for the detection of aCL IgG, aCL IgM, a β 2GPI IgG and a β 2GPI IgM antibodies in 1,168 samples from eight European centers. As in the Sydney criteria, all individual aPL are part of the laboratory criteria for APS, diagnostic accuracy was assessed for positivity for at least one aPL detected by a solid phase assay (aCL IgG, aCL IgM, a β 2GPI IgG or a β 2GPI IgM) (1). Positivity for at least one aPL detected by a solid phase assay was significantly correlated with thrombosis and/or pregnancy morbidity, independent from the solid phase assay used. The highest OR for thrombosis was obtained by detection of aPL by BioPlex2200 (2.56 [95%CI, 1.82-3.59]). Detection of aPL by ImmunoCapEliA resulted in the lowest OR (1.98 [95%CI, 1.46-2.69]), mainly because of lower specificity. aPL detection by BioPlex2200 and QUANTA Lite ELISA resulted in similar OR for thrombosis and/or pregnancy morbidity, higher than OR obtained by detection with ImmunoCapEliA and ACL AcuStar. Calculated sensitivity, NPV and AUC for thrombosis and/or pregnancy morbidity were low as expected, since we included patients with thrombosis and/or pregnancy morbidity without APS (negative for aPL). Inclusion of only APS patients would lead to a selection bias as diagnosis of APS is dependent on the aPL detection assays used by the local centre.

In our study, a maximum discrepancy of 36, 60, 53 and 36% for aCL IgG, aCL IgM, a β 2GPI IgG and a β 2GPI IgM positivity was found, respectively. Detection of aCL IgG and a β 2GPI IgM resulted in the best agreement. However, still a substantial number of samples were in disagreement. Conversely, a study comparing different kits detecting aCL and a β 2GPI IgG/IgM found better agreement between a β 2GPI ELISAs than aCL ELISAs (21). This might be a consequence of the small number of a β 2GPI positive samples in this study

(21). Another study compared the performance of aCL IgG and IgM antibody detection of the ImmunoCapEliA with their in-house aCL ELISA in 1,143 routine samples (22). The authors reached a good agreement (>90%) between the automated systems and their in-house ELISA assay (22). As expected, positivity for aPL proved to be relatively rare, because of which the majority of

TABLE 6. Threshold for positivity: titer of MoAB corresponding to a positive titer of aCL or aβ2GPI IgG

	aCL IgG				aβ2GPI IgG			
	BioPlex 2200	ImmunoCap EliA	ACL AcuStar	QUANTA Lite ELISA	BioPlex 2200	ImmunoCap EliA	ACL AcuStar	QUANTA Lite ELISA
P2-6 (μg/ml)	1.95	125	15.63	62.5	1.95	31.25	1.95	62.5
P1-117 (μg/ml)	1.95	Neg ^a	31.25	Neg ^a	1.95	125	7.81	125

Abbreviations: aCL, anti-cardiolipin; aβ2GPI, anti-β2 glycoprotein I; Ig, immunoglobulin; MoAB, monoclonal antibodies. ^aNegative results for all concentrations.

routine samples were classified as negative independent of the method used, resulting in a high agreement. Importantly, more samples were classified positive by only ImmunoCapEliA or only the in-house ELISA than by both methods (116/179 and 72/90 for aCL IgG and aCL IgM, respectively), suggesting a poor agreement comparable to our findings (22). We observed large differences in positivity across platforms, even between automated systems sharing the same solid phase (BioPlex2200 vs ACL AcuStar). In a collaborative study the inter-laboratory variability of aβ2GPI IgG and aβ2GPI IgM antibodies was assessed in 30 serum samples from 22 centres (22). Poor agreement was found between centers as positivity ranged from 50 to 93% and 13 to 70% for aβ2GPI IgG and aβ2GPI IgM detection, respectively (23). We excluded inter-laboratory variability and found the best agreement between BioPlex2200 and ACL AcuStar. However, OR for clinical features of APS obtained by BioPlex2200 were more comparable with an ELISA assay (QUANTA Lite ELISA) than an assay sharing the same solid phase (ACL AcuStar). BioPlex2200 and QUANTA Lite ELISA were in poor agreement but characterized by a comparable diagnostic accuracy. Our results highlight the importance of measuring both the agreement between assays as well as their diagnostic accuracy. It seems also important that in daily practice the four aPLs are measured with a same platform. Although some small differences exist in the diagnostic performance of the tested platforms, the values of sensitivity and specificity for APS related clinical symptoms and OR for clinical events are essentially comparable. This may be explained by the higher

agreement that was observed in samples with higher levels of aPL (Figure 1). That newer generation ELISA and automated systems show low agreement but comparable diagnostic accuracy was also illustrated in other studies (9,10,24–26).

In our study, guidelines from the SSC were followed by confirming manufacturer's cut-off values in at least 20 healthy volunteers (16). In practice, most laboratories transfer their cut-off values similarly, predominately due to practical difficulties to calculate the 99th percentiles in a population of at least 120 healthy volunteers. Positivity for aPL not in agreement across the assays were characterized by lower median aPL titers. The majority of samples positive for aCL and/or a β 2GPI IgG/IgM detected by one assay, but not for all solid phase assays fulfilled the clinical criteria of APS, suggesting that higher cut-off values result in reduced sensitivity for APS. The clinical relevance of aPL levels below the 99th percentile, needs to be further studied (27). Lower levels of antibodies are observed especially in obstetric APS (28,29).

Variability between aPL detecting assays is hypothesized to result from pre-analytical, analytical, and post-analytical conditions, calibration and assay-specific issues (30,31). In our study, detection of aPL and analysis was performed by a single operator, eliminating inter-laboratory and inter-operator variation. Traditionally, aCL and a β 2GPI antibodies are detected by ELISAs. Nowadays, automated systems become available which are hypothesized to improve agreement (8). Automation of assays indeed improved intra-laboratory and inter-laboratory reproducibility compared with non-automated ELISA. In general, ELISAs have shown large inter-laboratory variation and limited consensus in external quality control programs (11,12). The lack of international calibration standards makes the comparison between assays challenging. Efforts have been made for standardization by international reference materials, such as the Harris standards (pool of patient material and thus limited in production) and Koike standards (directed against a single epitope, thus decreasing the sensitivity of the assay in which it is used as aPL), although not reflecting the real life since aPL of patients are a heterogeneous group of antibodies [31]. Variation in aPL detection might be introduced by the heterogeneous origin of aPL, differences in assay design and a lack of standardization. All four tested a β 2GPI antibody detection assays make use of an antigen of human origin. However, for detection of aCL antibodies only BioPlex2200 uses human β 2GPI and non-animal derived cardiolipin. Human antigen source is considered more specific than animal CL and/or β 2GPI (6). Different preparations of human β 2GPI have shown not to influence agreement in a β 2GPI IgG and IgM detection (32). Indeed, BioPlex2200 and QUANTA Lite ELISA showed high specificity for thrombosis and/or pregnancy morbidity. However,

our results show that the QUANTA Lite ELISA using purified cardiolipin and bovine β 2GPI as antigen source resulted in the highest specificity for thrombosis and/or pregnancy morbidity.

Anti- β 2GPI immunoassays detect antibodies against all five domains of β 2GPI, including non-pathogenic antibodies, phospholipid-independent and low affinity a β 2GPI (6,33). The use of negative surface charge of the solid phase in a β 2GPI ELISAs have shown to increase the antigen density and exposure of cryptic epitopes of β 2GPI such as Gly40-Arg43 in domain I, mimicking the binding between negatively charged phospholipids and β 2GPI (6,13,14). Variation in solid phase may lead to variability in exposure of the epitope Gly40-Arg43 (15). Hence, antibodies against the Gly40-Arg43 epitope are considered pathogenic and have shown to highly correlate with thrombosis (13,14,34). We used patient derived MoAB P1-117 and P2-6 to verify and assess variability in exposure of epitopes of β 2GPI (7,15,17). We confirmed that the platform most sensitive in detecting P1-117 (the MoAB reactive against Gly40-Arg43 in domain I) by both the aCL and a β 2GPI IgG assay has the highest OR for thrombosis. Other platforms are less sensitive in detecting P1-117 compared to P2-6 (the MoAB recognizing β 2GPI irrespective of its conformation), resulting in lower OR for thrombosis.

In conclusion, we found poor agreement between commercially available immunoassays detecting aCL and a β 2GPI IgG/IgM antibodies, which may hamper uniformity in the classification of aPL positive samples. However, computed OR for thrombosis and/or pregnancy morbidity in our study, considering results of the four aPL together, were globally concordant among solid phase test systems. Since our comparison between systems is based considering measurement of the four aPL within one test system, classification and follow-up of patients for aPL is preferable when performed with the same system.

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SUPPLEMENTAL DATA

SUPPLEMENTAL TABLE 1. Technical specifications of the solid phase assay

Manufacturer	Platform	Method	Solid phase	aCL antigen source		aβ2GPI antigen source		Cut-off value
				Cardiolipin	β2GPI	β2GPI	β2GPI	
Bio-Rad Laboratories	BioPlex2200	Fluorescence	Beads	Synthetic	Human	Human	Human	20
Phadia/ ThermoFisher	ImmunoCap Elia	Fluorescence enzyme linked	Wells in carriers	Bovine	Bovine	Bovine	Human	10
Instrumentation Laboratories/Werfen	ACL AcuStar	Chemiluminescence	Beads	Bovine	Human	Human	Human	20
INOVA Diagnostics/Werfen	QUANTA Lite ELISA	Chromogenic enzyme linked	Poly-strene wells	NS	Bovine	Bovine	NS	20

Abbreviations: aCL, anti-cardiolipin; aβ2GPI, anti-β2 glycoprotein I; AU, arbitrary unit; NS, not specified.

3

Identification of high thrombotic risk triple-positive antiphospholipid syndrome patients is dependent on anti-cardiolipin and anti- β 2glycoprotein I antibody detection assays

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ABSTRACT

Background: The antiphospholipid syndrome (APS) is characterized by thrombosis and/or pregnancy morbidity with the persistent presence of antiphospholipid antibodies (aPL). Triple-positivity, (i.e. positivity for lupus anticoagulant [LAC], anti-cardiolipin [aCL] and anti- β 2glycoprotein I [a β 2GPI] antibodies) is associated with a high thrombotic risk.

Objectives: We investigated the variability in triple-positivity detection by measuring the same samples with four commercially available solid phase assays. In addition, the added clinical value of aPL in LAC positive patients was investigated, as well as the association of IgM triple-positivity and thrombosis.

Patients/Methods: We included 851 patients from seven European medical centers. Anti-CL and a β 2GPI IgG/IgM antibodies were determined by four platforms: BioPlex®2200, ImmunoCap®EliA, ACL AcuStar® and QUANTA Lite ELISA®.

Results: Triple-positivity detection by solid phase assays varied, ranging from 89 up to 118 in thrombotic APS patients (n=258) of which 86 were detected independent of the platform. Lupus anticoagulant positivity resulted in an odds ratio (OR) for thrombosis of 3.4; triple-positivity (irrespective of the isotype) increased the OR from 4.3 up to 5.2, dependent on the platform. Triple-positivity solely for the IgM isotype did not increase the OR for thrombosis compared with LAC positivity. The highest OR for thrombosis was reached for positivity for IgG and IgM a β 2GPI and aCL (8.6 up to 28.9).

Conclusions: Triple-positivity proved to be highly associated with thrombosis, but identification is assay dependent. Within triple-positivity, IgM antibodies only have an added clinical value in patients positive for IgG antibodies.

INTRODUCTION

The antiphospholipid syndrome (APS) is characterized by thrombosis and/or pregnancy morbidity with the persistent presence of antiphospholipid antibodies (aPL) [1]. Laboratory criteria include aPL detection by phospholipid-dependent coagulation tests referred to as lupus anticoagulant (LAC) or by quantitative solid phase assays measuring anti- β_2 glycoprotein I (a β_2 GPI) and anti-cardiolipin (aCL) IgG/IgM antibodies [1]. Given the high-frequency of thrombosis irrespective of the syndrome, laboratory tests are of utmost importance for the classification of APS. Detection of aPL by solid phase assays is associated with high inter-laboratory and inter-method variation [2, 3]. Reports from external quality control programs illustrate that commercially available aPL assays produce variable results [4-6].

In order to improve the identification of patients at risk, it was suggested that APS patients should be evaluated according to their aPL profile [1, 7]. Combined positivity for LAC, aCL and a β_2 GPI antibodies (i.e. triple-positivity) has shown to be associated with a high risk for both a first thrombotic event and recurrence [8-10]. In the NOH-APS study, a large observational study, triple-positivity was a predictor for thrombosis in purely obstetric APS patients [11]. However, computed risks for thrombosis of LAC positivity and triple-positivity were globally concordant, with the exception of pulmonary embolism [11]. Despite the high correlation of triple-positives with thrombosis, the predictive value is argued to originate from LAC positivity [12]. Recently, the detection of triple-positivity (i.e. positivity for LAC, aCL and a β_2 GPI antibodies) was suggested to be method and platform independent [13].

Looking at the isotype of aPL, both IgG and IgM antibodies directed against cardiolipin and β_2 GPI are included in the Sydney criteria [1]. However, the clinical value of IgM antibodies in thrombotic APS is debated [14]. To date, the thrombotic association of IgM antibodies in aPL profiles like triple-positivity is not known. In this study we included 851 patients from seven European medical centers. Four solid-phase assay platforms were selected based on frequency of use and the willingness of manufacturers to provide their assays. The samples were tested with all assays at one location by a single technician.

In a retrospective multicenter study we aimed to investigate the variability in triple-positivity detection between different aPL detection platforms and the impact of the platform on the association of triple-positivity with thrombosis. In addition, we aimed to assess the added value of aPL detection in LAC positive patients and the impact of the isotype with respect to the association with thrombosis.

MATERIALS AND METHODS

Study population

We included 851 patients from seven European medical centers. Classification of APS was based on the Sydney criteria [1]. Patients were classified by the local centers resulting in 258 thrombotic APS patients (APS thrombosis), 204 patients with a history of thrombosis and negative for laboratory criteria of APS (non-APS thrombosis), 196 patients with an autoimmune disease other than APS, like systemic lupus erythematosus (52%) and systemic sclerosis (27%), without thrombotic complications (AID controls) and 193 controls that were referred for aPL testing for other reasons than the clinical criteria of APS, including subfertility and prolonged activated partial thromboplastin time (controls). Patients were enrolled within a timespan of 1 year, with patient samples stored less than 5 years. Thrombosis was objectively confirmed according the Sydney criteria [1]. The majority of thrombotic APS patients received anticoagulant therapy including vitamin K antagonists (VKA) (46%), low molecular weight heparins (LMWHs) (5%) and direct oral anticoagulants (DOACs) (4%). Twenty-seven patients (10%) received anti-platelet therapy and only 2% of patients with thrombotic APS received both anticoagulant and anti-platelet therapy. Details on anti-coagulant and anti-platelet therapy of the remaining 84 patients are not available. Women classified with obstetrical APS were excluded. The study was approved by the local ethical committees. Lupus anticoagulant positivity was determined by the local center, according to the ISTH-SSC (International Society of Thrombosis and Haemostasis-Scientific Standardisation Subcommittee) guideline [15]

Solid phase assays

Commercially available solid phase assays (Supplemental Table 1) were selected based on frequently used assays in the external quality control program of the ECAT (External quality Control of diagnostic Assays and Tests, Leiden, The Netherlands) and the willingness of manufacturers for collaboration. Anti-CL IgG, aCL IgM, a β 2GPI IgG and a β 2GPI IgM antiphospholipid antibodies were detected by BioPlex®2200 (Bio-Rad, Bio-Rad Laboratories, Hercules, USA), ImmunoCap®EliA (Thermo Fisher Scientific/Phadia, Uppsala, Sweden), ACL AcuStar® (Werfen/Instrumentation Laboratories, Bedford, USA) and QUANTA Lite ELISA® (Inova Diagnostics, San Diego, USA) in the Ghent University Hospital (Ghent, Belgium). Reagents used for the detection of aCL and a β 2GPI IgG/IgM antibodies were measured with their corresponding instruments according to the manufacturer's instructions. All four methods were performed in parallel runs of 40 samples. Anti-phospholipid antibody titers were expressed in arbitrary units (GPL, MPL, U ml⁻¹, SGU or SMU). All samples were measured by the same technician and values

below the calculated limit of detection (LOD) were replaced by the LOD. Manufacturer's recommended cut-off values were used upon confirmation in 20 healthy volunteers, in accordance with the ISTH-SSC guideline [16].

Statistics

Significance of differences between aPL titers was determined with the Mann-Whitney U test. Diagnostic efficacy was assessed within the total population by sensitivity, specificity and odds ratios (ORs) using the statistical package for social sciences (SPSS 23.0; SPSS, Chicago, IL) and MedCalc Statistical Software version 17.7.2 (MedCalc Software bvba, Ostend, Belgium). Statistical significance was set at *P* value less than 0.05.

RESULTS

We measured aCL IgG/IgM and aβ2GPI IgG/IgM aPL in 833 individuals with a mean age of 46 years ranging from 16 to 87 years old (Table 1) with four commercially available assays (Supplemental Table 1). In our study population, venous thrombosis (VT), was more common than arterial thrombosis (AT), both in the APS and control group. In addition, primary APS (PAPS) was more prevalent than APS secondary to an underlying connective tissue disorder (SAPS).

From the 851 samples tested, 274 were LAC positive, and for these triple-positivity ranged from 106 (39%) up to 146 (53%) detected by QUANTA Lite ELISA® and BioPlex®2200, respectively (Table 2). In patients diagnosed with thrombotic APS (n=258), 202 were positive for LAC. From these 202 LAC positive samples 118, 101, 111 and 89 were defined as triple-positive (positivity for LAC, aCL IgG or IgM aβ2GPI IgG or IgM) detected with the solid phase assays: BioPlex® 2200, ImmunoCap® EliA, ACL AcuStar® and QUANTA Lite ELISA®, respectively (Table 2). By a comparison of two proportions, triple-positivity detection was found to be statistically different between BioPlex® 2200 and QUANTA Lite ELISA® (*P*=0.0122). Other combinations proved not to be statistically different, although the comparison of ACL AcuStar® and QUANTA Lite ELISA® almost reached statistical significance (*P*=0.0586). Of the LAC positive patients not defined as triple-positive, the majority was isolated LAC positive (Supplemental Table 2).

TABLE 1. Demographic and clinical characteristics of the study population

	Patients (n)	Female	Age [year,mean (range)]	Antiphospholipid syndrome			Thrombosis				
				Primary APS	Secondary APS	Not specified	Venous	Arterial	Venous and Arterial	Small vessel	Not specified
APS thrombosis	259	164 (63%)	50 (17-87)	150	45	64	160	55	26	4	14
Non-APS thrombosis	204	116 (57%)	46 (19-85)	NA	NA	NA	149	47	5	0	3
Autoimmune diseases	196	158 (81%)	46 (16-83)	NA	NA	NA	NA	NA	NA	NA	NA
Controls	194	170 (88%)	39 (18-82)	NA	NA	NA	NA	NA	NA	NA	NA
Total population	853	608 (71%)	46 (16-87)	150	45	64	309	102	31	4	17

APS, antiphospholipid syndrome; NA, not applicable.

TABLE 2. Triple-positive patients detected with BioPlex®2200, ImmunoCap®EliA, ACL AcuStar® and QUANTA Lite ELISA® in patients with thrombotic antiphospholipid syndrome (APS), patients with non-APS thrombosis, autoimmune disease (AID) controls and controls

	Lupus anticoagulant positive	Triple positives			
		BioPlex® 2200	Immuno Cap® EliA	ACL AcuStar®	QUANTA Lite ELISA®
APS Thrombosis (n = 258)	202	118	101	111	89
Non-APS Thrombosis (n = 204)	0	0	0	0	0
AID controls (n = 196)	56	22	18	19	14
Controls (n = 193)	16	6	6	4	3
Total patient population (n = 851)	274	146	125	134	106

TABLE 3. Discrepancies in triple-positivity detection with pairwise comparison. Number of LAC-positive samples and those positive for aCL and aβ2GPI antibodies (irrespective of the isotype) detected by BioPlex®2200, ImmunoCap®EliA, ACL AcuStar® and QUANTA Lite ELISA®.

		BioPlex® 2200		ImmunoCap® EliA		ACL AcuStar®	
		-	+	-	+	-	+
BioPlex® 2200	-						
	+						
ImmunoCap® EliA	-	696	30				
	+	9	116				
ACL AcuStar®	-	704	13	706	11		
	+	1	133	20	114		
Quanta Lite ELISA®	-	701	44	722	23	713	32
	+	4	102	4	102	4	102

Agreement of triple-positivity detection by solid phase assays was assessed by a 2 x 2 contingency table within the total population (Table 3). Discrepancies varied between 14 (BioPlex®2200 vs ACL AcuStar®) and 48 (BioPlex® 2200 vs QUANTA Lite ELISA®) individuals. In patients diagnosed with thrombotic APS (n=258) 118 triple-positives were detected by BioPlex® 2200 of which 86 patients were defined triple-positive,

independent of the solid phase assay used (Table 4). On the other hand, 32 patients were defined as triple-positive by BioPlex® 2200 but negative by all the other tested solid phase assays (Table 4). However, the majority of patients with discrepant results by the tested solid phase platforms have a history of thrombosis, illustrating clinical relevance (Table 4). Median aPL titers were calculated by aCL and aβ2GPI antibody titers above the cut-off. Triple-positives in agreement across all platforms displayed higher median aPL titers than triple-positive samples not positive for all tested platforms (Figure 1). Statistical difference was reached for BioPlex®2200 ($P < 0.001$), ACL AcuStar® ($P < 0.001$) and QUANTA Lite ELISA® ($P = 0.0029$), but not for ImmunoCap®EliA ($P = 0.5851$), as shown in Figure 1.

The sensitivity of triple-positivity for thrombosis was low compared to LAC alone and varied from 19% (95% confidence interval [95% CI], 16-23%) up to 26% (95% CI, 22-30%) between the tested assays (Figure 2A). However, higher specificity for thrombosis was obtained, ranging from 93% (95% CI, 90-95%) up to 96% (95% CI, 93-97%), as shown in Figure 2(B). Lupus anticoagulant positivity resulted in an OR of 3.63 (95% CI, 2.76-4.76). Triple-positivity was statistically correlated with thrombosis, independent of the solid phase assay used to detect aCL and aβ2GPI IgG/IgM antibodies (Figure 2B). Odds ratios for thrombosis ranged from 4.3 (95% CI, 2.7-6.8) up to 5.2 (95% CI, 3.0-8.9) among the platforms (Table 5).

TABLE 4. Discrepancies in triple-positivity detection between BioPlex®2200, ImmunoCap®EliA, ACL AcuStar® and QUANTA Lite ELISA®. Number of triple-positives in (dis)agreement among all four solid phase assays in patients with thrombotic APS, patients with non-APS thrombosis, autoimmune disease (AID) controls and controls

	BioPlex® 2200	ImmunoCap® EliA	ACL AcuStar®	QUANTA Lite ELISA®	In agreement among all solid phase assays
APS-thrombosis	32	15	25	3	86
AID	10	6	7	2	12
Controls	3	3	1	0	3
Total	45	24	33	5	101

FIGURE 1. Antiphospholipid antibody (aPL) titers of triple-positives in (dis)agreement. Log transformed aPL titers in agreement for all platforms tested are indicated by solid dots, disagreements are indicated by solid squares. Patients without the clinical criteria for the anti-phospholipid syndrome (APS) are indicated in red and patients with a history of thrombosis are indicated in black. Titers are expressed as the median value of positive aPL titers with interquartile ranges. AU, arbitrary units.

FIGURE 2. Diagnostic efficacy of lupus anticoagulant (LAC) and triple-positive patients detected by BioPlex®2200, ImmunoCap®EliA, ACL AcuStar® and QUANTA Lite ELISA®. (A) Sensitivity and (B) specificity for thrombosis (mean $\pm$ 95% confidence interval [95 % CI]).

Triple-positivity for aCL IgG and a β 2GPI IgG or aCL IgM and a β 2GPI IgG IgM was significantly correlated with thrombosis, independent of the solid phase assay used. However, positivity for the IgM isotype (aCL IgM and a β 2GPI IgM) was more correlated with thrombosis than triple-positivity for the IgG isotype (aCL IgG and a β 2GPI IgG) upon aCL and a β 2GPI detection by BioPlex® 2200, ImmunoCap® EliA and ACL AcuStar®. On the other hand, triple-positivity for the IgG isotype was stronger correlated with thrombosis than for the IgM isotype upon detection of aPL by QUANTA Lite ELISA®. However, the majority of triple-positives for the IgM isotype were also positive for the IgG isotype (data not shown). Isolated triple-positivity for the IgG isotype increased the OR for thrombosis compared to LAC positivity only in two out of the four tested aPL solid phase assays (ACL AcuStar® and QUANTA Lite ELISA®), as shown in Table 5. In triple-positivity, isolated IgM positivity did not increase OR compared with LAC. Moreover, isolated triple-positivity for the IgM isotype did not reach statistical difference when aPL were detected with BioPlex® 2200 and ACL AcuStar® (1.9 [95% CI, 0.6-5.4] and 2.0 [95% CI, 0.7-5.9], respectively). Positivity for all tested aPL (LAC, aCL IgG, aCL IgM, a β 2GPI IgG and a β 2GPI IgM) resulted in the highest OR for thrombosis ranging from 8.6 (95% CI 3.1-24.4) up to 28.9 (3.9-212.4) detected by BioPlex® 2200 and ACL AcuStar® respectively.

TABLE 5. Correlation of aPL profiles with thrombosis. Odds ratios (ORs) with 95% confidence intervals (CIs) are shown (significant ORs in bold).

Positive	Negative	BioPlex® 2200 (95% CI)	ImmunoCap® EliA (95% CI)	ACL AcuStar® (95% CI)	QUANTA Lite ELISA® (95% CI)
LAC	-	3.4 (2.5-4.7)	3.4 (2.5-4.7)	3.4 (2.5-4.7)	3.4 (2.5-4.7)
LAC+ aCL +a β 2GPI (irrespective of isotype)	-	4.4 (2.9-6.9)	4.3 (2.7-6.8)	5.0 (3.1-8.1)	5.2 (3.0-8.9)
LAC+ aCL IgG+ a β 2GPI IgG	-	4.8 (3.0-7.7)	4.8 (2.8-8.4)	6.0 (3.5-10.2)	6.2 (3.1-12.3)
LAC+ aCL IgM+ a β 2GPI IgM	-	5.0 (2.4-10.3)	5.0 (2.4-10.3)	6.7 (2.8-15.9)	4.6 (2.2-9.4)
LAC+ aCL IgG+ a β 2GPI IgG	aCL IgM+ a β 2GPI IgM	3.4 (2.0-5.8)	3.4 (1.9-6.2)	4.0 (2.3-6.9)	4.8 (2.2-10.3)
LAC+ aCL IgM+ a β 2GPI IgM	aCL IgG+ a β 2GPI IgG	1.9 (0.6-5.4)	2.7 (1.2-6.5)	2.0 (0.7-5.9)	2.7 (1.2-6.5)
LAC+ aCL IgG+ a β 2GPI IgG + aCL IgM+ a β 2GPI IgM	-	8.6 (3.1-24.4)	12.0 (2.8-50.8)	28.9 (3.9-212.4)	10.1 (2.4-43.3)

DISCUSSION

Detection of aPL antibodies is accompanied by large inter-method and inter-laboratory variation [2, 4, 5, 17, 18]. Traditionally, aCL and a β 2GPI antibodies are detected by ELISA. Nowadays, more advanced (automated) systems are available, which are suggested to reduce inter-laboratory variation [4, 17, 19-21]. We excluded inter-laboratory variation, by detecting aCL and a β 2GPI antibodies in the same samples at one laboratory (Ghent, Belgium), carried out by a single technician. Despite many efforts, standardization of antiphospholipid antibody (aPL) assays is far from reached as poor consensus is obtained between assays when measuring the same sample [2, 20]. The identification of triple-positive patients was recently suggested to be less affected by inter-method variation, thereby better classifying APS patients at risk [13]. In a retrospective cross-sectional study, the authors suggested that identification of patients with triple aPL positivity is platform and method independent, having compared two methods with a different solid phase from the same manufacturer [13]. However, they found a disparity of 6 or 9 triple-positives out of 220 patients (121 with APS and 99 with systemic lupus erythematosus), depending on the cut-off value used, already suggesting the presence of patients with low levels of a β 2GPI and aCL aPL titers and difficulties in reaching consensus in the classification of these patients [13, 22]. In our cohort, the highest discrepancy in number of triple-positive samples was found between BioPlex® 2200 and QUANTA Lite ELISA® with a discrepancy of 29 triple-positives out of 202 LAC-positive samples (14%). Similar to single positivity, identification of triple-positives was found to be assay dependent. In addition, we did not assess the variation of triple-positivity detection introduced by LAC assays. Indeed, an external quality control program concluded that inter-method and inter-laboratory variation is higher in solid phase assays than LAC detection by dilute Russell's Viper Venom Time (dRVVT) assay [3]. However, still difficulties persist in reaching consensus among weak-positive samples [2-4, 23]. The presented variation in triple-positivity detection may therefore be underestimated. A possible limitation of our study is that thrombotic patients under treatment during the time of blood collection, could result in an increased risk of false positive LAC tests.

Samples positive for LAC, aCL and a β 2GPI antibodies by one solid phase platform but not by all four tested platforms have lower median aPL titers, suggesting difficulties in consensus on positivity among low aPL titers (Figure 1) [24]. We accept that the cut-off calculated by the 99th percentile of a normal population is the best consensus between sensitivity and specificity, and the clinical relevance of aCL and/or a β 2GPI results that are below the 99th percentile needs to be further studied [25]. Few studies showed that low titers of aCL also were predictive for thrombotic recurrence [26]. In this study, we

transferred the manufacturer's cut-off after confirmation, as recommended by the ISTH-SSC guidelines [16]. In terms of clinical practice, a recent questionnaire raised by the SSC showed that the majority of laboratories use the same approach, because only a minority of laboratories have the resources to calculate a cut-off value from at least 120 normals. With this cut-off choice applied for all platforms, the majority of discrepant samples in our study were from patients that experienced a thrombotic event (Table 4).

A minority of non-thrombotic patients ($n=582$, auto-immune disease and controls) in our study population showed triple-positivity ($n=17-28/583$ or 2.9%-4.8%, depending on the platform). These should be regarded as asymptomatic carriers, what is in line with the findings of Mustonen *et al.* who found that 5% of asymptomatic triple-positives were carriers [27].

The association of thrombosis and single aPL positivity is debated because results are contrary. Recent studies showed that the risk of thrombotic events increases with the number of positive tests in APS patients and the creation of antibody profiles and test combinations increases the association with thrombosis [7, 8, 28-30]. On the other hand, another study showed a strong association between single aPL positivity and thrombosis in paediatric APS patients [31]. Although single positivity is not always significantly correlated with thrombosis, within the current guidelines all aPL have the same diagnostic value [1]. In our cohort, we confirmed the strong correlation between triple-positivity and thrombosis, as triple-positivity was significantly correlated with thrombosis independent of the platform used. A large observational study investigated the incidence of thrombosis in obstetric APS patients. Frequencies of thrombotic events were assessed in 517 APS patients, 279 women carrying a genetic thrombophilia polymorphism and 796 women with negative thrombophilia polymorphism results [11]. Computed risks for thrombosis of LAC positivity and triple-positivity were globally concordant [11]. However, triple-positivity was a predictor for pulmonary embolism, whereas LAC positivity alone was not [11]. In an Italian cohort, 618 patients were referred to aPL testing, of which 55% met the clinical criteria consistent with the Sapporo criteria [7]. A statistically significant correlation between LAC and thrombosis was found (OR 4.4, 95% CI 1.5-13.3) [7]. In triple-positive patients the association with thrombosis increased even further (OR 33.3, 95% CI 7.0-157.6), suggesting an additional value of triple-positivity detection in thrombotic risk stratification [7]. Patient population and aPL detection method may impact the correlation of thrombosis with triple-positivity and the role of aCL and $\text{a}\beta 2\text{GPI}$ antibodies in addition to LAC. It has already been shown that the presence of aCL and $\text{a}\beta 2\text{GPI}$ of the same isotype reinforces the clinical probability of APS [32]. We confirmed that ORs for all platforms are higher for triple-positivity with concordance of isotype compared to triple-positivity including combinations of aCL

and a β 2GPI irrespective of isotype, except for one platform (QUANTA Lite ELISA®) with lower OR for IgM triple-positivity compared with the OR for triple-positivity irrespective of the isotype.

Our results clearly illustrate the wide variation in thrombotic association introduced by aPL detection methods. In LAC positives, “isolated” IgM or “isolated” IgG aPL was less correlated with thrombosis than triple-positivity irrespective of the isotype. Interestingly, positivity for LAC, aCL and a β 2GPI antibodies for both isotypes resulted in the strongest correlation with thrombosis. Therefore, both IgG and IgM antibodies are of added value in stratification of risk of thrombosis in APS. However, IgM did not add any value in thrombotic association to LAC positives in the absence of IgG aPL.

In conclusion, detection of triple-positivity varied among commercially available solid phase assays detecting aCL and a β 2GPI antibodies. However, triple-positivity (irrespective of the isotype) was statistically correlated with thrombosis, independent of the solid phase assay used. Except for one platform, concordance of isotype resulted in the highest OR. Detection of IgM antibodies in triple-positivity was only of added clinical value in combination with LAC, aCL IgG and a β 2GPI IgG positivity. These data confirm the high association of triple-positivity with thrombosis and show that the isotype and solid phase assay used to detect aPL affect the association with thrombosis. As triple-positive APS patients have an increased risk of thrombotic recurrence, standardization in triple-positivity detection is urgently warranted.

ADDENDUM

K. M. J. Devreese, B. de Laat and H. Kelchtermans designed the study. K. M. J. Devreese organized the sample collection at the different centers. K. M. J. Devreese, D. Wahl, G. W. Moore and J. Musiał collected samples and identified sample characteristics. Samples were analyzed under supervision of K. M. J. Devreese. W. Chayoua, K. M. J. Devreese, B. de Laat and H. Kelchtermans interpreted data, performed statistical analyses and wrote the manuscript. D. Wahl, G. W. Moore and J. Musiał critically reviewed the manuscript.

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DISCLOSURE OF CONFLICT OF INTERESTS

G. W. Moore reports personal fees from DSM Pentapharm, outside the submitted work, and has been a member of the Coagulation Advisory Board for Roche Diagnostics. The other authors state that they have no conflict of interest.

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SUPPLEMENTAL DATA

SUPPLEMENTAL TABLE 1. Technical specifications of the solid phase assays. NS, Not specified.

Manufacturer	Platform	Method	Solid phase	aCL antigen source		aβ2GPI antigen source		Cut-off value
				Cardiolipin	β2GPI	β2GPI	β2GPI	
Bio-Rad Laboratories	BioPlex®2200	Fluorescence	Beads	Synthetic	Human	Human	Human	20
Phadia/ ThermoFisher	ImmunoCap® EliA	Fluorescence enzyme linked	Wells in carriers	Bovine	Bovine	Human	Human	10
Instrumentation Laboratories/ Werfen	ACL AcuStar®	Chemilumine-scence	Beads	Bovine	Human	Human	Human	20
Inova Diagnostics	QUANTA Lite ELISA®	Chromogenic enzyme linked	Poly-strene wells	NS	Bovine	NS	NS	20

SUPPLEMENTAL TABLE 2. Distribution of aCL and aβ2GPI antibodies in lupus anticoagulant (LAC) positive patients with thrombotic anti-phospholipid syndrome (APS).

	BioPlex®2200		ImmunoCap® EliA		ACL AcuStar®		QUANTA Lite ELISA®	
	LAC	202	202	202	202	202	202	202
Triple positive	118	118	101	111	111	89	89	89
Isolated LAC	82	82	79	76	76	89	89	89
LAC with aCL or aβ2GPI	2	2	22	15	15	24	24	24

4

The (non-)sense of detecting anti-cardiolipin and anti- β 2glycoprotein I IgM antibodies in the antiphospholipid syndrome

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ABSTRACT

Background: The antiphospholipid syndrome (APS) is characterized by thrombosis and/or pregnancy morbidity with the persistent presence of lupus anticoagulant (LAC), anti-cardiolipin (aCL) and/or anti- β 2glycoprotein I (a β 2GPI) antibodies of the immunoglobulin G/ immunoglobulin M (IgG/IgM) isotype. However, the role of aCL and a β 2GPI IgM as a serologic marker in APS is debated.

Objectives: We aimed to assess the diagnostic and clinical value of IgM antiphospholipid antibodies (aPL) in APS within the classification criteria.

Patients/Methods: Our multicenter study comprised of 1008 patients, including APS patients and controls. Anti-CL and a β 2GPI IgG and IgM antibodies were detected with four commercially available solid phase assays.

Results: Positivity for aCL and/or a β 2GPI antibodies was significantly correlated with thrombosis and pregnancy morbidity, independent of the isotype and solid phase assay. Higher odds ratios were obtained for IgG compared to IgM positivity. Isolated IgM was rare in thrombotic APS, but more frequent in obstetric APS, ranging from 3.5% to 5.4% and 5.7% to 12.3%, respectively, dependent on the solid phase assay. In a multivariate logistic regression analysis of aPL, IgM positivity was found to be associated with pregnancy morbidity. However, detection of IgM was not independently associated with thrombosis. Combined positivity for LAC, IgG and IgM was highly associated with thrombosis and pregnancy morbidity.

Conclusions: Our data support testing for aCL and a β 2GPI IgM in women suspected of obstetric APS. However, no added value was found for testing IgM in patients suspected of thrombotic APS. Still, IgM aPL might be useful as a second-line test to improve thrombotic risk stratification.

INTRODUCTION

The antiphospholipid syndrome (APS) is characterized by thrombosis and/or pregnancy morbidity with the persistent presence of antiphospholipid antibodies (aPL) [1]. Obstetrical complications include fetal loss after the 10th week of gestation, recurrent early miscarriages and premature birth due to severe preeclampsia or intrauterine growth restriction [1]. Thrombotic APS is characterized by venous, arterial and/or small vessel thrombosis [1]. Diagnosis of APS predominantly relies on aPL assays detecting lupus anticoagulant (LAC) by a functional assay or detection of anti-cardiolipin (aCL) immunoglobulin G/ immunoglobulin M (IgG/IgM) antibodies and anti- β 2glycoprotein I (a β 2GPI) IgG/IgM antibodies by solid phase assays [1–3]. Within the current criteria, persistent presence of either IgG or IgM aPL with associated thrombosis and/or pregnancy morbidity is sufficient for the classification of APS [1]. However, the value of IgM aCL and a β 2GPI antibodies in APS classification is debated [4–10].

Recently a meta-analysis aimed to assess the clinical value of IgM aCL and a β 2GPI antibodies in thrombotic APS and found a stronger thrombotic correlation for IgG than for IgM antibodies [4]. Studies that found a statistical association for aCL and a β 2GPI IgM antibodies with thrombosis mostly also found a significant association for the IgG isotype, suggesting a limited role of IgM in thrombotic APS [4]. In the meta-analysis, two studies were included that found a protective role of IgM antibodies in thrombosis [4,11,12]. In 2006, another meta-analysis assessed the association between aCL antibodies and recurrent fetal loss in women without an autoimmune disease and found a statistical association for both isotypes (IgM and IgG) [6]. However, in both meta-analyses authors were unable to evaluate IgM antibodies as a single serologic marker, because of unavailability of separate IgG and IgM results [4,6]. In addition, comparison of clinical studies is difficult as multiple study designs are used with a wide variety of aPL assays. Solid phase assays are poorly standardized and external quality control programs have shown that aPL assays produce variable results [13,14].

More evidence has become available that hints towards evaluation of the complete aPL profile to improve risk stratification of APS patients [2,15–17]. In an observational study among purely obstetric APS patients, aCL and a β 2GPI positivity together with LAC (triple positivity) was a predictor of pulmonary embolism while single LAC positivity was not [18]. Another study found that the presence of any aPL at any time did not result in a significant increase in the risk of thrombotic recurrence, while the persistent presence of the same type increased the risk of thrombotic recurrence significantly [15]. Multiple aPL positivity at the same time or at different time points even further increased the risk of recurrence [15]. We have previously shown that the thrombotic association of IgM

antibodies within triple positive (positivity for LAC, aCL and a β 2GPI) patients is limited [19]. Here, we aimed to assess the value of IgM antibodies as a single serologic marker in APS and its clinical value in aPL profiles within the current classification criteria. Four commercially available solid phase assays were used, reducing assay dependency on the association of IgM antibodies and thrombosis or obstetric complications.

METHODS

Study population

Patient samples were collected from eight medical European centers within a timespan of one year, with patient samples stored less than five years at or below -80°C. The Sydney classification criteria were followed for the diagnosis of thrombotic and obstetric APS [1]. Thrombosis was defined as one or more clinical episodes of arterial, venous, or small vessel thrombosis, in any tissue or organ, confirmed by objective validated criteria (e.g. unequivocal findings of appropriate imaging studies or histopathology) [1]. Pregnancy morbidity was defined as: (1) one or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation, with normal fetal morphology documented by ultrasound or by direct examination of the fetus; (2) one or more premature births of a morphologically normal neonate before the 34th week of gestation because of: eclampsia or severe pre-eclampsia defined according to standard definitions, recognized features of placental insufficiency; (3) three or more unexplained consecutive spontaneous abortions before the 10th week of gestation, with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded [1]. Patients were classified as thrombotic or obstetric APS by the local center. Control populations consisted of patients with an autoimmune disease other than APS (e.g. systemic lupus erythematosus and systemic sclerosis) without thrombotic complications (autoimmune disease [AID] controls); patients that were referred for aPL testing for other reasons than the clinical criteria of APS (e.g. subfertility and prolonged activated partial thromboplastin time [aPTT], controls); patients with a previous thrombotic event negatively tested for aPL (non-APS thrombosis); and patients that experienced obstetric complications in the absence of aPL (non-APS obstetric). The study was approved by the local ethical committees.

LABORATORY ASSAYS

Lupus anticoagulant

Lupus anticoagulant assays were performed by the local center, according to the International Society of Thrombosis and Haemostasis-Scientific Standardisation Subcommittee (ISTH-SSC) guideline [20]. Briefly, citrated blood was double centrifugated for 15 minutes at 2000 g (room temperature) [20]. After transferring the plasma in a non-activating plastic centrifuge tube, the plasma was centrifugated for an additional 10 minutes at > 2500 g (room temperature) [20]. The obtained platelet poor plasma was tested for a prolonged clotting time with two tests based on different principles (e.g. aPTT and dilute Russell viper venom time). Lupus anticoagulant testing was performed by a three-step procedure including screening, mixing, and confirmation.

Solid phase assays

Commercially available solid phase assays were selected based on frequently used assays in the external quality control program of the ECAT (External quality Control of diagnostic Assays and Tests, Voorschoten, The Netherlands) and the willingness of manufacturers for collaboration. Anti-CL IgG, aCL IgM, a β 2GPI IgG and a β 2GPI IgM aPL were detected by four solid phase assays: BioPlex®2200 (Bio-Rad, Bio-Rad Laboratories, Hercules, USA), Phadia® (Thermo Fisher Scientific/Phadia, Uppsala, Sweden), ACL AcuStar® (Instrumentation Laboratories, Bedford, USA) and QUANTA Lite® ELISA (Inova Diagnostics, San Diego, USA) in the Ghent University Hospital (Ghent, Belgium). Detection of aCL and a β 2GPI antibodies was performed according manufacturer's instructions. Manufacturer's recommended cut-off values were used upon confirmation in 20 healthy volunteers, in accordance with the ISTH-SSC guideline [21].

Statistical analyses

Crude odds ratios for thrombosis were calculated within patients with thrombotic APS, non-APS thrombosis, AID controls and controls. Crude odds ratios for pregnancy morbidity were calculated within women with obstetric APS, non-APS obstetric APS, normal pregnancy, AID controls and controls. Differences in IgM titers above the threshold were assessed with the Mann-Whitney U test. In a multivariate logistic regression analysis, a backward variable selection was performed with variables having a *P*-value < 0.2. In order to evaluate independent variables for thrombosis, analysis was performed on the total population with a model including age, sex, pregnancy morbidity, LAC, IgG and IgM. Within women, age, thrombosis, LAC, IgG and IgM were included in the multivariate model to evaluate independent variables for pregnancy

morbidity. Odds ratios with their respective 95% confidence interval was determined for each variable in the final model. A P -value < 0.05 was regarded as statistically significant. Statistical analyses were performed using the statistical package for social sciences (SPSS 23.0; SPSS, New York, USA) and MedCalc Statistical Software version 17.7.2 (MedCalc Software bvba, Ostend, Belgium).

RESULTS

Patient characteristics

We included 1008 patients of whom 75.7% were female with a mean age of 44 years (Table 1). Venous thrombosis was more prevalent compared to arterial thrombosis in thrombotic APS and non-APS thrombosis patients, 62% and 73% respectively (Table 1). The majority of thrombotic APS patients received anticoagulant therapy including vitamin K antagonists (46%), low molecular weight heparins (5%) and direct oral anticoagulants (4%). Twenty-seven patients (10%) received antiplatelet therapy and 2% of patients with thrombotic APS received both anticoagulant and antiplatelet therapy. Details on anticoagulant and anti-platelet therapy of the remaining 84 patients were not available.

Diagnostic role of aCL and a β 2GPI IgG/IgM antibodies in thrombosis

Lupus anticoagulant positivity resulted in an odds ratio (OR) for thrombosis of 3.4 (95% confidence interval [95% CI], 2.5-4.7) (Table 2). Positivity for aCL or a β 2GPI antibodies was significantly associated with thrombosis, independent of the isotype or solid phase assay used (Table 2). Positivity for IgG aCL or a β 2GPI antibodies was more strongly correlated with thrombosis than IgM aCL or a β 2GPI antibodies (Table 2). Lupus anticoagulant, IgG aCL or a β 2GPI and IgM aCL or a β 2GPI were analyzed in a multivariate model in which IgM aCL or a β 2GPI antibodies resulted not to be an independent variable for thrombosis, independent of the solid phase assay used. In addition, we performed a subanalysis separately on patients with venous or arterial thrombosis in which IgM aCL or a β 2GPI antibodies were not an independent variable for both types of thrombosis (data not shown for the separate subpopulations). Within the multivariate model, lupus anticoagulant and IgG aCL or a β 2GPI antibodies were independent variables with OR between 2.3 (95%CI, 1.6-3.3) and 2.4 (95%CI, 1.7-3.4) and 2.3 (95%CI, 1.6-3.5) and 3.2 (95%CI, 2.0-5.0), respectively (Table 2).

TABLE 1. Demographic and clinical characteristics of the study population

	Patients (n)	Female	Age [year,mean (range)]	Pregnancy morbidity	Thrombosis			
					Venous	Arterial	Venous and arterial	Small vessel
APS thrombosis	259	164 (63%)	50 (17-87)	22	160	55	26	4
Non-APS thrombosis	204	116 (57%)	46 (19-85)	0	149	47	5	0
APS obstetric	122	122 (100%)	35 (19-61)	122	4	2	0	0
Non-APS obstetric	33	33 (100%)	33 (20-52)	33	NA	NA	NA	NA
Autoimmune diseases	196	158 (81%)	46 (16-83)	NA	NA	NA	NA	NA
Controls	194	170 (88%)	39 (18-82)	NA	NA	NA	NA	NA
Total population	1008	763 (75.7%)	44 (16-87)	177	313	104	31	4

Abbreviation: APS, antiphospholipid syndrome; NA, not applicable

Diagnostic role of aCL and a β 2GPI IgG/IgM antibodies in pregnancy morbidity

Similar to thrombosis, positivity for aCL or a β 2GPI antibodies was significantly associated with pregnancy morbidity, independent of the isotype or solid phase assay used (Table 3). Positivity for IgG aCL or a β 2GPI antibodies clearly resulted in higher crude OR for obstetric complications than positivity for IgM aCL or a β 2GPI antibodies (Table 3). In contrast to thrombosis, IgM aCL or a β 2GPI antibodies was an independent variable for pregnancy morbidity with OR ranging between 1.7 (95% CI, 1.1-2.8) and 2.0 (95% CI, 1.1-3.5). The OR of LAC positivity varied between 3.6 (95% CI, 2.3-5.7) and 3.9 (95% CI, 2.5-6.1) (Table 3). IgG aCL or a β 2GPI antibodies was an independent variable with three out of the four tested solid phase assays, resulting in OR between 1.9 (95% CI, 1.1-3.2) and 2.5 (95% CI, 1.5-4.4).

Additional diagnostic value of aCL and a β 2GPI IgM antibodies in thrombotic patients

Positivity for IgM aCL or a β 2GPI antibodies ranged from 26% to 36% among thrombotic APS patients, 0% to 3.4% among non-APS thrombosis patients and 6.4% to 16% among patients with an autoimmune disease and controls (Table 4). Thrombotic APS patients with isolated IgM aCL or a β 2GPI positivity ranged from 3.5% to 5.4% (Table 4). In addition, up to 2.5% patients classified as non-APS thrombosis were found to be isolated positive for IgM aCL or a β 2GPI antibodies (Table 4). The prevalence of isolated IgM aCL or a β 2GPI antibodies in controls and AID patients was 1.8% to 10% as detected by BioPlex®2200 and Phadia®, respectively (Table 4).

TABLE 2. Correlation of lupus anticoagulant, IgG and IgM aCL, or a β 2GPI antibodies with thrombosis

	BioPlex®2200		Phadia®		AcuStar®		QUANTA Lite® ELISA	
	Crude OR	Adjusted OR*	Crude OR	Adjusted OR	Crude OR	Adjusted OR	Crude OR	Adjusted OR
Lupus anticoagulant	3.4 (2.5-4.7)	2.4 (1.7-3.4)	3.4 (2.5-4.7)	2.4 (1.7-3.4)	3.4 (2.5-4.7)	2.3 (1.6-3.3)	3.4 (2.5-4.7)	2.4 (1.7-3.4)
IgG antibodies	2.7 (1.9-3.9)	2.3 (1.6-3.5)	3.7 (2.5-5.5)	2.9 (1.9-4.4)	2.7 (1.9-3.8)	2.4 (1.6-3.3)	4.7 (2.9-7.5)	3.2 (2.0-5.0)
IgM antibodies	2.5 (1.5-4.0)	NS	1.5 (1.1-2.1)	NS	1.7 (1.1-2.5)	NS	1.8 (1.2-2.7)	NS

Odds ratios (ORs) are shown with their respective 95% confidence interval. IgG antibodies, aCL, or a β 2GPI IgG antibodies; IgM antibodies, aCL, or a β 2GPI IgM antibodies; NS, nonsignificant

Multivariate analysis, adjusted for age, sex, and pregnancy morbidity.

TABLE 3. Correlation of lupus anticoagulant, IgG and IgM aCL or a β 2GPI antibodies with pregnancy morbidity.

	BioPlex®2200		Phadia®		AcuStar®		QUANTA Lite® ELISA	
	Crude OR	Adjusted OR	Crude OR	Adjusted OR	Crude OR	Adjusted OR	Crude OR	Adjusted OR
Lupus anticoagulant	4.2 (2.8-6.4)	3.6 (2.3-5.7)	4.2 (2.8-6.4)	3.7 (2.4-5.7)	4.2 (2.8-6.4)	3.9 (2.5-6.1)	4.2 (2.8-6.4)	3.6 (2.4-5.6)
IgG antibodies	3.2 (2.0-5.0)	1.9 (1.1-3.2)	4.1 (2.5-6.8)	2.2 (1.3-3.6)	2.8 (1.8-4.4)	1.5 (0.9-2.5)	5.5 (3.1-9.9)	2.5 (1.5-4.4)
IgM antibodies	2.9 (1.6-5.4)	2.0 (1.1-3.5)	2.2 (1.4-3.5)	1.7 (1.1-2.8)	2.1 (1.3-3.5)	2.0 (1.2-3.4)	2.8 (1.7-4.6)	2.0 (1.2-3.3)

Odds ratios (ORs) are shown with their respective 95% confidence interval. IgG antibodies, aCL, or a β 2GPI IgG antibodies; IgM antibodies, aCL, or a β 2GPI IgM antibodies; NS, nonsignificant

Multivariate analysis, adjusted for age and thrombosis.

FIGURE 1. Titers of patients with thrombosis or pregnancy morbidity positive for IgM antibodies versus controls (autoimmune disease and controls) positive for IgM antibodies. Log transformed IgM titers of patients with (A) thrombosis or (B) pregnancy morbidity are indicated in red and patients without the clinical criteria for APS are indicated in black. Titers are expressed as the median value of positive IgM titers with their 95% confidence interval. * P -value < .05. NS, not significant; AU, arbitrary units

TABLE 4. Additional diagnostic value of aCL and a β 2GPI IgM antibodies

A		Overall population (n=1008)		Non-APS thrombosis (n=204)		APS-thrombosis (n=259)		Non-APS obstetric (n=33)		APS-obstetric (n=122)		AID (n=196)		Controls (n=194)	
		LAC/IgG		LAC/IgG		LAC/IgG		LAC/IgG		LAC/IgG		LAC/IgG		LAC/IgG	
		+	-	+	-	+	-	+	-	+	-	+	-	+	-
IgM	+	95	23	0	0	58	9	0	0	19	7	12	6	6	1
	-	304	586	3	201	157	35	0	33	72	24	56	122	16	171
Total		399	609	3	201	215	44	0	33	91	31	68	128	22	172

B		Overall population (n=1008)		Non-APS thrombosis (n=204)		APS-thrombosis (n=259)		Non-APS obstetric (n=33)		APS-obstetric (n=122)		AID (n=196)		Controls (n=194)	
		LAC/IgG		LAC/IgG		LAC/IgG		LAC/IgG		LAC/IgG		LAC/IgG		LAC/IgG	
		+	-	+	-	+	-	+	-	+	-	+	-	+	-
IgM	+	135	72	2	5	80	14	1	2	30	12	15	25	7	14
	-	273	528	10	187	136	29	1	29	62	18	51	105	13	160
Total		408	600	12	192	216	43	2	31	92	30	66	130	20	174

C		Overall population (n=1008)		Non-APS thrombosis (n=204)		APS-thrombosis (n=259)		Non-APS obstetric (n=33)		APS-obstetric (n=122)		AID (n=196)		Controls (n=194)	
		LAC/IgG		LAC/IgG		LAC/IgG		LAC/IgG		LAC/IgG		LAC/IgG		LAC/IgG	
		+	-	+	-	+	-	+	-	+	-	+	-	+	-
IgM	+	123	37	0	1	71	10	0	1	24	9	21	10	7	6
	-	294	554	7	196	153	25	0	32	70	19	50	115	14	167
Total		417	591	7	197	224	35	0	33	94	28	71	125	21	173

D		Overall population (n=1008)		Non-APS thrombosis (n=204)		APS-thrombosis (n=259)		Non-APS obstetric (n=33)		APS-obstetric (n=122)		AID (n=196)		Controls (n=194)	
		LAC/IgG		LAC/IgG		LAC/IgG		LAC/IgG		LAC/IgG		LAC/IgG		LAC/IgG	
		+	-	+	-	+	-	+	-	+	-	+	-	+	-
IgM	+	113	56	0	1	70	14	0	2	24	15	15	19	4	5
	-	276	563	5	198	143	32	1	30	66	17	47	115	14	171
Total		389	619	5	199	213	46	1	32	90	32	62	134	18	176

Note: figures indicate the number of patients testing positive or negative for IgM antibodies or LAC/IgG.

A BioPlex®2200 **B**, Phadia® **C**, ACL AcuStar® and **D**, QUANTA Lite® ELISA. AID, autoimmune disease; APS antiphospholipid syndrome; IgG antibodies, aCL, or a β 2GPI IgG antibodies; IgM antibodies, aCL, or a β 2GPI IgM antibodies; LAC, lupus anticoagulant.

Additional diagnostic value of aCL and a β 2GPI I IgM antibodies in patients with pregnancy morbidity

Positivity for IgM aCL or a β 2GPI antibodies ranged from 21% to 34% among obstetric APS patients, 0% to 9.1% among non-APS obstetric patients (Table 2), and 6.4% to 16% among female patients with an autoimmune disease and controls. Isolated IgM aCL or a β 2GPI positivity ranged from 5.7% to 12.3% in APS-obstetric patients (Table 2). Up to 6% of the patients classified as non-APS obstetric were found to be isolated positive for IgM aCL or a β 2GPI and up to 10% of female patients with an autoimmune disease and controls were only positive for IgM aCL or a β 2GPI antibodies but not LAC or IgG aCL or a β 2GPI antibodies.

IgM antibody titers in thrombotic patients and controls

Positive IgM aCL or aβ2GPI antibody titers were compared between patients with thrombosis and controls (AID and control) within one solid phase assay. No significant difference in IgM aCL or aβ2GPI antibody titers was found between patients with thrombosis and our control population with the exception of one solid phase assay (Phadia®) (Figure 1A).

IgM antibody titers in obstetric patients and controls

IgM aCL or aβ2GPI antibody titers were not significantly different between patients with pregnancy morbidity and the control population with all solid phase assays tested (Figure 1B). Of note, maximum IgM aCL or aβ2GPI antibody titers measured were higher in thrombotic patients versus obstetric patients: 3708 versus 995 (BioPlex®2200), 1056 versus 410 (Phadia®), 2798 versus 785 (ACL AcuStar®) and 320 versus 250 (QUANTA Lite® ELISA) of the respective units.

Additional clinical value of aCL and aβ2GPI I IgM antibodies in thrombotic patients

Within the current criteria positivity for either LAC, IgG aCL/aβ2GPI or IgM aCL/aβ2GPI is sufficient for classification of APS. In our cohort, these classification criteria resulted in OR between 2.4 (95% CI, 1.8-3.2) and 2.9 (95% CI, 2.2-3.9) (Table 5). Combined positivity for LAC and IgG aCL or aβ2GPI antibodies resulted in higher OR than combined positivity for LAC and IgM aCL or aβ2GPI antibodies with two out of the four tested solid phase assays. With the other solid phase assays, combined positivity for LAC and IgM aCL or aβ2GPI reached higher OR than positivity for LAC and IgG aCL or aβ2GPI. The variation in OR for thrombosis among solid phase assays increased in combined positivity for IgG aCL or aβ2GPI and IgM aCL or aβ2GPI antibodies from 2.6 (95% CI, 1.6-4.5) up to 7.1 (95% CI, 3.0-16.7) (Table 5). The combination of positive LAC, IgG aCL or aβ2GPI and IgM aCL or aβ2GPI results is highly associated with thrombosis with OR between 5.5 (95% CI, 2.6-11.8) and 9.6 (95% CI, 3.4-27.1) (Table 5).

TABLE 5. Additional value of aCL and a β 2GPI IgM antibodies in thrombosis

	BioPlex®2200	Phadia®	AcuStar®	QUANTA Lite® ELISA
LAC and/or IgG and/or IgM	2.9 (2.2-3.9) n=324	2.4 (1.8-3.2) n=372	2.9 (2.1-3.8) n=350	2.8 (2.1-3.7) n=337
LAC and IgG	3.8 (2.5-5.9) n=138	4.7 (2.8-7.6) n=118	3.3 (2.2-4.9) n=148	5.9 (3.4-10.5) n=104
LAC and IgM	4.5 (2.3-8.7) n=64	3.5 (2.1-5.8) n=93	3.5 (2.0-6.3) n=77	3.6 (2.1-6.3) n=83
IgG and IgM	3.3 (1.7-6.2) n=60	5.8 (2.8-11.9) n=65	2.6 (1.6-4.5) n=78	7.1 (3.0-16.7) n=52
LAC and IgG and IgM	6.4 (2.7-15.2) n=48	6.2 (2.8-13.8) n=54	5.5 (2.6-11.8) n=56	9.6 (3.4-27.1) n=46

Note: Odds ratios (ORs) were calculated in a subpopulation of 853 patients consisting of thrombotic APS (n=259), non-APS thrombosis (n=204), AID controls (n=196) and controls (n=194). ORs are shown with their respective 95% confidence interval. IgG antibodies, aCL, and/or a β 2GPI IgG antibodies; IgM antibodies, aCL and/or a β 2GPI IgM antibodies; LAC, lupus anticoagulant

TABLE 6. Additional value of aCL and a β 2GPI IgM antibodies in pregnancy morbidity

	BioPlex®2200	Phadia®	AcuStar®	QUANTA Lite® ELISA
LAC and/or IgG and/or IgM	5.1 (3.4-7.7) n=181	4.9 (3.2-7.4) n=213	5.3 (3.5-8.0) n=195	6.6 (4.3-10.0) n=193
LAC and IgG	3.3 (1.9-5.6) n=62	3.7 (2.0-6.8) n=50	2.5 (1.5-4.2) n=67	4.3 (2.2-8.6) n=39
LAC and IgM	3.5 (1.5-8.3) n=23	3.4 (1.7-6.4) n=41	2.7 (1.3-5.6) n=33	2.7 (1.4-5.5) n=35
IgG and IgM	3.8 (1.7-8.2) n=29	6.5 (2.9-14.3) n=33	2.7 (1.4-5.2) n=39	8.0 (3.1-20.2) n=26
LAC and IgG and IgM	5.9 (2.1-16.9) n=18	5.6 (2.3-13.9) n=24	4.9 (2.0-12.3) n=22	8.7 (2.8-26.6) n=19

Note: Odds ratios (ORs) were calculated in a subpopulation of 483 patients consisting of, obstetric APS (n=122), non-APS obstetric (n=33), female AID controls (n=158) and female controls (n=170). ORs are shown with their respective 95% confidence interval. IgG antibodies, aCL, and/or a β 2GPI IgG antibodies; IgM antibodies, aCL and/or a β 2GPI IgM antibodies; LAC, lupus anticoagulant

Additional clinical value of aCL and a β 2GPI IgM antibodies in patients with pregnancy morbidity

Positivity for either LAC, IgG and/or IgM antibodies resulted in OR between 4.9 (95% CI, 3.2-7.4) and 6.6 (95%CI, 4.3-10.0) (Table 6). In contrast to thrombosis, combined positivity for LAC and IgG aCL or a β 2GPI or LAC and IgM aCL or a β 2GPI antibodies did not increase the association with pregnancy morbidity compared to the classification criteria (Table 6). Positivity for LAC and IgG aCL or a β 2GPI antibodies resulted in OR between 2.5 (95% CI, 1.5-4.2) and 4.3 (95% CI, 2.2-8.6) (Table 6). Positivity for LAC and IgM aCL or a β 2GPI antibodies resulted in OR between 2.7 (95% CI, 1.3-5.6) and 3.5 (95% CI, 1.5-8.3) (Table 6). The association between pregnancy morbidity and LAC, IgG and/or IgM aCL or a β 2GPI positivity were similar (Table 6). However, positivity for IgG aCL or a β 2GPI and IgM aCL or a β 2GPI antibodies resulted in a large variation of OR for pregnancy morbidity among solid phase assays. Combined positivity for IgG aCL or a β 2GPI and IgM aCL or a β 2GPI antibodies resulted in OR between 2.7 (95% CI, 1.4-5.2) and 8.0 (95% CI, 3.1-20.2). IgM aCL or a β 2GPI positivity in combination with LAC and IgG aCL or a β 2GPI positivity is highly associated with pregnancy morbidity with OR between 4.9 (95% CI, 2.0-12.3) and 8.7 (95% CI, 2.8-26.6).

DISCUSSION

The role of IgM aCL or a β 2GPI antibodies as a serologic marker in APS is debated [1,4,7,8,22]. Although most studies were unable to demonstrate an association between IgM aCL or a β 2GPI antibodies and thrombosis, a questionnaire by the SSC of the ISTH indicated that most of the respondents found it too early to eliminate IgM aCL or a β 2GPI antibodies from the criteria [8]. We demonstrated a significant correlation for both IgG and IgM aCL and a β 2GPI antibodies with thrombosis as well as pregnancy morbidity. However, IgM positivity was mostly associated with positivity for LAC and/or IgG aPL. The presence of isolated IgM antibodies in thrombotic APS patients was rare (3.5%-5.4%, Table 4), and not an independent variable for thrombosis. In addition, IgM aCL or a β 2GPI was not found to be an independent variable for arterial nor venous thrombosis. These results are in line with prospectively collected data from patients referred to Italian thrombosis centers in which only a minority of APS patients was found to be positive for predominantly aCL or a β 2GPI IgM antibodies (>40 MPL units and < 40 GPL units) [16]. A thrombotic association of true isolated IgM has not been reported in literature. One study concluded that "isolated" IgG antibodies are more prevalent in deep venous thrombosis and pulmonary embolism while "isolated" aCL IgM antibodies were frequently found in patients with a cerebrovascular infarction [23]. However, patients were only tested for aCL and a β 2GPI IgG/IgM antibodies and not for

LAC, rendering the identification of true isolated IgG or IgM antibodies impossible [23]. While we showed that IgM was not an independent variable of thrombosis along with LAC and IgG, another study even found that in SLE patients $\alpha\beta 2$ GPI IgM antibodies were protective against lupus nephritis and renal damage [24].

Within the current aPL-panel, LAC is considered the strongest predictor of thrombosis and pregnancy morbidity [25,26]. In 2003, a meta-analysis assessed the role of LAC and aCL antibodies in thrombosis. Computed ORs for both LAC and aCL positivity were only available in five studies of which none was able to illustrate a significant association between thrombosis and IgG or IgM aCL antibodies [26]. We found an association between LAC as well as aCL antibodies and thrombosis, independent of the isotype and solid phase assay used. As argued by de Groot et al most of the studies included in the meta-analysis did not follow the ISTH-SSC guidelines, in which it is stated that aCL antibodies should bind to cardiolipin in a $\beta 2$ GPI-dependent manner [1,3,27–29]. A more recent meta-analysis included studies between 2001 and 2014 and showed that aCL antibodies are associated with thrombosis, especially aCL IgG antibodies [4]. In our cohort, LAC was an independent variable for thrombosis, illustrating its importance in thrombotic APS. In addition, LAC showed the highest association with pregnancy morbidity within the current aPL panel.

Thrombotic and obstetric APS are hypothesized to have a distinct aPL profile with more frequent IgM positivity in obstetrical APS [30]. Our results suggest comparable IgM positivity in obstetrical APS to thrombotic APS, 21%-34% and 26%-36%, respectively (dependent on the solid phase assay). However, LAC, aCL, and $\alpha\beta 2$ GPI IgG/IgM antibodies were found to be independently associated with pregnancy morbidity. In contrast, IgM was not independently associated with thrombosis. In agreement, a systematic review and meta-analysis found a significant association of LAC, aCL IgG/IgM, and $\alpha\beta 2$ GPI IgG/IgM antibodies with pre-eclampsia and fetal death [31]. Another study found that positivity for LAC, aCL IgG, and aCL IgM were associated with recurrent fetal loss in women without autoimmune disease [6]. In the same study, no significant association was found between $\alpha\beta 2$ GPI positivity and recurrent loss, presumably due to the small study population [6]. In a population-based, case-control study, aPL were measured in 582 stillbirth deliveries and 1547 live birth deliveries [32]. Women with a stillbirth were found to be more likely to have elevated aCL and $\alpha\beta 2$ GPI IgG levels, but not aCL and $\alpha\beta 2$ GPI IgM levels compared to those with term live births [32]. We found a significant association between aCL IgM or $\alpha\beta 2$ GPI IgM antibodies and pregnancy morbidity, but were not able to differentiate in subgroups due to unavailability of detailed description of obstetric complications. The PROMISSE (Predictors of pRegnancy Outcome: bioMarkers In antiphospholipid antibody Syndrome and

Systemic lupus Erythematosus) study has shown that LAC is the primary predictor of obstetric complications after 12 weeks of gestation in aPL associated pregnancies [25]. Positivity for aCL and a β 2GPI antibodies did not predict adverse pregnancy outcomes if LAC was absent [25]. In contrast, we found that positivity for aCL and a β 2GPI IgG and IgM were independently associated with a history of pregnancy morbidity. However, LAC showed the highest association with pregnancy morbidity within the current aPL-panel. In addition, the presence of isolated IgM antibodies in obstetric APS patients was rare (5.7%-12.3%, Table 4), dependent on the solid phase assay.

Some studies found a strong correlation of high aPL titers with clinical manifestations of APS [9,33–35]. However, previous reports on the association between aPL titers and pregnancy morbidity are conflicting [32,36,37]. In a retrospective study a higher cut-off value did not result in a stronger association with stillbirth [32]. Another study assessed whether there is a relationship between low aPL levels and obstetrical complications [36]. The authors found that patients with low aPL titers have similar obstetrical outcomes compared to confirmed APS patients [36]. In contrast, the risk of adverse fetal/neonatal outcome was found to be higher in APS-obstetrical patients with high aPL titers (≥ 4 times upper limit of normal) compared to APS-obstetrical patients with aPL titers < 4 times the upper limit of normal [37]. Our results showed that IgM titers were not significantly different between obstetrical patients and our control population. Of note, the maximum IgM titer measured in obstetric patients was lower than the maximum titers measured in thrombotic patients. In addition, patients with a history of thrombosis did not have significant differently IgM titers than patients without thrombosis. Future studies are needed to investigate the relationship between aPL titers and clinical manifestation of APS in more detail. Categorizing aPL titers into low, medium and high positivity might help to study the correlation between aPL titers and thrombosis or pregnancy morbidity.

In APS, multiple aPL positivity (aPL profiles) have shown to be of added value in thrombotic risk stratification [15,17,38]. In agreement we found that OR for thrombosis were highest in patients positive for LAC, IgG, and IgM. One study assessed the role of a β 2GPI and aCL antibodies in 87 LAC positive patients and found that the presence of IgG but not IgM antibodies in LAC-positives predicts an increased risk of thrombosis [39]. We found that combined positivity for LAC and IgG as well as combined positivity for LAC and IgM were significantly associated with thrombosis. However, in two out of four solid phase assays OR of combined LAC and IgG aCL or a β 2GPI was higher than positivity for LAC and IgM aCL or a β 2GPI. Within pregnancy morbidity, combined positivity for LAC and IgG or positivity for LAC and IgM did not increase the OR compared

to the current criteria aPL-panel. Combined positivity for LAC, IgG, and IgM was more associated with pregnancy morbidity than the current aPL-panel with three out of the four tested solid phase assays.

Diagnosis of APS is dependent on laboratory tests detecting aPL antibodies [10,14,40]. Currently no gold standard exists for the detection of aPL antibodies. Solid phase assays are poorly standardized and many studies have shown that different results are obtained by measuring the same sample [10,14]. In addition, studies have shown that solid phase assays are associated with a large inter-laboratory variation [10,41]. In order to be independent of the solid phase assay used and minimize the interlaboratory and interindividual variation in aPL detection, a single operator performed all assays within one laboratory. Although we demonstrated a largely similar clinical performance for all platforms, these data indicate that results for single parameters may vary between platforms. One of the four tested solid phase assay was unable to demonstrate a significant association between IgG aCL or $\alpha\beta 2\text{GPI}$ antibodies and pregnancy morbidity within a multivariate model, while IgG aCL or $\alpha\beta 2\text{GPI}$ was an independent variable within the other tested solid phase assays. In addition, combinations of IgG and IgM showed higher variation of OR among the solid phase assays. Combined positivity for LAC, IgG, and IgM aCL or $\alpha\beta 2\text{GPI}$ antibodies was highly associated with thrombosis and pregnancy morbidity. However, calculated OR varied strongly among solid phase assays, accompanied with wide 95% confidence intervals.

In conclusion, in a large multicenter study aCL and $\alpha\beta 2\text{GPI}$ IgM antibodies were significantly correlated with clinical features of APS. However, IgM is only independently associated with pregnancy morbidity and not with thrombosis. Altogether, our data support the usage of two separate decision trees for thrombosis versus pregnancy morbidity, in which aCL and $\alpha\beta 2\text{GPI}$ IgM is tested in women suspected of obstetric APS but not in patients suspected of thrombotic APS (Figure 2A-B). However, IgM aPL might be useful as a second line test to improve thrombotic risk stratification in LAC- and/or IgG-positive patients (Figure 2A).

FIGURE 2. Decision tree for the detection and risk stratification of (A) thrombotic and (B) obstetric antiphospholipid syndrome

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CONFLICTS OF INTEREST

G. W. Moore reports personal fees from DSM Pentapharm, outside the submitted work. The other authors state that they have no conflict of interest.

AUTHORSHIP CONTRIBUTIONS

K.M.J.D., B.d.L and H.K designed the study. K.M.J.D. organized the sample collection of the different centers. K.M.J.D., G.W.M., J-C.G., J.M. and D.W. collected samples and identified sample characteristics. Samples were analyzed under supervision of K.M.J.D. W.C., K.M.J.D, B.d.L and H.K interpreted data, performed statistical analyses and wrote the manuscript. G.W.M., J-C.G., J.M., D.W and P.G.d.G. critically reviewed the manuscript.

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5

Is there an additional value in detecting anticardiolipin and anti- β 2glycoprotein I IgA antibodies in the antiphospholipid syndrome?

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ABSTRACT

Background: Anticardiolipin (aCL) and anti- β 2glycoprotein I (a β 2GPI) immunoglobulin A (IgA) antiphospholipid antibodies (aPL) have shown to associate with thrombosis and pregnancy morbidity. However, inclusion of IgA aPL in the classification criteria of the antiphospholipid syndrome (APS) has been debated.

Objectives: We investigated the value of aCL and a β 2GPI IgA aPL in the detection of thrombosis and pregnancy morbidity in addition to the current aPL panel for APS.

Methods: We included 1,608 patients from eight European medical centers: 259 thrombotic APS patients, 122 obstetric APS patients, 204 non-APS thrombosis patients, 33 non-APS obstetric patients, 60 APS patients with unspecified clinical manifestations, 196 patients with autoimmune diseases and 194 controls. aCL and a β 2GPI IgG/M/A were detected with four commercial assays and lupus anticoagulant was determined by the local center.

Results: Positivity for IgA aPL was found in 17 to 26% of the patients with clinical manifestations of APS and in 6 to 13% of the control population. Both aCL and a β 2GPI IgA were significantly associated with thrombosis and pregnancy morbidity. Isolated IgA positivity was rare in patients with clinical manifestations of APS (0.3-5%) and not associated with thrombosis and/or pregnancy morbidity. Addition of IgA to the current criteria panel did not increase odds ratios for thrombosis nor pregnancy morbidity.

Conclusions: aCL and a β 2GPI IgA are associated with clinical manifestations of APS. However, isolated IgA positivity was rare and not associated with thrombosis or pregnancy morbidity. These data do not support testing for aCL and a β 2GPI IgA subsequent to conventional aPL assays in identifying patients with thrombosis or pregnancy morbidity.

INTRODUCTION

Clinical manifestations of the antiphospholipid syndrome (APS) include thrombosis and pregnancy morbidity (1). However, these manifestations occur frequently and often independent of APS. Therefore, classification of APS predominantly relies on antiphospholipid (aPL) assays detecting lupus anticoagulant (LAC) by coagulation tests or detection of anticardiolipin (aCL) and anti- β 2glycoprotein I (a β 2GPI) antibodies from the immunoglobulin G (IgG) or immunoglobulin M (IgM) isotype by solid-phase assays (1,2). aCL and a β 2GPI antibodies from the IgA isotype are not included in the current classification criteria (1,2). Multiple studies have illustrated an association of aCL and a β 2GPI immunoglobulin A (IgA) antibodies with thrombosis and pregnancy morbidity (3–12). Still, aCL and a β 2GPI IgA are not included in the current guidelines (1,2). The main reason is unavailability of data to show the clinical significance of aCL or a β 2GPI IgA with clinical manifestations of APS in addition to the current aPL panel (1,13).

In a mouse model for thrombosis, IgA antibodies isolated from APS patients resulted in increased thrombus area, faster thrombus formation and decreased time of thrombus disappearance compared with control IgA (14). Outcomes of clinical studies have been contradictory regarding the association of aCL and/or a β 2GPI IgA with clinical symptoms of APS and their role in identifying additional APS patients with thrombosis or pregnancy morbidity (3–12,15,16). Multiple studies have shown that IgA is associated with thrombosis and pregnancy morbidity (3–12), other studies report no association of aCL or a β 2GPI IgA with thrombosis or pregnancy morbidity (15,16). Isolated aCL and/or a β 2GPI IgA positivity was often found not to be associated with thrombosis or pregnancy morbidity (4,9,15–17). However, some studies concluded that aCL and/or a β 2GPI IgA is an independent risk factor for thrombosis (6,10,18). In addition to the clinical manifestations of APS, IgA positivity has also been associated with systemic lupus erythematosus (SLE), thrombocytopenia, heart valve disease, livedo reticularis and epilepsy (5,19). Comparison of clinical studies is difficult as multiple study designs are used with a wide variety of aPL assays and aPL cut-off values. Furthermore, solid-phase assays detecting aCL and a β 2GPI antibodies are poorly standardized and produce variable results in a head-to-head comparison (20–22).

In this multicenter study, we used four commercially available solid-phase assays to detect aCL and a β 2GPI IgG/M/A, to assess for an association of aCL and a β 2GPI IgA and thrombosis or obstetric complications. A total of 1,608 patients were included of which 678 patients with clinical manifestations of APS and 390 patients who served

as controls. We aimed to assess the added value of aCL and a β 2GPI IgA antibodies as a biomarker for thrombosis and pregnancy morbidity within the current aPL panel consisting of LAC, aCL IgG/M, and a β 2GPI IgG/M.

MATERIALS AND METHODS

Study population

A total of 1,608 patient samples were collected from eight medical European centers. The Sydney classification criteria were followed for the classification of thrombotic and obstetric APS (Table 1) (1). Classification of thrombotic or obstetric APS was determined by the local center. In addition to APS patients, we included patients with an AID other than APS (e.g., 54% SLE and 29% systemic sclerosis) without thrombotic complications (AID controls); patients that were referred for aPL testing for other reasons (e.g., subfertility and prolonged activated partial thromboplastin time [aPTT]) than the clinical criteria of APS (controls); patients with a previous thrombotic event and negatively tested for aPL (non-APS thrombosis); and patients who experienced obstetric complications in the absence of aPL (non-APS obstetric). The study was approved by the local ethical committees.

LABORATORY ASSAYS

Lupus anticoagulant

LAC assays were performed by the local center, according to the International Society of Thrombosis and Haemostasis-Scientific Standardization Subcommittee (ISTH-SSC) guideline (23). Briefly, citrated plasma was tested in a multiple-step procedure (screening, mixing, confirmation step) with two test systems based on different principles (LAC-sensitive aPTT and dilute Russell viper venom time) (1,23).

Solid-phase assays

Commercially available solid-phase assays were selected based on frequently used assays in the external quality control program of the ECAT (External quality Control of diagnostic Assays and Tests, Voorschoten, The Netherlands) and the willingness of manufacturers for providing the reagents. aCL and a β 2GPI antibodies from the IgG, IgM and IgA isotype were detected at one occasion by four solid-phase assays: BioPlex2200 (Bio-Rad, Bio-Rad Laboratories, Hercules, USA), Phadia (Thermo Fisher Scientific/Phadia, Uppsala, Sweden), HemosIL AcuStar (Instrumentation Laboratories, Bedford, USA) and

TABLE 1. Demographic and clinical characteristics of the study population

	Patients (n)	Female	Age [year,mean (range)]	Pregnancy morbidity	Thrombosis			
					Venous	Arterial	Venous and arterial	Small vessel
APS thrombosis	259	164 (63%)	50 (17-87)	22	160	55	26	4
Non-APS thrombosis	204	116 (57%)	46 (19-85)	0	149	47	5	0
APS obstetric	122	122 (100%)	35 (19-61)	122	4	2	0	0
Non-APS obstetric	33	33 (100%)	33 (20-52)	33	NA	NA	NA	NA
Autoimmune diseases	196	158 (81%)	46 (16-83)	NA	NA	NA	NA	NA
Controls	194	170 (88%)	39 (18-82)	NA	NA	NA	NA	NA
APS ^a	60	60 (100%)	48 (24-70)	Not available	Not available	Not available	Not available	Not available
Total population	1068	823 (77%)	44 (16-87)	177	313	104	31	4

Abbreviation: NA, not applicable.

^aThe medical center did not specify if the patient suffered from obstetric or thrombotic complications.

QUANTA Lite ELISA (Inova Diagnostics, San Diego, USA) in the Ghent University Hospital (Ghent, Belgium). Detection of aCL and a β 2GPI antibodies was performed according to manufacturer's instructions. Manufacturer's recommended cut-off values were used upon confirmation in 20 healthy volunteers, in accordance with the ISTH-SSC guideline (Supplemental Table 1) (24).

Statistical analyses

Associations of aCL and a β 2GPI IgA positivity and clinical manifestations of APS were assessed by calculating odds ratios (ORs) with their respective 95% confidence intervals (CIs). Thrombotic association with IgA aPL was assessed within patients with thrombotic APS, non-APS thrombosis, AID controls and, controls (n = 853). Obstetric association with IgA aPL was assessed within female patients with obstetric APS, non-APS obstetric APS, AID controls and controls (n = 483). The additional diagnostic value of aCL and a β 2GPI IgA antibodies was assessed by 2 x 2 contingency tables. Kruskal–Wallis test was used to evaluate differences in IgA titers. To compare numbers (percentages) of positive tests between systems, the comparison of two proportions (from independent samples) was used. Statistical analyses were performed using the statistical package for social sciences (SPSS 23.0; SPSS, Chicago, IL) and MedCalc Statistical Software version 17.7.2 (MedCalc Software bvba, Ostend, Belgium). Statistical significance was set at *P*-value less than 0.05.

RESULTS

Patient characteristics

We included 1,608 patients of which 678 patients had clinical manifestations of APS and 390 controls (Table 1). In thrombotic APS, venous thrombosis was more prevalent than arterial thrombosis (160 [62%] versus 55 [21%] patients, respectively; Table 1). From the 204 non-APS thrombotic patients, 149 (73%) had a history of venous thrombosis and 47 (23%) had a history of arterial thrombosis (Table 1). From the 259 thrombotic APS patients, the majority (40%) received Vitamin K antagonists, 5% received low-molecular-weight heparin, 4% direct oral anticoagulants, 10% antiplatelet therapy, 8% a combination of oral anticoagulants and antiplatelet therapy. Details on anticoagulant and antiplatelet therapy of the remaining 83 (32%) patients are not available. Patients classified as non-APS thrombosis and non-APS obstetric were negatively tested for criteria aPL by the local medical center. However, retesting of the 204 non-APS thrombosis patients resulted in 1.5 to 8% positives for aCL and/or a β 2GPI IgG/IgM,

depending on the solid-phase assay (Table 2). Retesting of 33 non-APS obstetric patients resulted in 0 to 12% positives for aCL and/or a β 2GPI IgG/IgM, depending on the solid-phase assay (Table 2).

Prevalence of (isolated) aCL and/or a β 2GPI IgA

Among patients with clinical manifestations of APS, 17 to 26% tested positive for aCL and/or a β 2GPI IgA antibodies (Table 2). Positivity for aCL and/or a β 2GPI IgA antibodies in thrombotic APS ranged between 26 and 37%, depending on the solid-phase assay used to detect IgA antibodies (Table 2). Within obstetric APS, positivity for aCL and/or a β 2GPI IgA ranged between 16 and 34%, depending on the solid-phase assay (Table 2). In the control group, consisting of AID patients and controls 6 to 8% were positive for aCL and/or a β 2GPI IgA aPL (Table 2). Isolated positivity for aCL and/or a β 2GPI IgA (positive for IgA with negative LAC, IgG and IgM results) was rare in thrombotic and obstetric patients with a prevalence of 0 to 3% and 1 to 5%, respectively (Table 2). Within AID and controls, isolated aCL and/or a β 2GPI IgA positivity ranged between 1 and 4% (Table 2). With two (BioPlex2200 and HemosIL AcuStar) out of the four tested solid-phase assays, only 1% of the total study population was positive for IgA with negative LAC, IgG, and IgM results (Table 2). The other two solid-phase assays detected more isolated IgA patients, 2% (Phadia) and 3% (QUANTA Lite ELISA) (Table 2).

Correlation of aCL and/or a β 2GPI IgA with criteria aPL

Within the total population consisting of 1,608 patients, 408 (38%) were positive for LAC, thereby the most prevalent aPL (Supplemental Table 2). IgG was the most prevalent aCL or a β 2GPI antibody isotype in the total study population (Supplemental Table 2). Only 6% of the total patient population was positive for aCL IgA, detected with QUANTA Lite ELISA (Supplemental Table 2). By a comparison of two proportions, a significant difference ($P < 0.0001$) was found for the number of patients positive for aCL IgA and aCL IgM, detected with either BioPlex2200 or Phadia. Detection of a β 2GPI IgM and IgA resulted in a significant difference in number of positive samples with the BioPlex2200 system only ($P = 0.0001$). a β 2GPI IgG was more prevalent than a β 2GPI IgA antibodies, except when detected with BioPlex2200 ($P = 0.0007$). By a comparison of two proportions, significant differences were found between aCL IgG and aCL IgA for all solid-phase assays. Prevalence of aPL in the subgroups (controls, AID, APS thrombosis, non-APS thrombosis, APS obstetric, non-APS obstetric, and APS patients) are shown in Supplemental Table 2. LAC and aCL or a β 2GPI IgA were highly correlated, as 63 to 80% IgA-positive patients were also characterized by positive LAC results (data not shown). Similarly, 72 to 83% of the patients positive for aCL IgG, were also positive for LAC. a β 2GPI IgG and LAC showed an overlap of 77 to 87%. Positive results for aCL IgM

TABLE 2. Additional diagnostic value of aCL and aβ2GPI IgA antibodies. Figures indicate the number of patients testing positive or negative for LAC, aCL and aβ2GPI IgG/M antibodies versus aCL and aβ2GPI IgA, for each solid-phase assay

		Overall population (n=1068)	Non-APS thrombosis (n=204)	APS- thrombosis (n=259)	Non-APS obstetric (n=33)	APS- obstetric (n=122)	APS* (n=60)	AID (n=196)	Controls (n=194)
		LAC, aCL IgG, aβ2GPI IgG, aCL IgM or aβ2GPI IgM	LAC, aCL IgG, aβ2GPI IgG, aCL IgM or aβ2GPI IgM	LAC, aCL IgG, aβ2GPI IgG, aCL IgM or aβ2GPI IgM	LAC, aCL IgG, aβ2GPI IgG, aCL IgM or aβ2GPI IgM	LAC, aCL IgG, aβ2GPI IgG, aCL IgM or aβ2GPI IgM	LAC, aCL IgG, aβ2GPI IgG, aCL IgM or aβ2GPI IgM	LAC, aCL IgG, aβ2GPI IgG, aCL IgM or aβ2GPI IgM	LAC, aCL IgG, aβ2GPI IgG, aCL IgM or aβ2GPI IgM
BioPlex 2200	aCL and/or aβ2GPI IgA	+ 197 10 1 2 97 0 0 1 39 2 31 1 22 3 7 1	- 106 755 2 199 45 117 0 32 20 61 9 19 22 149 8 178						
	Total	303 765 3 201 142 117 0 33 59 63 40 20 44 152 15 179							
Phadia	aCL and/or aβ2GPI IgA	+ 121 26 2 7 60 4 1 2 17 4 21 1 17 7 3 3	- 247 674 15 180 91 104 3 27 49 52 22 16 44 128 23 165						
	Total	368 700 17 187 151 108 4 29 66 56 43 17 61 135 26 168							
HemosIL AcuStar	aCL and/or aβ2GPI IgA	+ 147 6 0 0 77 0 0 0 24 1 26 1 15 4 5 0	- 208 707 8 196 81 101 1 32 41 56 18 15 44 133 15 174						
	Total	355 713 8 196 158 101 1 32 65 57 44 16 59 137 20 174							
QUANTA Lite ELISA	aCL and/or aβ2GPI IgA	+ 102 34 1 7 62 5 0 1 14 6 15 1 8 10 2 4	- 210 658 5 191 76 116 3 29 45 57 31 13 40 138 10 178						
	Total	312 756 6 198 138 121 3 30 59 63 46 14 48 148 12 182							

Abbreviations: aβ2GPI, anti-β2 glycoprotein I; aCL, anticardiolipin; AID, autoimmune diseases; APS, antiphospholipid syndrome; IgA, immunoglobulin A; IgM, immunoglobulin M; LAC, lupus anticoagulant. *The medical center did not specify if the patient suffered from obstetric or thrombotic complications.

or aβ2GPI IgM were less correlated with LAC, 59-74% and 72-75%, respectively. Upon detection with BioPlex2200, positivity for IgA aPL also highly correlated with triple positivity. Within the total population, the BioPlex2200 identified 221 triple positive patients of which 162 (73%) were positive for aCL and/or aβ2GPI IgA antibodies. Other solid-phase assays tested showed less correlation between IgA aPL and triple positivity (Phadia [49%], HemosIL AcuStar [60%] and QUANTA Lite ELISA [48%]).

Association of aCL and/or aβ2GPI IgA with thrombosis and pregnancy morbidity

Both aCL and aβ2GPI IgA positivity were significantly associated with thrombosis (Table 3). Positivity for aCL IgA reached ORs for thrombosis of 3.0 (95% CI, 1.9-4.9) to 9.9 (95% CI, 3.5-27.8) (Table 3). aβ2GPI IgA positivity showed ORs for thrombosis between 2.4 (95% CI, 1.5-3.9) and 3.1 (95% CI, 1.9-5.2) (Table 3). A stronger thrombotic association was found with aCL IgA assays than with the aβ2GPI IgA assay (Table 3). Detection of aCL IgA with QUANTA Lite ELISA reached the highest OR for thrombosis (9.9; 95% CI, 3.5-27.8) (Table 3). However, from the 47 patients positive for aCL IgA, only 43 patients with a history of thrombosis were detected with this assay, while the aCL IgA assay from BioPlex2200 detected 130 patients of which 100 patients with a history of thrombosis (Table 3). Positivity for aCL and/or aβ2GPI IgA reached comparable ORs for thrombosis, between the tested assays with ORs between 2.2 (95% CI, 1.4-3.5) and 3.0 (95% CI, 2.0-4.5) (Table 3).

Positivity for both aCL and aβ2GPI from the same isotype is considered to be more reliable for diagnosis of APS. ORs for thrombosis did not increase in patients positive for aCL and aβ2GPI IgA aPL, compared with patients positive for aCL IgA or aβ2GPI IgA when detected with the BioPlex2200 and HemosIL AcuStar (Table 3). The other two tested solid-phase assay showed an increase of OR for thrombosis in patients positive for aCL and aβ2GPI IgA compared with ORs obtained from the aCL and aβ2GPI IgA assay separately, most pronounced for QUANTA Lite ELISA (Table 3). In comparison, patients positive for both aCL and aβ2GPI IgG were characterized with a similar or slightly higher OR than positivity for aCL IgG or aβ2GPI IgG (Supplemental Table 3). However, the increase in OR for double IgG positivity for QUANTA Lite ELISA was less pronounced compared with the increase observed in double IgA positivity for that platform. Also, for the IgM isotype similar results were obtained (Supplemental Table 3). Patients positive for both aCL and aβ2GPI IgM antibodies were characterized with similar ORs for thrombosis than OR from an aCL IgM or aβ2GPI IgM assay alone (Supplemental Table 3).

TABLE 3. Thrombotic and obstetric association of aCL and a β 2GPI IgA antibodies

	BioPlex2200	Phadia	HemosIL AcuStar	QUANTA Lite ELISA
Thrombosis				
aCL	3.3 (2.1-5.1)	3.1 (1.6-6.1)	3.0 (1.9-4.9)	9.9 (3.5-27.8)
n	130	49	101	47
a β 2GPI	2.8 (1.8-4.3)	2.4 (1.5-3.9)	2.5 (1.5-4.0)	3.1 (1.9-5.2)
n	124	94	88	91
aCL and/or a β 2GPI	3.0 (2.0-4.5)	2.2 (1.4-3.5)	3.0 (1.9-4.9)	3.0 (1.8-4.8)
n	133	103	101	99
aCL and a β 2GPI	3.1 (2.0-4.8)	4.2 (1.8-9.6)	2.4 (1.5-4.0)	35.8 (4.8-254.5)
n	121	40	88	39
Pregnancy morbidity				
aCL	3.6 (2.1-6.1)	3.5 (1.5-8.3)	2.4 (1.3-4.5)	5.0 (1.5-16.5)
n	67	23	47	13
a β 2GPI	2.9 (1.7-4.9)	1.9 (1.0-3.5)	2.2 (1.2-4.1)	2.7 (1.4-5.3)
n	65	42	45	37
aCL and/or a β 2GPI	3.6 (2.1-5.9)	1.8 (1.0-3.4)	2.6 (1.4-4.7)	2.4 (1.3-4.6)
n	73	49	48	41
aCL and a β 2GPI	2.9 (1.7-5.0)	4.9 (1.7-14.5)	2.1 (1.1-3.9)	17.8 (2.2-143.6)
n	59	16	44	9

Abbreviations: a β 2GPI, anti- β 2 glycoprotein I; aCL, anticardiolipin; CI, confidence interval; n, number of patients positive for aCL or a β 2GPI IgA within the population; OR, odds ratio. Note: Odds ratios for thrombosis are calculated in a subpopulation including controls, AID, non-APS thrombosis, and APS thrombosis patients (n = 853). Odds ratios for pregnancy morbidity are calculated in a female subpopulation including controls, AID, non-APS obstetric, and APS obstetric patients (n = 483).

IgA aPL was significantly correlated with pregnancy morbidity, independent of the solid-phase platform (Table 3). Similar to thrombosis, the aCL IgA assay from QUANTA Lite ELISA reached the highest OR (5.0 (95% CI, 1.5-16.5)) for pregnancy morbidity (Table 3). Double positivity for IgG or IgM resulted in slightly higher OR for three out of the four platforms (supplemental Table 4). Double positivity for IgA (Table 3) resulted in significant higher OR for one platform (QUANTA Lite ELISA), in agreement with the thrombosis results.

Association of aCL and a β 2GPI IgA was calculated separately for venous and arterial thrombosis (Supplemental Table 5). ORs for venous thrombosis (n = 309) ranged from 1.8 (95% CI, 1.1-2.8) to 7.0 (95% CI, 2.4-20.7) and 1.8 (95% CI, 1.1-3.0) to 2.4 (95% CI, 1.4-4.2)

for aCL and aβ2GPI IgA, respectively (Supplemental Table 5). ORs for arterial thrombosis (n = 102) ranged from 2.2 (95% CI, 1.2-4.0) to 14.1 (95% CI, 4.5-44.3) and 2.9 (95% CI, 1.5-5.6) to 3.8 (95% CI, 1.9-7.4) for aCL and aβ2GPI IgA, respectively (Supplemental Table 5).

TABLE 4. Thrombotic and obstetric association of isolated aCL and aβ2GPI IgA antibodies

	BioPlex2200	Phadia	HemosIL AcuStar	QUANTA Lite ELISA
Thrombosis				
aCL	0.6 (0.1-3.4)	1.3 (0.4-4.5)	0.5 (0.4-0.5)	1.7 (0.2-18.7)
n	5	10	4	3
aβ2GPI	0.5 (0.4-0.5)	1.1 (0.4-2.9)	0.5 (0.4-0.5)	0.6 (0.3-1.5)
n	4	16	4	23
aCL and/or aβ2GPI	0.4 (0.08-2.3)	0.9 (0.4-2.2)	0.5 (0.4-0.5)	0.7 (0.3-1.6)
n	6	21	4	26
aCL and aβ2GPI	0.5 (0.4-0.5)	3.4 (0.4-30.5)	0.5 (0.4-0.5)	-
n	3	5	4	0
Pregnancy morbidity				
aCL	2.1 (0.3-15.3)	2.1 (0.4-10.7)	0.7 (0.1-6.8)	2.1 (0.1-34.2)
n	4	6	4	2
aβ2GPI	2.1 (0.4-10.7)	1.7 (0.5-6.5)	0.7 (0.6-0.7)	1.5 (0.6-4.0)
n	6	9	3	17
aCL and/or aβ2GPI	2.1 (0.4-10.7)	1.1 (0.3-3.6)	0.7 (0.1-6.8)	1.4 (0.5-3.6)
n	6	12	4	18
aCL and aβ2GPI	2.1 (0.3-15.3)	0.3 (0.3-0.4)	0.7 (0.6-0.7)	0.3 (0.3-0.4)
n	4	3	3	1

Abbreviations: aβ2GPI, anti-β2 glycoprotein I; aCL, anticardiolipin; CI, confidence interval; n, number of patients positive within the indicated subpopulation; OR, odds ratio. Note: Odds ratios for thrombosis are calculated in a subpopulation including controls, AID, non-APS thrombosis, and APS thrombosis patients (n = 853). Odds ratios for pregnancy morbidity are calculated in a female subpopulation including controls, AID, non-APS obstetric, and APS obstetric patients (n = 483).

Association of isolated aCL and/or aβ2GPI IgA with thrombosis and pregnancy morbidity

Isolated aCL and/or aβ2GPI IgA positivity was not associated with thrombosis (Table 4). ORs varied between 0.4 (95% CI, 0.08-2.3) and 0.9 (95% CI, 0.4-2.2) (Table 4). In a more detailed analysis, no significant association was found for arterial or venous thrombosis

and isolated IgA aCL or a β 2GPI (Supplemental Table 5). Isolated aCL or a β 2GPI IgA positivity was also not significantly associated with pregnancy morbidity with OR ranging from 0.7 (95% CI, 0.1-6.8) to 2.1 (95% CI, 0.4-10.7) (Table 4).

Titers of isolated aCL and/or a β 2GPI IgA-positive patients

Within the total population, the QUANTA Lite ELISA assay detected the highest number of isolated IgA samples ($n = 34$ patients). Only 12, 47, and 6% of these 34 patients were also positive for isolated IgA with the BioPlex2200, Phadia and HemosIL AcuStar assay, respectively. Titers of isolated IgA aPL patients were low (Figure 1). Isolated aCL and a β 2GPI IgA titers of patients with clinical manifestation were similar to IgA titers in the control population. Of note, upon aPL detection with Phadia, one patient with a history of thrombosis and one patient with known pregnancy morbidity had an aCL and a β 2GPI IgA titer >100 AU, while all patients in the control population had titers <100 AU (Figure 1C-D).

aCL and/or a β 2GPI IgA titers of triple positive patients

The majority of triple positive patients (LAC, aCL, and a β 2GPI IgG/M positivity, from the same isotype) had a history of thrombosis and/or pregnancy morbidity (Figure 2). aCL and a β 2GPI IgA titers were found to be significantly different between triple positives and nontriple positives, independent of the solid-phase assay (Figure 2). Triple positives for the IgG isotype (LAC, aCL IgG and a β 2GPI IgG) had higher aCL and a β 2GPI IgA titers than non-triple positive patients, independent of the solid-phase assay (Figure 2). aCL and a β 2GPI IgA titers from triple positives for the IgM isotype (LAC, aCL IgM, and a β 2GPI IgM) were also significantly higher than nontriple-positives, independent of the solid-phase assay (Figure 2). aCL and a β 2GPI IgA titers of triple positives for the IgG isotype and triple positives for the IgM isotype were not significantly different for all platforms (Figure 2).

Additional value in thrombosis of aCL and a β 2GPI IgA in the current aPL panel

Inclusion of aCL and a β 2GPI IgA antibodies in the current aPL panel consisting of LAC, IgG and/or IgM aCL, and/or a β 2GPI did not increase ORs for thrombosis (Table 5). Replacement of IgM by IgA aPL resulted in similar ORs for thrombosis compared with the current classification criteria with most solid-phase assays tested (Table 5). However, an aPL panel consisting of LAC, aCL IgG/M/A, and a β 2GPI IgG/M/A resulted in similar ORs for thrombosis (2.4 [95% CI, 1.8-3.1] to 2.8 [95% CI, 2.1-3.7]) in comparison to calculated OR with an aPL panel consisting of LAC, aCL IgG/M, and a β 2GPI IgG/M (2.4 [95% CI, 1.8-3.2] to 2.9 [95% CI, 2.2-3.9]) (Table 5).

Additional value in pregnancy morbidity of aCL and a β 2GPI IgA in the current aPL panel

Positivity for at least one of the criteria aPL of APS resulted in significant ORs for pregnancy morbidity between 4.9 (95% CI, 3.2-7.4) and 6.6 (95% CI, 4.3-10.0), depending on the solid-phase assay (Table 6). An aPL panel for the classification of APS, consisting of LAC, IgG aCL or a β 2GPI, aCL or a β 2GPI IgA reached similar ORs, between 4.6 (95% CI, 3.0-6.9) and 5.1 (95% CI, 3.4-7.7) (Table 6). Positivity for LAC, IgG, IgM or IgA did not increase the OR for pregnancy morbidity as ORs between 4.6 (95% CI, 3.1-7.0) and 6.2 (95% CI, 4.1-9.4) were obtained (Table 6).

FIGURE 1. Isolated aCL or aβ2GPI IgA titers of controls and AID (control), APS thrombosis and non-APS thrombosis (thrombosis), and APS obstetric and non-APS obstetric (pregnancy morbidity). **(A)** Isolated aCL IgA titers detected with BioPlex 2200; **(B)** isolated aβ2GPI IgA titers detected with BioPlex 2200; **(C)** isolated aCL IgA titers detected with Phadia; **(D)** isolated aβ2GPI IgA titers detected with Phadia; **(E)** isolated aCL IgA titers detected with HemosIL AcuStar; **(F)** isolated aβ2GPI IgA titers detected with HemosIL AcuStar; **(G)** isolated aCL IgA titers detected with QUANTA Lite ELISA; **(H)** and isolated aβ2GPI IgA titers detected with QUANTA Lite ELISA. Represented *p*-values were calculated using the Kruskal–Wallis test. aβ2GPI, anti-β2 glycoprotein I; aCL, anticardiolipin; APS, antiphospholipid syndrome; IgA, immunoglobulin A.

FIGURE 2. aCL or a β 2GPI IgA titers of triple-positive patients and non-triple-positive patients. Solid dots in black indicate aCL or a β 2GPI IgA titers of patients without clinical manifestations of APS. Solid dots in red indicate aCL or a β 2GPI IgA titers of patients with clinical manifestations of APS. (A) aCL IgA titers detected with BioPlex 2200; (B) a β 2GPI IgA titers detected with BioPlex 2200; (C) aCL IgA titers detected with Phadia; (D) a β 2GPI IgA titers detected with Phadia; (E) aCL IgA titers detected with HemosIL AcuStar; (F) a β 2GPI IgA titers detected with HemosIL AcuStar; (G) aCL IgA titers detected with QUANTA Lite ELISA; (H) a β 2GPI IgA titers detected with QUANTA Lite ELISA. Mean IgA titers with their 95% confidence intervals are shown. Significance was calculated using the Kruskal–Wallis test followed by Mann–Whitney U test. *, p -value < 0.0001. a β 2GPI, anti- β 2 glycoprotein I; aCL, anticardiolipin; APS, antiphospholipid syndrome; IgA, immunoglobulin A.

TABLE 5. Additional value of aCL and a β 2GPI IgA antibodies in thrombosis

	LAC, aCL IgG, aβ2GPI IgG, aCL IgM and/or aβ2GPI IgM	LAC, aCL IgG, aβ2GPI IgG, aCL IgA and/or aβ2GPI IgA	LAC, aCL IgG, aβ2GPI IgG, aCL IgM, aβ2GPI IgM, aCL IgA and/or aβ2GPI IgA
	Odds ratio (95% CI)	Odds ratio (95% CI)	Odds ratio (95% CI)
BioPlex2200	2.9 (2.2-3.9)	2.8 (2.1-3.7)	2.8 (2.1-3.7)
Phadia	2.4 (1.8-3.2)	3.1 (2.3-4.2)	2.4 (1.8-3.1)
HemosIL AcuStar	2.9 (2.1-3.8)	3.0 (2.3-4.1)	2.7 (2.0-3.6)
QUANTA Lite ELISA	2.8 (2.1-3.7)	3.1 (2.3-4.2)	2.6 (2.0-3.5)

Abbreviations: a β 2GPI, anti- β 2 glycoprotein I; aCL, anticardiolipin; CI, confidence interval; IgA, immunoglobulin A; IgM, immunoglobulin M; LAC, lupus anticoagulant.

TABLE 6. Additional value of aCL and a β 2GPI IgA antibodies in pregnancy morbidity

	LAC, aCL IgG, aβ2GPI IgG, aCL IgM and/or aβ2GPI IgM	LAC, aCL IgG, aβ2GPI IgG, aCL IgA and/or aβ2GPI IgA	LAC, aCL IgG, aβ2GPI IgG, aCL IgM, aβ2GPI IgM, aCL IgA and/or aβ2GPI IgA
	Odds ratio (95% CI)	Odds ratio (95% CI)	Odds ratio (95% CI)
BioPlex2200	5.1 (3.4-7.7)	4.6 (3.0-6.9)	5.0 (3.3-7.6)
Phadia	4.9 (3.2-7.4)	4.7 (3.1-7.0)	4.6 (3.1-7.0)
HemosIL AcuStar	5.3 (3.5-8.0)	4.8 (3.2-7.2)	5.2 (3.4-7.8)
QUANTA Lite ELISA	6.6 (4.3-10.0)	5.1 (3.4-7.7)	6.2 (4.1-9.4)

Abbreviations: a β 2GPI, anti- β 2 glycoprotein I; aCL, anticardiolipin; CI, confidence interval; IgA, immunoglobulin A; IgM, immunoglobulin M; LAC, lupus anticoagulant.

DISCUSSION

In this study we evaluated the prevalence of aCL and a β 2GPI IgA antibodies, tested with four solid-phase platforms, in 1,608 patients. We also investigated the added value of IgA aPL in APS classification.

Of the 1,608 included patients 6 to 19% tested positive for aCL IgA aCL and 12 to 18% for a β 2GPI IgA antibodies, dependent on the solid-phase assay used. LAC was by far the most prevalent aPL (38%) in our study population consisting of 1,608 APS patients and controls. In comparison, a retrospective study included 472 patients with aPL testing and found a similar distribution of aCL IgA and a β 2GPI IgA antibodies, 6 and 19%

respectively (10). Another study included 314 patients suspected for APS or related AIDs (e.g., SLE) and found 28% patients positive for a β 2GPI IgA using the QUANTA Lite ELISA (15). We only found 12% of the patients positive for a β 2GPI IgA in the total population using the QUANTA Lite ELISA. The lower prevalence of a β 2GPI IgA antibodies might be due to a different patient population as we included a large number of patients with a history of thrombosis or pregnancy morbidity, but negative for any consensus aPL as control population. Another cross-sectional study included 156 patients that fulfilled the clinical criteria of APS and found a prevalence of 5 and 29% for aCL IgA and a β 2GPI IgA positivity, respectively (8).

In our study, aCL as well as a β 2GPI IgA antibodies were correlated with clinical manifestations of APS (thrombosis and pregnancy morbidity), independent of the solid-phase platform used. In agreement with our results, multiple studies have shown an association between clinical manifestations of APS and aCL and/or a β 2GPI IgA antibodies (3–12). However, some studies were only able to show a clinical association for either aCL or a β 2GPI IgA aPL detected with an in-house ELISA (3,9). One retrospective study including 439 patient samples within a timespan of 6 years found an association between a β 2GPI IgA and thrombosis, but not for aCL IgA (3). Another retrospective study including 130 SLE patients and 35 patients with primary APS demonstrated a correlation of aCL IgA aPL with a history of thrombosis and recurrent fetal loss, but was unable to show any correlation of a β 2GPI IgA aPL with clinical manifestations of APS (9).

An external quality control program illustrated that aCL and a β 2GPI IgG/M assays produce variable results (20,25). We have previously shown that even within commercially available solid-phase assays, detection of aCL and a β 2GPI IgG/M differs between platforms (21). Within our total population consisting of 1,608 patients, 19.4, 13.8, 14.3, and 12.7% patients were found to be positive for aCL and/or a β 2GPI IgA antibodies detected with BioPlex2200, Phadia, HemosIL AcuStar and QUANTA Lite ELISA, respectively. These data indicate that the detection of IgA aPL also depends on the solid-phase assay that is used. Other studies also showed the lack of standardization in IgA aPL detecting assays (26–28). In a subpopulation from the PROMISSE cohort, a β 2GPI IgA was detected in 18.9% and 55.6% of the patients, detected with QUANTA Lite ELISA and BioPlex2200, respectively (26). Taken together, these results indicate that the detection and association of IgA aPL is dependent on the solid-phase assay and study population.

In agreement with a previous cross-sectional study, we confirmed the association of aCL and a β 2GPI IgA with arterial and venous thrombosis (8). However, some studies found an association between IgA aPL and venous thrombosis, but were unable to show an

association with arterial thrombosis and vice versa (17,18,29). In contrast, a recent study could not demonstrate an association between a β 2GPI IgA aPL and thrombosis (15). Positivity for a β 2GPI IgA aPL was found in 31% of the included APS patients (15), as well as a large portion (30%) of SLE patients was found to be positive for a β 2GPI IgA aPL (15). Upon exclusion of SLE patients, a significant association was found between a β 2GPI IgA and venous thrombosis (OR 2.5; 95% CI, 1.1-4.9) (15).

In our cohort, positive aCL and/or a β 2GPI IgA results were significantly associated with pregnancy morbidity, as defined in the Sydney criteria. However, the non-APS obstetric group was relatively small ($n = 33$), compared with obstetric APS patients ($n = 122$) which might have affected the association of aPL and pregnancy morbidity. Limited data are available on the association between IgA aPL and obstetric complications (4,8,9,15). Most studies found a value for aCL or a β 2GPI IgA testing in pregnancy morbidity (4,8,9). However, a study that included 314 patients suspected from APS or related autoimmune diseases (e.g., SLE) did not find a significant association for aCL or a β 2GPI IgA aPL with pregnancy morbidity (15). Interestingly, no association of aCL IgG/M and a β 2GPI IgG/M with pregnancy morbidity was found within the same study (15).

Pathogenicity of IgA aPL has been shown in animal models (14,18). In mice, IgA antibodies from APS patients increased the mean thrombus area and mean thrombus disappearance time upon induced thrombus formation of a nonocclusive thrombus by pinch injury (14). IgA antibodies were purified from two APS patients of which one was also positive for aCL IgG and LAC. The other patient was positive for aCL IgA, but negative for aCL IgG, aCL IgM and LAC (14). However, the authors did not demonstrate that aCL is solely responsible for the observed effect (14). Another study also showed that IgA isolated from APS patients is pathogenic as thrombus formation and tissue factor activity in mice injected with IgA from APS was increased compared with control IgA (18). However, the plasma of the mice injected with IgA from APS patients showed LAC activity (18). These data indicate that IgA aPL can be pathogenic (14,18). However, pathogenic IgA aPL seem to correlate with LAC.

Despite the association of aCL and/or a β 2GPI IgA aPL with thrombosis shown in multiple studies, only some have suggested an added value for IgA testing in patients suspected of thrombotic APS (6,8,10,12,18,30). Although an added value was suggested for so called "isolated IgA" by some studies, many studies did not include LAC testing, hampering assessment of true isolated IgA aPL (8,12,18,30). In a retrospective study testing 472 patients with suspected or confirmed thrombophilia, an autoimmune disease or pregnancy morbidity (10) IgA (aCL, a β 2GPI and/or antiphosphatidylserine

[aPS]) aPL positivity was found to be an independent risk factor for thrombosis using a multivariate analysis [10]. Detection of aCL and aPS IgG, IgM, and IgA antibodies were performed with another platform than detection of a β 2GPI IgG, IgM and IgA antibodies (10). Considering the high variability between solid-phase platforms in antibody detection, classification of APS patients and the association of aPL might be affected. It is suggested to detect all aPL within the same system for the classification of APS patients (21). In addition, the method used to determine the cut-off value for an aPL assay has also shown to affect between-assay performance (31). Also, antibody heterogeneity has been suggested to attribute to variability in test results (32).

We defined isolated aCL and/or a β 2GPI IgA positivity for aCL and/or a β 2GPI IgA aPL, as negative for aCL and/or a β 2GPI IgG/M and/or LAC. Isolated IgA positivity was rare in the total population. Overall, the prevalence of isolated IgA was comparable between patients with clinical manifestations of APS and patients without a history of thrombosis or pregnancy morbidity. Positivity for isolated IgA aPL was not associated with thrombosis or pregnancy morbidity as calculated OR did not reach statistical significance. Titers of IgA positive patients were characterized by low aCL or a β 2GPI IgA titers, around the cut-off value. Other noncriteria aPL like anti-phosphatidylethanolamine, negatively charged phospholipids (other than cardiolipin), anti-vimentin/cardiolipin, Annexin A5, and aPST/prothrombin (PT) antibodies have been suggested to be clinical relevant in patients suspected of APS, but negatively tested for consensus aPL (33). A recent systematic review showed a strong association between aPS/PT aPL and clinical manifestations of APS, with a high association with LAC (34). However, this needs to be validated in a large multicenter study.

In this study, we aimed to investigate the value of aCL and a β 2GPI IgA aPL in addition to the current aPL panel (LAC, aCL IgG/M, and a β 2GPI IgG/M). Positivity for IgA aPL was found to be associated with thrombosis and pregnancy morbidity. However, isolated IgA positivity was rare and not correlated with clinical manifestations of APS. Our results do not support testing for aCL and/or a β 2GPI IgA in addition to conventional aPL for the identification of patients with clinical manifestations of APS.

CONFLICT OF INTEREST

G. W. Moore reports consultancy fees from Technoclone, outside the submitted work. W. Chayoua, D. Yin and B. de Laat are employees of Synapse Research Institute, part of Diagnostica Stago SAS. The other authors state that they have no relevant conflict of interest.

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SUPPLEMENTAL DATA

SUPPLEMENTAL TABLE 1. Cut off values of aCL IgG/M/A and aβ2GPI IgG/M/A detected with BioPlex2200, Phadia, HemosILAcuStar and QUANTA Lite ELISA

	aCL IgG	aβ2GPI IgG	aCL IgM	aβ2GPI IgM	aCL IgA	aβ2GPI IgA
BioPlex2200	20	20	20	20	20	20
Phadia	10	10	10	10	20	10
HemosIL AcuStar	20	20	20	20	20	20
QUANTA Lite ELISA	20	20	20	20	20	20

Abbreviations: aβ2GPI, anti-β2 glycoprotein I; aCL, anticardiolipin; AID, autoimmune diseases; APS, antiphospholipid syndrome; IgA, immuno- globulin A; IgM, immunoglobulin M.

SUPPLEMENTAL TABLE 2. Prevalence of LAC, aCL, and aβ2GPI IgG/M/A in the total population, controls, AID, APS thrombosis, non-APS thrombosis, APS obstetric, non-APS obstetric, and APS patients

		Total population (n=1068)	Controls (n=193)	AID (n=196)	APS thrombosis (n=258)	Non-APS thrombosis (n=204)	APS obstetric (n=121)	Non-APS obstetric (n=33)	APS ^a (n=60)
aCL IgG	LAC	408 (38%)	16 (8%)	56 (29%)	202 (78%)	0 (0%)	75 (62%)	0 (0%)	59 (98%)
	BioPlex2200	248 (23%)	10 (5%)	30 (15%)	120 (47%)	3 (1%)	50 (41%)	0 (0%)	35 (58%)
	Phadia	215 (20%)	7 (4%)	20 (10%)	101 (39%)	9 (4%)	45 (37%)	1 (3%)	32 (53%)
	HemosIL AcuStar	222 (21%)	5 (3%)	24 (12%)	112 (43%)	3 (1%)	46 (38%)	0 (0%)	32 (53%)
	QUANTA Lite ELISA	191 (18%)	3 (2%)	19 (10%)	96 (37%)	3 (1%)	38 (31%)	1 (3%)	31 (52%)
aβ2GPI IgG	BioPlex2200	252 (24%)	9 (5%)	35 (18%)	120 (47%)	2 (1%)	50 (41%)	0 (0%)	13 (22%)
	Phadia	202 (19%)	6 (3%)	24 (12%)	97 (38%)	7 (3%)	39 (32%)	3 (9%)	18 (30%)
	HemosIL AcuStar	284 (27%)	11 (6%)	42 (21%)	135 (52%)	5 (2%)	52 (43%)	1 (3%)	18 (30%)
	QUANTA Lite ELISA	142 (13%)	3 (2%)	11 (6%)	75 (29%)	2 (1%)	23 (19%)	2 (6%)	32 (53%)
	BioPlex2200	118 (11%)	6 (3%)	16 (8%)	60 (23%)	0 (0%)	23 (19%)	0 (0%)	13 (22%)
aCL IgM	Phadia	202 (19%)	19 (10%)	34 (17%)	87 (34%)	7 (3%)	34 (28%)	3 (9%)	18 (30%)
	HemosIL AcuStar	169 (16%)	12 (6%)	30 (15%)	76 (29%)	1 (0.5%)	31 (26%)	1 (3%)	18 (30%)
	QUANTA Lite ELISA	170 (16%)	8 (4%)	25 (13%)	71 (28%)	1 (0.5%)	31 (26%)	2 (6%)	32 (53%)
	BioPlex2200	131 (12%)	6 (3%)	16 (8%)	64 (25%)	0 (0%)	26 (21%)	0 (0%)	19 (32%)
	Phadia	138 (13%)	6 (3%)	22 (11%)	65 (25%)	1 (0.5%)	29 (24%)	0 (0%)	15 (25%)
aβ2GPI IgM	HemosIL AcuStar	113 (11%)	5 (3%)	14 (7%)	55 (21%)	0 (0%)	24 (20%)	0 (0%)	15 (25%)
	QUANTA Lite ELISA	152 (14%)	4 (2%)	26 (13%)	67 (26%)	0 (0%)	29 (24%)	0 (0%)	26 (43%)
	BioPlex2200	200 (19%)	7 (4%)	23 (12%)	97 (38%)	3 (1%)	38 (31%)	1 (3%)	31 (52%)
	Phadia	174 (16%)	8 (4%)	31 (16%)	76 (29%)	12 (6%)	25 (21%)	3 (9%)	19 (32%)
	HemosIL AcuStar	151 (14%)	5 (3%)	19 (10%)	77 (30%)	0 (0%)	24 (20%)	0 (0%)	26 (43%)

SUPPLEMENTAL TABLE 2. Continued

	Total population (n=1068)	Controls (n=193)	AID (n=196)	APS thrombosis (n=258)	Non-APS thrombosis (n=204)	APS obstetric (n=121)	Non-APS obstetric (n=33)	APS ^a (n=60)
aβ2GPI IgA	QUANTA Lite ELISA	67 (6%)	2 (1%)	41 (16%)	2 (1%)	9 (7%)	0 (0%)	11 (18%)
	BioPlex2200	187 (18%)	8 (4%)	91 (35%)	1 (0.5%)	34 (28%)	1 (3%)	28 (47%)
	Phadia	131 (12%)	6 (3%)	61 (24%)	7 (3%)	18 (15%)	1 (3%)	18 (30%)
	HemosIL AcuStar	132 (12%)	5 (3%)	64 (25%)	0 (0%)	22 (18%)	0 (0%)	22 (37%)
	QUANTA Lite ELISA	126 (12%)	5 (3%)	64 (25%)	6 (3%)	19 (16%)	1 (3%)	15 (25%)

Abbreviations: aβ2GPI, anti-β2 glycoprotein I; aCL, anticardiolipin; AID, autoimmune diseases; APS, antiphospholipid syndrome; IgA, immuno- globulin A; IgM, immunoglobulin M. ^aThe medical center did not specify if the patient suffered from obstetric or thrombotic complications.

SUPPLEMENTAL TABLE 3. Thrombotic association of aCL and aβ2GPI IgG/M antibodies

	BioPlex2200	Phadia	HemosIL AcuStar	QUANTA Lite ELISA
aCL IgG	3.2 (2.2-4.7)	4.2 (2.7-6.5)	4.1 (2.7-6.3)	4.5 (2.8-7.4)
n	163	137	144	121
aβ2GPI IgG	2.8 (1.9-4.1)	3.5 (2.3-5.4)	2.8 (1.9-3.9)	5.4 (3.0-9.6)
n	166	134	193	91
aCL and aβ2GPI IgG	3.3 (2.2-5.0)	4.1 (2.5-6.7)	4.3 (2.8-6.8)	5.3 (2.9-9.6)
n	157	113	140	84
aCL IgM	2.5 (1.5-4.1)	1.6 (1.1-2.3)	1.7 (1.1-2.5)	2.0 (1.3-3.1)
n	82	147	119	105
aβ2GPI IgM	2.7 (1.6-4.4)	2.2 (1.4-3.4)	2.6 (1.5-4.5)	2.0 (1.3-3.2)
n	86	94	74	97
aCL and aβ2GPI IgM	2.7 (1.6-4.7)	2.7 (1.6-4.6)	2.7 (1.5-4.7)	2.4 (1.4-4.2)
n	76	79	67	74

Abbreviations: aβ2GPI, anti-β2 glycoprotein I; aCL, anticardiolipin; AID, autoimmune diseases; APS, antiphospholipid syndrome; IgM, immunoglobulin M. Note: Odds ratios are calculated in a subpopulation including controls, AID, non-APS thrombosis, and APS thrombosis patients (n = 853).

SUPPLEMENTAL TABLE 4. Obstetric association of aCL and aβ2GPI IgG/M antibodies

Pregnancy morbidity	BioPlex2200	Phadia	HemosIL AcuStar	QUANTA Lite ELISA
aCL IgG	3.5 (2.2-5.7)	5.1 (3.0-8.7)	4.5 (2.7-7.6)	5.2 (2.9-9.2)
n	89	71	74	59
aβ2GPI IgG	3.3 (2.1-5.3)	3.6 (2.1-6.1)	2.8 (1.8-4.4)	4.2 (2.1-8.6)
n	91	69	102	36
aCL and aβ2GPI IgG	3.7 (2.3-6.1)	4.6 (2.6-8.2)	4.6 (2.7-7.7)	3.8 (1.8-7.8)
n	85	58	72	34
aCL IgM	3.0 (1.6-5.7)	2.0 (1.2-3.3)	2.0 (1.2-3.4)	2.9 (1.7-5.0)
n	41	81	69	61
aβ2GPI IgM	3.1 (1.7-5.8)	3.2 (1.8-5.8)	3.6 (1.8-6.9)	2.7 (1.5-4.7)
n	46	51	40	55
aCL and aβ2GPI IgM	3.2 (1.6-6.2)	3.3 (1.6-6.5)	3.7 (1.8-7.5)	2.9 (1.5-5.6)
n	40	36	36	38

Abbreviations: aβ2GPI, anti-β2 glycoprotein I; aCL, anticardiolipin; AID, autoimmune diseases; APS, antiphospholipid syndrome; IgM, immunoglobulin M. Note: Odds ratios are calculated in a female subpopulation including controls, AID, non-APS obstetric, and APS obstetric patients (n = 483).

SUPPLEMENTAL TABLE 5. Association of aCL and aβ2GPI IgA with venous or arterial thrombosis

	Venous n=309					Arterial n=102				
	IgA OR (95% CI)		Isolated IgA OR (95% CI)		aCL	IgA OR (95% CI)		aCL	Isolated IgA OR (95% CI)	
	aCL	aβ2GPI	aCL	aβ2GPI		aCL	aβ2GPI		aCL	aβ2GPI
BioPlex2200	2.7 (1.7-4.3)	2.2 (1.4-3.5)	0.4 (0.04-4.0)	0.6 (0.5-0.6)		3.7 (2.0-6.7)	2.9 (1.6-5.3)	1.3 (0.1-12.4)	0.8 (0.8-0.8)	
Phadia	1.8 (1.1-2.8)	1.8 (1.1-3.0)	0.8 (0.4-1.6)	1.5 (0.5-4.1)		2.2 (1.2-4.0)	3.0 (1.6-5.7)	0.7 (0.2-2.0)	0.8 (0.8-0.8)	
HemosIL AcuStar	2.3 (1.4-4.0)	1.8 (1.1-3.2)	0.6 (0.5-0.6)	0.6 (0.5-0.6)		3.5 (1.8-6.7)	2.9 (1.5-5.6)	0.8 (0.8-0.8)	0.8 (0.8-0.8)	
QUANTA Lite ELISA	7.0 (2.4-20.7)	2.4 (1.4-4.2)	2.5 (0.2-28.1)	0.7 (0.3-1.7)		14.1 (4.5-44.3)	3.8 (1.9-7.4)	0.8 (0.8-0.8)	0.3 (0.04-2.2)	

Abbreviations: aβ2GPI, anti-β2 glycoprotein I; aCL, anticardiolipin; CI, confidence interval; IgA, immunoglobulin A; OR, odds ratio. Note: Odds ratios are calculated with their respective 95% confidence intervals for each solid-phase assay. Significant odds ratios are shown in bold.

6

The significance of antibodies against domain I of beta-2 Glycoprotein I in antiphospholipid syndrome

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ABSTRACT

The antiphospholipid syndrome (APS) is characterized by vascular thrombosis and/or pregnancy morbidity with the persistent presence of antiphospholipid antibodies (aPLs). Progress is being made in understanding the pathogenesis of the syndrome, but difficulties persist in the identification of patients at risk for thrombosis and/or pregnancy morbidity. Beta-2 glycoprotein I (β_2 GPI), a plasma protein consisting of five sushi domains, is thought to be the main antigenic target of aPLs. Antibodies recognizing domain I of β_2 GPI are predominantly present in patients with an elevated risk of thrombosis, whereas antidomain IV/V antibodies are found in nonthrombotic autoimmune diseases. Indeed, domain I antibodies proved to be pathogenic in multiple studies. Retrospective studies have provided evidence for an added clinical value of antidomain I antibodies in the risk stratification of patients with APS. Still, wide ranges of odds ratio exist between studies, probably due to differences in the study and control population, and detection methods used. Despite the proven pathogenicity of antidomain I antibodies and their correlations with clinical manifestations of APS, heterogeneity of the current studies has prohibited their acceptance in the official diagnostic criteria. Well-designed large longitudinal prospective studies with available and new, preferentially functional, assays for the risk stratification of patients with APS are required.

INTRODUCTION

Because of the high incidence of thrombosis and pregnancy morbidity independent from the antiphospholipid syndrome (APS), the diagnosis of APS predominantly relies on laboratory results.¹⁻³ As a consequence, the quality of the assays used to detect these antibodies is of utmost importance. The Sydney update of the classification criteria for definite APS led to a substantial improvement of the APS diagnosis, but several difficult issues remain unsolved.^{4,5} Huge progress is being made in understanding the pathogenesis, but difficulties persist in the identification of patients at risk for thrombosis. In the Sydney criteria, three different types of assays to detect antiphospholipid antibodies (aPL) are included: (1) phospholipid-dependent prolongation of coagulation assays (Lupus anticoagulant[LA]); (2) detection of IgM/IgG antibodies against beta2glycoprotein I (β_2 GPI) antibodies; (3) detection of immunoglobulin M (IgM)/immunoglobulin G (IgG) antibodies against cardiolipin (CL).³ While the role of these criteria can be debated, all assays have a place in the diagnostic work-up for APS.

Especially IgG anti-CL/anti- β_2 GPI antibodies have been shown to correlate with thrombosis. A prospective study confirmed that only a minority of patients with APS carry IgM anti-CL/anti- β_2 GPI antibodies and these patients displayed other thrombotic risk factors.⁶ Nonetheless, discrepancies have been found in literature concerning the role of IgM antibodies in the pathogenesis of APS. A recent review of the literature clearly found more significant correlations with thrombosis for the IgG compared with the IgM isotype, but failed to identify the added value of isolated IgM positivity due to the unavailability of paired results of IgG and IgM for each separate patient.⁷

Several other autoantibodies have been proposed to be involved in the pathogenesis of APS, and this review will focus on the possible added clinical value of a subset of antibodies directed against domain I of β_2 GPI.

Discovery of domain I as an immunodominant epitope

Upon the general acceptance that a main antigenic target of aPL is phospholipid-bound β_2 GPI, a 50 kDa plasma protein composed of five short consensus repeat domains, many studies tried to identify which domains of β_2 GPI are involved in the binding of anti- β_2 GPI antibodies. Although more domains of β_2 GPI have been indicated as the target location of anti- β_2 GPI antibodies,⁸⁻¹¹ strong evidence points towards the first domain of β_2 GPI (domain I).^{12,13} By using domain-deletion mutants, Iverson *et al* were the first to show that anti- β_2 GPI antibodies recognize an epitope on domain I.¹⁴ Later on, this finding was confirmed by others, and especially a positively charged epitope,

TABLE 1. Performance characteristics of different anti-domain I β_2 GPI IgG assays

Publication		Patients		Controls		Anti-domain I β_2 GPI		Anti- β_2 GPI		Non-domain I anti- β_2 GPI	
Endpoint	First author	Year	Number	Type	Number	Type	OR ^a	Type of assay	OR ^a	Type of assay	Type of assay
T	de Laat ²⁸	2009	511	anti- β_2 GPI positive	NA	NA	3.5	2-step ELISA	-	-	0.4 2-step ELISA
T	Pengo ³³	2015	65	anti- β_2 GPI positive	NA	NA	7.25	CLIA	-	-	-
T	De Craemer ⁶⁴	2016	101	clinical manifestations APS	325	AID+HC	14.4	CLIA	29.2	CLIA	-
T	Mahler ⁶⁰	2016	106	clinical manifestations APS	272	HC+ID	8.7^b	CLIA	4.1^c	CLIA	-
							3.7^d	ELISA			
T	Agmon-Levin ³⁵	2013	178	APS	97	HC+ Sepsis	2.54	CLIA	-	-	-
T	Zhang ⁵⁶	2016	190	APS + non-APS T + non-APS P	39	HC	3.27^e	CLIA	2.75	CLIA	1.37 CLIA
T	de Laat ¹⁶	2005	198	APS + SLE + LLD	NA	NA	18.9	2-step ELISA	-	-	1.1 2-step ELISA
T	Andreoli ⁵⁷	2015	159	APS + AIRD + aPL carriers	NA	NA	1.67	ELISA	-	-	0.8 ELISA (DIV/V)
T	Zuily ⁵⁸	2015	137	aPL carriers + SLE	NA	NA	-	-	ns	ELISA	-
T	Akhter ⁵⁹	2013	164	SLE	162	NT	1.22	ELISA	3.21	ELISA	-
P	de Laat ²⁸	2009	352	anti- β_2 GPI positive	NA	NA	2.4	2-step ELISA	1.5	ELISA	-
P	Zhang ⁵⁶	2016	190	APS + non-APS T + non-APS P	39	HC	1.55^{**}	CLIA	1.27	CLIA	0.75 CLIA
P	Andreoli ⁵⁷	2015	73	APS + AIRD + aPL carriers	NA	NA	2.39	ELISA	-	-	1.93 ELISA (DIV/V)
APS	Mondejar ⁵²	2014	39	clinical manifestations APS	77	RD+HC	21	CLIA	8.6	CLIA	-
APS	De Craemer ⁶	2016	101	clinical manifestations APS	325	AID+HC	93.1	CLIA	-	-	-
APS	Pericleous ⁴⁸	2016	111	APS	319	HC+SLE	14.3	ELISA	30.87	ELISA	-
APS	Meneghel ⁵¹	2015	151	APS + seronegative APS	166	HC+RD	5	CLIA	-	-	-

Abbreviations: –, not specified; AID, autoimmune disorder; AIRD, autoimmune rheumatic diseases; aPL, antiphospholipid antibody; APS, antiphospholipid syndrome; β_2 GPI, beta-2 glycoprotein I; CLIA, chemiluminescence assay; DIV/V, domain IV/V; ELISA, enzyme-linked immunosorbent assay; HC, healthy controls; ID, infectious disease; IgG, immunoglobulin G; LLD, lupuslike disease; NA, not available; (n)s, (not) significant; NT, no thrombosis; OR, odds ratio; P, pregnancy morbidity; SLE, systemic lupus erythematosus; T, thrombosis.

^aSignificant ORs are shown in bold; ORs were calculated with a cut-off of: ^a190.2 units, ^b164.6 chemiluminescence units, ^c7.6 units, or ^d20 units.

spanning amino acids 39/40-43, proved to be important in the binding of anti- β_2 GPI antibodies.¹⁵⁻¹⁸ Further research revealed a possible contribution of the domain I-II linker, suggesting that antibodies may bind to discontinuous epitopes in this area.¹⁹ β_2 GPI has been shown to exist in different conformations, “open” and “closed”.^{20,21} β_2 GPI is present in plasma in its “closed” native conformation and interaction with anionic surfaces such as phospholipids has been found to induce an activated J-shaped conformation. This conformational change proved to be mandatory for the exposure of the pathogenic epitope G40-R43 on domain I and hence to enable anti- β_2 GPI antibodies to interact with the epitope.^{22,23} The closed and open conformations of β_2 GPI were suggested to resemble two different redox states of β_2 GPI, and patients with APS have a significantly elevated antigen load in the oxidized/open state.²⁴ Interestingly, patient-derived anti- β_2 GPI antibodies display greater avidity to oxidized versus reduced β_2 GPI.²⁴

Evidence for domain I pathogenicity: *in vivo/in vitro*

The pathogenicity of antidomain I antibodies has been demonstrated by both *in vivo* and *in vitro* studies. *In vitro*, antidomain I antibodies were found to display LA activity.^{16,25} Furthermore, antidomain I antibodies have been shown to increase resistance against the anticoagulant properties of annexin A5 and against activated protein C.²⁵⁻²⁹ LA-positive anti- β_2 GPI antibodies, isolated from a subset of patients with APS, were found to neutralize the inhibitory function of β_2 GPI on von Willebrand Factor induced platelet aggregation.³⁰ Given the fact that antidomain I antibodies display LA activity, this effect may be mediated by antidomain I antibodies. Ninivaggi *et al* have postulated that antidomain I antibodies may interfere with the natural anticoagulant action of “open” β_2 GPI, evidenced by its inhibitory effect on thrombin generation.³¹ Our unpublished data using antidomain I antibodies confirm this hypothesis.

A first indication of domain I pathogenicity *in vivo* comes from the fact that human β_2 GPI becomes immunogenic only after incubation with apoptotic cells or phospholipids.^{32,33} De Laat *et al* confirmed the necessity of a conformational change for immunogenicity of β_2 GPI and demonstrated that antibodies displaying LA activity developed in mice injected with domain I but not domain II to IV.²² However, another study showed that aPLs were induced by immunization with the phospholipid binding site of β_2 GPI (domain V), and these proved to be thrombogenic in a mouse model.³⁴ Importantly, both affinity purified IgG antidomain I from APS serum and human monoclonal antidomain I IgG have been shown to induce thrombosis and/or fetal loss in mice APS models of venous thrombosis.³⁵⁻³⁷ Interestingly, recombinant human domain I was found to abolish antibody-induced thrombosis.³⁸ Accordingly, antidomain I fractions from an APS patient induced a higher increase in tissue factor activity and larger thrombi

compared with antidomain I poor fractions obtained after affinity purification.³⁹ Given the high degree of homology between domain I and an extracellular epitope of toll-like receptor 4 (TLR4), the pathogenicity of antidomain I antibodies has been suggested to result from stimulation of the TLR4-nuclear factor (NF κ B) pathway.⁴⁰ In this study, antibodies directed against a cryptic epitope in domain I were found to engage TLR4 directly, in the absence of β_2 GPI, inducing a proinflammatory phenotype in monocytes and endothelial cells.

Detection of domain I antibodies

Given the cryptic nature of the domain I epitope, detection of domain I antibodies in the current available full-length anti- β_2 GPI assays depends highly on the availability of the epitope. As a sufficiently large fraction of β_2 GPI needs to be in its “open” conformation, the purification/preparation methods of β_2 GPI and the selection of negatively charged Enzyme-Linked Immuno Sorbent Assay (ELISA) plates play an important role in the detection of antibodies against the cryptic epitope.^{15,41} Interestingly, by using monoclonal antibody P1-117 recognizing specifically epitope G40-R43, exposed only when β_2 GPI is in the open conformation, versus antibody P2-6 recognizing β_2 GPI regardless of its conformation, we previously demonstrated clear differences in exposure of epitope G40-R43 on domain I of β_2 GPI in commercially available anti- β_2 GPI IgG assays.^{42,43} Importantly, we found that a significant number of samples positive for domain I reactivity are falsely assigned negative in assays characterized by a decreased availability of this epitope. These findings have been extended to two automated panels for anti-CL and anti- β_2 GPI IgG.⁴⁴ In agreement with these results, a line immunoassay was able to discriminate patients with APS from infectious patients and asymptomatic carriers, likely through the way β_2 GPI is oriented on the matrix, resulting in the exposure of domain I.⁴⁵

Apart from these full-length β_2 GPI assays, specific assays for the detection of domain I antibodies have been developed. First reports on domain I reactivity were using a baculovirus system to express β_2 GPI that lacked one or more domains.¹⁴ Next, a two-step approach was optimized by coating domain I on both hydrophobic and hydrophilic ELISA plates.¹⁶ The ratio in optical density (OD) obtained on both plates provides an estimation of the relative amount of antibodies reactive with the cryptic epitope, as interactions between the positive charge of the epitope and the negative charge of the hydrophilic plate hamper interactions of antibodies with that epitope. Additionally, other ELISAs based on human recombinant domain I expressed in bacteria that was refolded after purification; recombinant domain I expressed in insect cells; an N-terminally biotin-(PEG)₂ derivative of domain I and competitive inhibition

between domain I and full-length β_2 GPI have been developed.^{15,46-49} More recently, chemiluminescence immunoassays for the detection of domain I antibodies have been described.^{50,51} Good correlations were demonstrated between the available methods, although small differences in specificity and sensitivity were observed.^{52,53}

Clinical significance of antidomain I positivity

Using the different methods described previously, multiple studies have shown a significant association between the presence of antibodies against the cryptic epitope of domain I and thrombosis/APS classification (Table 1). Using an antidomain I antibody as competitor for patient antibody binding to β_2 GPI, Arvieux *et al* already demonstrated the presence of antidomain I antibodies in patients with APS with thrombosis, versus, probably nonpathogenic, antibodies against other domains in leprosy patients in which thrombosis is uncommon.⁶⁰ In a systematic review of the literature, the prevalence of antidomain I antibodies in 548 patients with APS from 11 different centers, proved to be 44%.⁶¹ In a large retrospective multicenter study on 442 anti- β_2 GPI-positive patients, antidomain I positivity was reported in 55% of the patients, and the presence of these antibodies associated significantly with an increased risk for thrombosis and pregnancy complications.²⁸ The detection of antibodies against the cryptic epitope in domain I frequently proved to be superior over the detection of anti- β_2 GPI antibodies recognizing other domains or the full-length β_2 GPI, with a higher specificity for both thrombosis and pregnancy morbidity.^{16,28,50} These results are in contrast with two recent studies, in which the clinical performances of anti- β_2 GPI and antidomain I assays proved to be equal.^{54,56} Both studies agree that antidomain I IgG do not provide added clinical value to the current classification criteria, but disagree about the potential of antidomain I IgG as replacement of anti- β_2 GPI assays. Other studies confirmed a correlation of antidomain I IgG antibodies and thrombosis, but failed to establish an association with pregnancy morbidity.^{48,53,56} In another study, measuring antidomain I antibodies yielded higher odds ratio (OR) for clinical manifestations of APS compared with anti- β_2 GPI antibodies (21.0 versus 8.6, respectively) and proved to be suitable to replace anti- β_2 GPI assays in an earlier described antiphospholipid score, aPL-S.^{52,62}

Importantly, looking at aPL profiles, patients at higher clinical risk, i.e. triple positive patients⁶, were shown to display higher levels of antidomain I antibodies compared with single or double positive patients.^{47,51,53,54,56,57} IgG antidomain I antibodies were also reported in seronegative patients with APS that fulfilled clinical APS criteria, suggesting that detection of antidomain I antibodies potentially improves the classification of patients with APS.^{63,64} Although most studies investigating antidomain I antibodies

detect IgG, antidomain I antibodies of all three isotypes have been shown to associate with APS with high specificity.^{48,61} Inconsistently, another study did not find a significant association between antidomain I IgA antibodies and thrombosis.⁶⁶

Looking at the titers of antidomain I antibodies, de Craemer *et al* demonstrated significantly reduced levels in clinically unaffected patients.⁵⁴ Antidomain I antibodies proved to be highly prevalent in patients with a history of thrombosis, whereas antibodies targeting other domains were shown in patients with and without thrombosis. Interestingly, antidomain I IgG titers proved to be stable over a 12-week period, suggesting that a single antidomain I measurement is sufficient to classify patients with APS.^{53,54}

Of note, the reported percentage of antidomain I positivity, as well as the OR for clinical manifestations of APS varies within the different studies presented in Table 1 and this probably can be explained by several causes. First, the patient and control population varies widely in the different studies. Although at first sight the obtained ORs for the risk on thrombosis of 18.9 and 3.5 in two studies performed in our laboratory seem to be contradictory,^{16,28} the difference is easily explained by the starting populations, being broad (APS, systemic lupus erythematosus and lupus like disease patients) versus narrow (anti- β_2 GPI positive patients), respectively. Indeed, recalculating the OR for thrombosis of domain I positivity in the first study specifically for the anti- β_2 GPI positive patients, yields an OR of 2.8, which closely resembles the obtained OR of 3.5 in the second study. Apart from the starting population, differences in exposure of the pathogenic domain I in the domain I detection platforms may add up to the variability.^{28,42,44,53,56} Additionally, differences in ORs may result from the selection of cut-off values, as Zhang *et al* recently demonstrated an increase in OR with raising cut-off values.⁵⁶ As with the assays currently included in the official APS classification criteria, antidomain I assays urgently require standardization concerning domain I production and coating, cut-off determination and inclusion of reference material. Nonetheless, most studies agree that a significant proportion of patients positive for anti- β_2 GPI antibodies are negative for antidomain I antibodies and the existence of autoantibodies reacting with non-domain I epitopes has been demonstrated.^{53,57,66} As a result, to avoid false-negative results, replacement of available full-length anti- β_2 GPI assays with specific antidomain I assays may not be advised at this stage. Nevertheless, side by side application may improve risk stratification of clinical events.

Correlations of antidomain I antibodies with clinical symptoms apart from APS have been assessed. Mullen *et al* performed a prospective study with patients with transient ischemic attack (TIA), in which antidomain I antibodies could not be used as biomarkers

of risk on stroke or death after TIA, in contrast to anti-phosphatidylserine/prothrombin (anti-PS/PT) antibodies.⁶⁷ Antidomain I antibodies were observed in patients with multiple sclerosis and systemic autoimmune rheumatic disease with no clinical features of APS.^{57,68} However, this may be related to the fact that MS patients display a higher risk of thrombosis compared with healthy individuals.^{69,70} Additionally, antidomain I antibodies have been shown to associate with non-criteria-related manifestations of APS, including livedo reticularis and heart valve disease.⁷¹

Evidence for other noncriteria antibodies

Evidence for the pathogenicity of other non-criteria antibodies is growing. Although IgG antibodies against domain IV/V from β_2 GPI have been shown to be increased in single positive anti- β_2 GPI positive patients, their presence did not correlate with thrombosis.⁷² Epitopes on domain IV or V have shown to be preferentially recognized by IgG antibodies in sera from antiphospholipid-positive asymptomatic carriers, individuals with leprosy or children with atopic dermatitis.^{73,74} *In vivo*, anti- β_2 GPI antibodies from leprosy patients do not show thrombogenic effects in mice.⁷⁵ These findings arise the possibility that antidomain IV/V antibodies are present in non-thrombotic autoimmune diseases. In the same line, Andreoli *et al* provided evidence that the ratio of antidomain I to antidomain IV/V antibodies can be used to determine the pathogenic potential of anti- β_2 GPI antibodies and discriminate between autoimmune diseases such as APS and other pathologies.⁵⁷

Anti-PS/PT were suggested to be a good alternative to estimate thrombotic risk in patients where LA results are difficult to interpret due to the intake of anticoagulants.⁵⁹ The clinical significance of these antibodies in APS classification is beyond the scope of this review, but has been reviewed before,⁷⁶ and is the topic of another chapter in this issue of the journal. Given their association with both thrombosis and pregnancy morbidity, their presence may represent a new risk stratification tool, especially in patients negative for the traditional antibodies included in the APS criteria.

Additionally, although IgA antibodies so far have not been included in the current classification criteria, measurement of (isolated) IgA anti- β_2 GPI antibodies, often a subclass of IgA antibodies targeting domain IV/V, previously showed to identify additional patients with clinical features of APS.^{48,59,65,77-79} The pathogenicity of purified IgA anti- β_2 GPI antibodies, reactive against domain I and IV/V, was demonstrated in a mouse model of thrombosis.⁷⁷ As positivity for IgA antibodies has been shown to largely overlap with other antiphospholipid antibodies, it may be advisable to only measure IgA (particularly anti- β_2 GPI) in patients negative for IgG/IgM but in whom APS is suspected.^{79,80}

Future perspectives

As misdiagnosis has major implications regarding patient treatment, we believe efforts should be taken to improve existing assays for the detection of known pathogenic antibodies, including but not limited to the antidomain I antibodies. Of note, a possible threat that may delay the inclusion of assays detecting antidomain I antibodies in the classification criteria of APS is the wide availability of different assays and their lack of standardization, resulting in large inter-assay variability. Apart from the known standardization that is currently ongoing for the anti- β_2 GPI and anti-CL antibodies, including preparation of standards, reference material, defining international units and establishing cut-off values, additional measures may be required for the antidomain I assays. Taking into account that one of its pathogenic epitopes is cryptic and negatively charged, one can imagine the importance of strict guidelines regarding the production of domain I and the coating procedures. Of note, our antibodies P1-117 and P2-6 may be included in the different antidomain I and full-length β_2 GPI assays as positive controls to ensure satisfactory exposure of the cryptic epitope.^{42,44}

Investigation of domain I pathogenicity paved the way for novel therapeutic approaches for APS. The idea to specifically block pathogenic antidomain I antibodies without interfering with host biology seems to be interesting. The development of recombinant domain I or a variant as a potential therapeutic agent has been suggested based on its ability to inhibit pathogenic effects of antidomain I antibodies.³⁸ A monoclonal antibody against domain I, not able to activate complement was reported as a potential therapeutic tool, in both thrombotic and obstetric complications.³⁷ Tolerogenic dendritic cells specific for domain I but not domain V resulted in an efficient reduction of the fetal loss rate in a mouse model, by induction of regulatory T cells.⁸¹

Importantly, anti- β_2 GPI and anti-CL antibodies, and in the same line antidomain I antibodies, are mainly measured by quantitative endpoint assays. Taking into account that not all antibodies directed against β_2 GPI, cardiolipin/ β_2 GPI and possibly even domain I are pathogenic, such non-functional assays will result in a relatively high false positivity.⁵ To reduce the risk of false positive results, we feel efforts should be made in the development of functional assays. In contrast with the quantitative anti- β_2 GPI/CL assays, such functional assays should only detect pathogenic antibodies, including the antidomain I antibodies, but possibly also other (non-discovered) aPL. Currently, LA is the only functional assay included in the Sydney criteria. Probably due to the fact that LA measures a functional effect of the antibodies, LA is a better predictor of thrombosis than quantitative solid-phase immunoassays. These LA tests are far from ideal since they require an adequate plasma preparation and are complicated and labor-intensive, as well as being sensitive to the presence of anticoagulants used as therapy in patients

with APS.⁵ Their specificity and sensitivity proved to largely depend on the calculation of cut-off values and the choice of the reagents. The relevance of a weak-positive LA, and the influence of oral anticoagulants need to be further addressed.

Other functional assays have been developed as research tool, including β_2 GPI-dependent LAC test and thrombin generation-based assays, and proved to be promising for the diagnosis of APS.^{82,84} Recently, we combined both assays in one functional ellagic acid-triggered thrombin generation assay performed in the absence and presence of cardiolipin. Our assay proved to be sensitive to monoclonal anti- β_2 GPI antibodies and was able to discriminate between antidomain I positive patients with APS and healthy controls in a small patient cohort. Further validation of this assay in a multi-center study is required.

CONCLUSION

With the increasing evidence that not all anti- β_2 GPI antibodies are pathogenic, the detection of anti- β_2 GPI in the absence of anti-CL and LA may not be sufficient for the classification of patients with APS. For this reason, an update of the Sydney criteria focusing on aPL antibody profiles rather than single positivity seems to be a logical next step. Prospective studies on large population cohorts, including appropriate control groups, are urgently required to further evaluate the significance of both criteria and non-criteria (isolated) antibodies in the classification and risk stratification of patients with APS. To be able to compare results within different centers, a consensus has to be reached first on the standardization of the assays. As to the criteria antibodies, using the different available methodologies will allow to solidly determine the impact of the variable exposure of domain I in the currently available assays on the classification of patients with APS. Although diverse retrospective studies have provided evidence for an added clinical value of antidomain I antibodies in the risk stratification of patients with APS, large longitudinal prospective studies are lacking. In the meanwhile, the search for new, preferentially functional, assays should continue. The risk stratification potential of thrombin generation assays needs to be properly assessed. Large patient studies hopefully will lead to an update of the classification criteria for APS determined in Sydney. The accompanied reduction in the false-positive and false-negative rate and optimized risk stratification may eventually lead to an improved treatment strategy in APS.

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Detection of anti-domain I antibodies by chemiluminescence enables the identification of high-risk antiphospholipid syndrome patients: A multicenter multiplatform study

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ABSTRACT

Background: Classification of the antiphospholipid syndrome (APS) relies predominantly on detecting antiphospholipid antibodies (aPLs). Antibodies against a domain I (DI) epitope of anti- β 2 glycoprotein I (β 2GPI) proved to be pathogenic, but are not included in the current classification criteria.

Objectives: Investigate the clinical value of detecting anti-DI IgG in APS.

Patients/Methods: From eight European centers 1005 patients were enrolled. Anti-cardiolipin (CL) and anti- β 2GPI were detected by four commercially available solid phase assays; anti-DI IgG by the QUANTA Flash® β 2GPI domain I assay.

Results: Odds ratios (ORs) of anti-DI IgG for thrombosis and pregnancy morbidity proved to be higher than those of the conventional assays. Upon restriction to patients positive for anti- β 2GPI IgG, anti-DI IgG positivity still resulted in significant ORs. When anti-DI IgG was added to the criteria aPLs or used as a substitute for a β 2GPI IgG/anti-CL IgG, ORs for clinical symptoms hardly improved. Upon removing anti-DI positive patients, LAC remained significantly correlated with clinical complications. Anti-DI IgG are mainly present in high-risk triple positive patients, showing higher levels. Combined anti-DI and triple positivity confers a higher risk for clinical symptoms compared to only triple positivity.

Conclusions: Detection of anti-DI IgG resulted in higher ORs for clinical manifestations than the current APS classification criteria. Regardless of the platform used to detect anti- β 2GPI/anti-CL, addition of anti-DI IgG measured by QUANTA Flash® did not improve the clinical associations, possibly due to reduced exposure of the pathogenic epitope of DI. Our results demonstrate that anti-DI IgG potentially helps in identifying high-risk patients.

INTRODUCTION

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by recurrent thrombosis and/or pregnancy morbidity in combination with the persistent presence of antiphospholipid antibodies (aPLs).¹ Due to the high prevalence of thrombosis and pregnancy morbidity in the general population, classification of APS mainly relies on laboratory results.² Five different assays that detect aPLs are included in the current revised laboratory criteria: lupus anticoagulant (LAC), detecting a phospholipid-dependent prolongation of in vitro clotting times and two immunological quantitative assays measuring immunoglobulin (Ig) G and/or IgM anti-cardiolipin antibodies (anti-CL) and anti- β 2glycoprotein I (anti- β 2GPI) antibodies. Positive tests should be repeated with an interval of at least 12 weeks, to avoid transient positivity due to infections.³

Although laboratory testing of aPLs is critical to the classification of APS, the application and interpretation of these tests remain challenging.^{4,5} The variable clinical performance in conventional commercial and home-made aPL assays not only results from the lack of standardization,^{6,7} but also from the heterogeneity in aPLs.⁸

Accumulating evidence revealed that aPLs are directed against phospholipid-bound plasma proteins, of which β 2GPI proved to be the main target.⁹⁻¹¹ β 2GPI consists of five homologous domains (Domain [D]I-DV). In the native circular or S-shaped conformation, the critical DI epitope is not exposed. Upon binding to an anionic phospholipid (PL) surface through the positively charged patch on DV, β 2GPI undergoes a conformational change.¹² Consequently, the DI-IV spreads out resulting in a more open J shape, exposing a cryptic epitope G40-R43 on DI and allowing a subset of anti-DI β 2GPI autoantibodies to bind.¹³⁻¹⁵ Various subsets of anti- β 2GPI antibodies targeting different domains of the protein have been described with clear differences in clinical potential. The subpopulation of aPLs that recognize this epitope comprising at least G40-R43 on DI proved to be pathogenic in vitro/in vivo, and in clinical studies,¹⁶⁻²⁰ while aPLs that recognize other domains of β 2GPI seem to be benign.²¹⁻²⁴

The aim of this study is to assess the clinical relevance of antibodies against DI of β 2GPI in APS patients in an international multicenter study and evaluate the added value of detecting anti-DI IgG compared to the conventional assays, as well as whether the added value of the anti-DI IgG assay measured by this QUANTA Flash® depends on the platform used to detect anti- β 2GPI and anti-CL IgG. The commercially available chemiluminescence (CIA) assay for anti-DI IgG was used in combination with anti-CL and anti- β 2GPI assays of different manufacturers. Assays were selected based on

frequently used assays in the external quality control program of the ECAT (External quality Control of diagnostic Assays and Tests, Voorschoten, the Netherlands) and willingness of manufacturers for collaboration.

METHODS

Study population

Patient and control samples were collected from eight European medical centers. The study was approved by the local ethics committees. General characteristics (eg, age, gender, ethnicity), clinical characteristics (eg, thrombotic events with specification of the type, pregnancy morbidity, autoimmune disease), and previous laboratory determinations (eg, LAC tests, IgG/IgM anti-CL, and anti- β 2GPI reactivity) were recorded. In total, the database enrolled 1005 samples and samples were allocated to six different groups according to the information of the centers.

The classification of APS was based on the Sydney criteria.³ Classification was determined using the local aPL assay panel for LAC and IgG/IgM anti-CL or anti- β 2GPI antibodies. Control populations consisted of patients with an autoimmune disease other than APS (eg, systemic lupus erythematosus and systemic sclerosis), meeting the criteria for the classification of autoimmune disease without Sydney criteria thrombotic or pregnancy morbidity complications (autoimmune disease other than APS [AID] controls); patients that were referred for aPLs testing for other reasons than the clinical criteria of APS (eg, subfertility and prolonged activated partial thromboplastin time [aPTT], normal controls); patients with a previous thrombotic event negatively tested for aPLs (non-APS thrombosis); and patients that experienced obstetric complications in the absence of aPLs (non-APS obstetric). In the normal control-female population ($n = 169$) there was no history of pregnancy morbidity. Of the 169 women 119 (70.4%) were characterized by subfertility without previous pregnancy; of the 50 other control females information on whether they were (successfully) pregnant before was not available.

Methodology

Anti-CL IgG, anti-CL IgM, anti- β 2GPI IgG, and anti- β 2GPI IgM were detected by four solid phase assays: BioPlex®2200 (Bio-Rad, Bio-Rad Laboratories), Phadia® (Thermo Fisher Scientific/Phadia), HemosIL AcuStar® (Instrumentation Laboratories), and QUANTA Lite® ELISA (Inova Diagnostics). Anti-DI IgG was detected by the CIA of QUANTA Flash® β 2GPI domain I assay (Inova Diagnostics) on the ACL AcuStar® platform. All tests were performed between February 2016 and October 2016 by a single technician in the Ghent University Hospital following the instructions of the manufacturer. Values below

the calculated limit of detection (LOD) were replaced by the LOD. The cutoff values from the manufacturers' recommendation were confirmed in 20 healthy individuals according to the Clinical and Laboratory Standards Institute (CLSI) guidelines²⁵ and guidance from the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis (ISTH).⁵ Based on the cut-off values (20 arbitrary units ie U/mL, GPL, MPL, SGU, SMU on platforms of HemosIL AcuStar®, BioPlex®2200, and QUANTA Lite® ELISA; 10 arbitrary units on Phadia® for anti-CL IgG/IgM and anti-β2GPI IgG/IgM; 20 chemiluminescence units [CU] on QUANTA Flash® for anti-DI IgG), positive samples were identified. LAC positivity was determined by the individual center, according to the ISTH guidelines.⁷

Statistics

Statistical analyses were performed using IBM SPSS® Statistics 24 (IBM SPSS). To avoid a possible bias induced by the classification performed by the eight individual centers, we selected "clinically affected versus clinically not affected" as outcome variable instead of "APS versus non-APS classification." Relationships between the different laboratory assays and the clinical events (thrombosis or pregnancy morbidity) of the patients were investigated by calculating odds ratios (ORs) and 95% confidence intervals (95% CI) in the respective subpopulations. To determine the association with thrombosis and pregnancy morbidity, analysis was restricted to the thrombosis subpopulation (thrombotic APS, non-APS thrombosis, AID, and normal controls [n = 851]) and the pregnancy morbidity subpopulation (obstetric APS, non-APS obstetric, female AID, and female normal controls [n = 481]), respectively. Subsequently, the chi-squared test was used for the comparison of dichotomous variables, and anti-DI IgG titers were compared between groups by the Kruskal-Wallis H test (more than two groups) or Mann-Whitney U test (two groups). Finally, the kappa agreement of anti-DI IgG and conventional aPL tests was studied via the chi-squared test, and the correlation between the titer of anti-DI IgG, anti-β2GPI IgG, and anti-CL IgG was performed by a Spearman rank correlation test. $P < .05$ (two-tailed) were considered statistically significant.

RESULTS

Characteristics of the study population

The patients' demographic data and clinical characteristics are summarized in Table 1. To evaluate associations with thrombotic events and pregnancy morbidity separately, the characteristics of two subpopulations are shown in Table 1.

TABLE 1. Demographic and clinical characteristics of the study population

	A. The thrombosis subpopulation (N = 851)			
	Thrombotic APS	AID	Non-APS thrombosis	Normal controls
Patients (N)	258	196	204	193
Gender				
Female [N(%)]	165 (64.0)	158 (80.6)	116 (56.9)	169 (87.6)
Age (Mean $\pm$ SD, years)	49.6 $\pm$ 14.7	46.4 $\pm$ 14.2	46.5 $\pm$ 14.1	39.4 $\pm$ 11.0
Clinical features [N (%)]				
Thrombosis	258 (100.0)	0	204 (100)	0
AT	54 (20.9)	0	47 (23.0)	0
VT	160 (62.0)	0	149 (73.0)	0
AT + VT	26 (10.1)	0	5 (2.5)	0
Small vessel	4 (1.6)	0	0 (0)	0
Pregnancy morbidity	23 (8.9)	2 (1.0)	0	0
A	6 (2.3)	0	0	0
B	6 (2.3)	0	0	0
C	6 (2.3)	0	0	0
NS/ non-Sydney criteria	5 (1.9)	2 (1.0)	0	0

	B. The pregnancy morbidity subpopulation (N = 481)			
	Obstetric APS	AID-female	Non-APS obstetric	Normal controls-female
Patients (N)	121	158	33	169
Age (Mean $\pm$ SD, years)	34.3 $\pm$ 6.6	45.4 $\pm$ 14.5	32.7 $\pm$ 5.6	38.4 $\pm$ 9.6
Clinical features [N (%)]				
Thrombosis	9 (7.4)	0	0	0
AT	3 (2.5)	0	0	0
VT	5 (4.1)	0	0	0
AT + VT	1 (0.8)	0	0	0
Small vessel	0 (0)	0	0	0
Pregnancy morbidity	121 (100.0)	2 (1.3)	33 (100.0)	0
A	35 (28.9)	0	9 (27.3)	0
B	10 (8.3)	0	4 (12.1)	0
C	67 (55.4)	0	6 (18.2)	0
B+C	2 (1.7)	0	1 (3.0)	0
A+C	3 (2.5)	0	1 (3.0)	0
NS/non-Sydney criteria	4 (3.3)	2 (1.3)	12 (36.4)	0

Note: A, history of three or more consecutive unexplained losses <10 weeks gestation; B, history of fetal death after 10 weeks before gestation; C, history of premature birth(s) before 34 weeks due to preeclampsia or placental insufficiency based on the Sydney classification criteria. NS, non-specified pregnancy complications; non-Sydney criteria, not fulfilling Sydney criteria for pregnancy morbidity. Abbreviations: AID, autoimmune disease other than APS; APS, antiphospholipid syndrome; AT, arterial thrombosis; N, number of patients; SD, standard deviation; VT, venous thrombosis.

Association between anti-DI IgG and clinical symptoms of APS

Independent of the platform, a significant association with clinical events was found for all tested aPL assays, with ORs varying from 2.7 (95% CI 1.9-3.9) to 5.4 (95% CI 3.0-9.6) for thrombosis, 2.3 (95% CI 1.5-3.3) to 4.1 (95% CI 2.5-6.6) for arterial thrombosis, 2.0 (95% CI 1.4-2.8) to 2.8 (95% CI 1.9-4.2) for venous thrombosis, and 2.7 (95% CI 1.7-4.3) to 5.6 (95% CI 3.1-9.9) for pregnancy morbidity (Table 2A). Compared to the criteria aPL assays, detection of anti-DI IgG was less sensitive (21.9%/26.6% for thrombosis/pregnancy morbidity), but more specific (94.9%/93.9% for thrombosis/pregnancy morbidity), overall resulting in higher ORs for both clinical criteria of APS compared to LAC, anti- β 2GPI, or anti-CL IgG except for the QUANTA Lite® ELISA platform. Similar ORs of anti-DI were found for thrombosis and pregnancy morbidity, and no difference in association was observed between venous versus arterial thrombosis. Interestingly, upon restriction to patients positive for anti- β 2GPI IgG, anti-DI IgG positivity still resulted in significant—albeit lower—ORs for both clinical criteria of APS except for the QUANTA Lite® ELISA platform (Table 2B).

Additional clinical value of detecting anti-DI IgG on top of the currently used laboratory tests

Table 3 shows the number of patients testing positive or negative for anti-DI IgG in relation to positivity for LAC or anti- β 2GPI IgG or at least one of the criteria aPL panel measured by HemosIL AcuStar® considering the previously defined subpopulations. The results of the other three solid phase assays can be found in Table S1 in supporting information.

From Table 3, looking at the thrombosis subpopulation, 13 out of the 19 LAC negative anti-DI positive patients had thrombosis. Additionally, the four samples testing negative for anti- β 2GPI IgG by HemosIL AcuStar® but positive for anti-DI IgG all had thrombosis. Similarly, the two individuals testing negative for the criteria aPL panel by HemosIL AcuStar® and positive for anti-DI IgG both suffered from thrombosis. Concerning the pregnancy morbidity subpopulation, 12 out of the 18 LAC negative anti-DI IgG positive patients experienced pregnancy morbidity. One patient testing negative for anti- β 2GPI IgG by HemosIL AcuStar® and positive for anti-DI IgG had pregnancy morbidity. Moreover, only one individual negative for the criteria aPL panel by HemosIL AcuStar® tested positive for anti-DI IgG and suffered from pregnancy morbidity. Looking at Table S1, both for the thrombosis subpopulation or the pregnancy morbidity subpopulation, similar results were found when anti- β 2GPI or anti-CL IgG/IgM were detected by BioPlex®2200 although more samples tested negative for anti- β 2GPI IgG by Phadia®

TABLE 2. Additional diagnostic value of aCL and $\alpha\beta 2\text{GPI}$ IgA antibodies. Figures indicate the number of patients testing positive

A. In the total population									
	Manufacturer	Thrombosis ^a				Arterial thrombosis ^{a,d}			
		N	Sensitivity (%)	Specificity (%)	OR	95%CI	N#	Sensitivity (%)	Specificity (%)
LAC		851	43.7	81.5	3.4	2.5-4.7	834	47.0	71.8
Anti-CL IgG	HemosIL AcuStar®	851	24.7	92.5	4.1	2.6-6.3	834	31.8	86.2
	BioPlex® 2200	851	26.4	89.7	3.1	2.1-4.6	834	34.1	83.9
	Phadia®	851	23.6	93.1	4.1	2.7-6.5	834	31.1	87.2
	QUANTA Lite ELISA®	851	21.4	94.3	4.6	2.8-7.4	834	28.8	88.6
Anti- $\beta 2\text{GPI}$ IgG	HemosIL AcuStar®	851	30.1	86.4	2.7	1.9-3.9	834	36.4	80.2
	BioPlex® 2200	851	26.2	88.7	2.8	1.9-4.1	834	34.1	83.5
	Phadia®	851	22.5	92.3	3.5	2.6-5.4	834	29.5	87.0
	QUANTA Lite ELISA®	851	16.7	96.4	5.4	3.0-9.6	834	25.8	92.2
Anti-DI IgG	QUANTA Flash®	851	21.9	94.9	5.2	3.1-8.5	834	27.3	88.5
Triple positivity	HemosIL AcuStar®	851	24.0	94.1	5.0	3.1-8.1	834	28.8	87.2
	BioPlex® 2200	851	25.5	92.8	4.4	2.9-6.9	834	31.1	86.2
	Phadia®	851	21.9	93.8	4.3	2.7-6.8	834	26.5	88.0
	QUANTA Lite ELISA®	851	19.3	95.6	5.2	3.0-8.9	834	24.2	90.3
Anti-DI IgG plus triple positivity	HemosIL AcuStar®	851	18.8	96.7	6.7	3.7-12.2	834	24.2	90.9
	BioPlex® 2200	851	19.0	96.7	6.8	3.7-12.4	834	24.2	90.7
	Phadia®	851	16.9	96.9	6.4	3.4-11.9	834	22.0	91.9
	QUANTA Lite ELISA®	851	15.6	97.4	7.0	3.6-13.8	834	21.2	92.7
B. ORs of anti-DI IgG QUANTA Lite ELISA® for the indicated clinical manifestation of APS in anti-$\beta 2\text{GPI}$ IgG positive individuals									
	Manufacturer for anti- $\beta 2\text{GPI}$	Thrombosis ^a				Pregnancy complications ^b			
		N	Sensitivity (%)	Specificity (%)	OR	95%CI	N	Sensitivity (%)	Specificity (%)
anti-DI IgG	HemosIL AcuStar®	192	69.8	62.3	3.8	2.0-7.4	101	78.4	60.0
	BioPlex® 2200	165	79.3	54.5	4.6	2.2-9.6	90	81.6	51.2
	Phadia®	134	82.7	40.0	3.2	1.3-7.8	68	87.2	37.9
	QUANTA Lite ELISA®	91	89.6	14.3	1.4	0.3-7.6	35	95.5	7.7

Note: Significant ORs are shown in bold and calculated according to the outcome variable "clinically affected versus clinically not-affected patients". Abbreviations: aPLs, antiphospholipid antibodies; APS, antiphospholipid syndrome; $\beta 2\text{GPI}$, $\beta 2\text{glycoprotein I}$; CL, cardiolipin; DI, domain I; IgG, immunoglobulin G; LAC, lupus anticoagulant; N, number of patients; OR, odds ratio; 95% CI, 95% confidence interval; Triple positivity, LAC positive, anti-CL IgG/IgM positive and anti- $\beta 2\text{GPI}$ IgG/IgM positive. ^aThrombotic APS + non-APS thrombosis versus non-thrombotic population (AID + normal controls). ^bObstetric APS + non-APS obstetric versus non-obstetric population (AID-female + normal controls-female). ^cIn the thrombosis subpopulation, 17 individuals without specified thrombosis type were defined as missing data. ^dArterial thrombosis versus non-arterial thrombosis. ^eVenous thrombosis versus non-venous thrombosis.

or negative for LAC, aCL and aβ2GPI IgG/M antibodies versus aCL and aβ2GPI IgA, for each solid-phase assay.

Venous thrombosis ^{a,e}						Pregnancy complications ^b					
OR	95%CI	N#	Sensitivity (%)	Specificity (%)		OR	95%CI	N	Sensitivity (%)	Specificity (%)	
2.3	1.5-3.3	834	43.2	77.1		2.6	1.9-3.5	481	48.7	81.7	4.2
2.9	1.9-4.4	834	24.4	88.7		2.5	1.7-3.7	481	29.2	91.4	4.4
2.7	1.8-4.1	834	25.9	85.8		2.1	1.5-3.0	481	31.8	88.1	3.4
3.1	2.0-4.7	834	22.6	89.1		2.4	1.6-3.5	481	29.2	92.4	5.0
3.1	2.0-4.9	834	20.9	90.5		2.5	1.7-3.7	481	24.7	93.9	5.0
2.3	1.6-3.5	834	30.0	82.8		2.1	1.5-2.9	481	33.1	84.7	2.7
2.6	1.7-3.9	834	25.6	85.0		2.0	1.4-2.8	481	31.8	87.5	3.3
2.8	1.8-4.3	834	21.8	88.7		2.2	1.5-3.2	481	25.3	91.1	3.5
4.1	2.5-6.6	834	15.3	92.5		2.2	1.4-3.5	481	14.3	96.0	4.0
2.9	1.8-4.5	834	21.5	91.1		2.8	1.9-4.2	481	26.6	93.9	5.6
2.7	1.8-4.3	834	24.1	90.7		3.1	2.1-4.6	481	22.1	93.3	3.9
2.8	1.8-4.3	834	24.7	89.1		2.7	1.8-3.9	481	22.7	91.7	3.3
2.7	1.7-4.2	834	21.5	90.7		2.7	1.8-4.0	481	19.5	93.6	3.5
3.0	1.9-4.8	834	18.5	92.5		2.8	1.8-4.3	481	13.0	95.1	2.9
3.2	2.0-5.1	834	18.2	93.1		3.0	1.9-4.7	481	18.8	96.0	5.6
3.1	2.0-5.0	834	18.5	93.1		3.1	2.0-4.8	481	18.8	96.0	5.6
3.2	1.9-5.2	834	16.2	93.7		2.9	1.8-4.6	481	16.2	96.3	5.1
3.4	2.1-5.7	834	14.7	94.1		2.8	1.7-4.5	481	12.3	96.9	4.5

OR	95%CI
5.5	2.3-13.1
4.7	1.8-12.0
4.2	1.3-13.8
1.8	0.1-30.6

TABLE 3. Number of patients testing positive (+) or negative (-) for anti-DI IgG in relation to positivity for LAC or anti-β2GPI IgG or at least one of the criteria aPL panel measured by HemosIL AcuStar®, see Supplementary Table 1 for the other platforms

		Thrombosis subpopulation (N = 851)		With T (N = 462)		Without T (N = 389)		Pregnancy morbidity subpopulation (N = 481)		With P (N = 154)		Without P (N = 327)	
		LAC	Anti-CL IgG	LAC	Anti-CL IgG	LAC	Anti-CL IgG	LAC	Anti-CL IgG	LAC	Anti-CL IgG	LAC	Anti-CL IgG
Anti-DI IgG	+	102	19	88	13	14	6	43	18	29	12	14	6
	-	172	558	114	247	58	311	92	328	46	67	46	261
Total		274	577	202	260	72	317	135	346	75	79	60	267
Anti-β2GPI IgG													
	+		Anti-CL IgG		Anti-CL IgG		Anti-CL IgG		Anti-CL IgG		Anti-CL IgG		Anti-CL IgG
Anti-DI IgG	+	114	7	95	6	19	1	59	2	40	1	19	1
	-	29	701	19	342	10	359	14	406	5	108	9	298
Total		143	708	114	348	29	360	73	408	45	109	28	299
Anti-β2GPI IgG													
	+		Anti-β2GPI IgG		Anti-β2GPI IgG		Anti-β2GPI IgG		Anti-β2GPI IgG		Anti-β2GPI IgG		Anti-β2GPI IgG
Anti-DI IgG	+	117	4	97	4	20	0	60	1	40	1	20	0
	-	75	655	42	319	33	336	41	379	11	102	30	277
Total		192	659	139	323	53	336	101	380	51	103	50	277
Criteria aPL panel													
	+		Criteria aPL panel		Criteria aPL panel		Criteria aPL panel		Criteria aPL panel		Criteria aPL panel		Criteria aPL panel
Anti-DI IgG	+	119	2	99	2	20	0	60	1	40	1	20	0
	-	230	500	142	219	88	281	134	286	63	50	71	236
Total		349	502	241	221	108	281	194	287	103	51	91	236

Abbreviations: aPL, antiphospholipid antibody; β2GPI, β2glycoprotein I; DI, domain I; IgG (M), immunoglobulin G (M); LAC, lupus anticoagulant; N, number of patients; P, pregnancy morbidity; T, thrombosis. ^aCriteria aPL panel positivity: patients testing positive for at least one of the criteria aPL panel (i.e. LAC, anti-CL IgG/M and/or anti-β2GPI IgG/M).

TABLE 4. ORs of the current criteria aPL panel and two newly defined aPL panels including anti-DI IgG for clinical manifestations of APS

Manufacturer		Thrombosisb				Pregnancy morbidityc					
		N	Sensitivity (%)	Specificity (%)	OR	95% CI	N	Sensitivity (%)	Specificity (%)	OR	95% CI
HemosIL AcuStar®	Criteria aPL panela	851	52.2	72.2	2.8	2.1-3.8	481	66.9	72.2	5.2	3.5-7.9
	Criteria aPL panel with addition of anti-DI IgG	851	52.6	72.2	2.9	2.2-3.8	481	67.5	72.2	5.4	3.6-8.2
	Criteria aPL panel with anti-DI IgG replacing anti-β2GPI IgG and anti-CL IgG	851	49.6	73.5	2.7	2.0-3.6	481	64.3	73.4	5.0	3.3-7.5
BioPlex® 2200	Criteria aPL panela	851	48.9	75.1	2.9	2.2-3.9	481	63.0	74.6	5.0	3.3-7.5
	Criteria aPL panel with addition of anti-DI IgG	851	49.6	75.1	3.0	2.2-4.0	481	63.0	74.6	5.0	3.3-7.5
	Criteria aPL panel with anti-DI IgG replacing anti-β2GPI IgG and anti-CL IgG	851	48.5	76.6	3.1	2.3-4.1	481	61.7	76.5	5.2	3.5-7.9
Phadia®	Criteria aPL panela	851	53.2	68.1	2.4	1.8-3.2	481	69.5	68.2	4.9	3.2-7.4
	Criteria aPL panel with addition of anti-DI IgG	851	54.1	68.1	2.5	1.9-3.3	481	70.1	68.2	5.0	3.3-7.6
	Criteria aPL panel with anti-DI IgG replacing anti-β2GPI IgG and anti-CL IgG	851	51.1	69.9	2.4	1.8-3.2	481	66.9	70.0	4.7	3.1-7.1
QUANTA Lite ELISA®	Criteria aPL panela	851	50.4	73.5	2.8	2.1-3.8	481	69.5	74.3	6.6	4.3-10.1
	Criteria aPL panel with addition of anti-DI IgG	851	51.7	73.0	2.9	2.2-3.9	481	70.1	73.7	6.6	4.3-10.1
	Criteria aPL panel with anti-DI IgG replacing anti-β2GPI IgG and anti-CL IgG	851	51.3	73.3	2.9	2.2-3.9	481	66.9	74.0	5.8	3.8-8.7

Note: Significant ORs are shown in bold and calculated according to the outcome variable "clinically affected versus clinically not-affected patients." Abbreviations: aPL, antiphospholipid antibody; APS, antiphospholipid syndrome; β2GPI, β2glycoprotein I; CL, cardiolipin; DI, domain I; IgG(M), immunoglobulin G(M); LAC, lupus anticoagulant; N, number of patients; OR, odds ratio; 95% CI, 95% confidence interval. ^aCriteria aPL panel positivity: patients testing positive for at least one of the criteria aPL panel (i.e. LAC, anti-CL IgG/M and/or anti-β2GPI IgG/M). ^bThrombotic APS + non-APS thrombosis versus non-thrombotic population (AID + normal controls). ^cObstetric APS + non-APS obstetric versus non-obstetric population (AID-female + normal controls-female).

and QUANTA Lite® ELISA but positive for anti-DI IgG, and most of them had thrombosis or pregnancy morbidity. Including LAC, fewer individuals negative for the criteria aPL panel tested positive for anti-DI IgG and suffered from clinical events.

To assess the additional clinical value of anti-DI IgG on top of the currently used aPL measured by different platforms, anti-DI IgG was added to the criteria aPL panel or used as a substitute for anti-β2GPI IgG and anti-CL IgG in the criteria aPL panel. For all platforms, the addition of anti-DI IgG to the current criteria aPL panel hardly resulted in an increase of the OR for thrombosis or pregnancy morbidity. Replacement of anti-β2GPI IgG and anti-CL IgG by anti-DI IgG resulted in comparable ORs for both clinical symptoms. For all platforms, a small increase was observed in specificity when replacing anti-β2GPI IgG and anti-CL IgG by anti-DI IgG, mostly accompanied by a decrease in sensitivity (Table 4).

To investigate if anti-DI antibodies are the only pathogenic antibodies, we re-evaluated the correlation of LAC, anti-CL IgG, and anti-β2GPI IgG with clinical manifestations of APS in anti-DI IgG negative patients. Interestingly, upon removal of anti-DI positive samples, positivity for LAC still resulted in significant ORs for thrombosis (2.5, 95% CI 1.7-3.5) and for pregnancy morbidity (3.9, 95% CI 2.4-6.4) while correlations for anti-β2GPI IgG and anti-CL IgG antibodies were no longer statistically significant except for when the anti-CL IgG was measured by Phadia® and QUANTA Lite® ELISA (OR for thrombosis of 2.3 (95% CI 1.1-4.8) and 2.4 (95% CI 1.0-5.5), respectively; Table 5).

Distribution of anti-DI IgG titers according to the patient's antibody profile

For both the thrombosis and pregnancy morbidity subpopulation, all individuals were grouped according to their aPL profile into triple positive (LAC +, anti-CL IgG/IgM +, anti-β2GPI IgG/IgM +), double positive (LAC –, anti-CL IgG/IgM +, anti-β2GPI IgG/IgM + or LAC +, anti-CL IgG/IgM +, anti-β2GPI IgG/IgM – or LAC +, anti-CL IgG/IgM –, anti-β2GPI IgG/IgM +), and single positive (isolated positive for LAC, anti-CL IgG/IgM, or anti-β2GPI IgG/IgM). Results were compared with those of patients without any aPL reactivity (negative control). The different antibody profiles with their characteristics are shown in Table 6 for anti-β2GPI and anti-CL measured by HemosIL AcuStar® and in Table S2-S4 in supporting information for anti-β2GPI and anti-CL measured by the three other solid-phase assays.

Furthermore, we compared anti-DI IgG titers in the triple positive, solid-phase double-positive (anti-CL IgG/IgM positive and anti-β2GPI IgG/IgM positive but LAC negative), combined single-positive group (isolated LAC +, and isolated anti-CL IgG/IgM +, and

isolated anti- β 2GPI IgG/IgM +), and the negative control. Comparison of anti-DI IgG titers between four aPL profile groups for anti- β 2GPI and anti-CL measured by four assays is shown in Figure 1. Looking at HemosIL AcuStar®, in both the thrombosis subpopulation and pregnancy morbidity subpopulation anti-DI titers were significantly higher in triple positive samples compared to the other aPL profile groups, followed by the solid-phase double-positive samples. No difference was observed between anti-DI titers of combined single positive and negative samples. Similar results were found for the three other assays, except that there was a significant difference between anti-DI titers of combined single positive and negative samples for anti- β 2GPI and anti-CL measured by QUANTA Lite® ELISA (Figure 1).

TABLE 5. ORs of LAC, anti-CL IgG and anti- β 2GPI IgG with clinical manifestations of APS upon the removal of anti-DI IgG positive patients

		Thrombosis ^a				Pregnancy morbidity ^b			
Manufacturer		Population (N [#])	Positive (N [§])	OR	95% CI	Population (N [#])	Positive (N [§])	OR	95% CI
LAC		730	172	2.5	1.7-3.5	420	92	3.9	2.4-6.4
Anti-CL IgG	HemosIL AcuStar®	730	29	2.0	0.9-4.4	420	14	1.5	0.5-4.7
	BioPlex® 2200	730	47	1.3	0.7-2.3	420	29	1.2	0.5-2.8
	Phadia®	730	35	2.3	1.1-4.8	420	16	2.2	0.8-6.0
	QUANTA Lite ELISA®	730	26	2.4	1.0-5.5	420	11	2.3	0.7-7.8
Anti- β 2GPI IgG	HemosIL AcuStar®	730	75	1.3	0.8-2.2	420	41	1.0	0.5-2.1
	BioPlex® 2200	730	49	1.1	0.6-1.9	420	30	1.2	0.5-2.7
	Phadia®	730	30	1.6	0.7-3.3	420	16	1.2	0.4-3.7
	QUANTA Lite ELISA®	730	10	4.2	0.9-19.7	420	2	2.7	0.2-44.1

Note: Significant ORs are shown in bold and calculated according to the outcome variable "clinically affected versus clinically not-affected patients." Abbreviations: APS, antiphospholipid syndrome; β 2GPI, β 2glycoprotein I; CL, cardiolipin; DI, domain I; IgG, immunoglobulin G; LAC, lupus anticoagulant; N[#], number of patients; N[§], number of biomarker positive cases; OR, odds ratio; 95% CI, 95% confidence interval. ^aThrombotic APS + non-APS thrombosis versus non-thrombotic population (AID + normal controls).

^bObstetric APS + non-APS obstetric versus non-obstetric population (AID - female + normal controls - female).

Association of triple positivity with anti-DI positivity

The association between DI positivity and triple positivity was studied in detail for anti- β 2GPI and anti-CL measured by the Acustar® (Table 6). As to the thrombosis subpopulation (Table 6), positive values of anti-DI IgG were found in 100 out of 134 individuals with triple positivity (74.6%), compared with 44.1% (15/34) in the solid-phase double-positive group (Pearson chi-square, $P = .002$). Combined single-positive (2/146 = 1.4%) and aPL-negative patients (2/502 = 0.4%) hardly showed any anti-DI IgG

TABLE 6 Number of cases with distinct antibody profiles (anti-CL IgG/IgM and anti-β2GPI IgG/IgM measured by HemosIL AcuStar®), see Supplementary Table 2-4 for the other platforms

In the thrombosis subpopulation	aPL profile		Anti-DI IgG positive				Anti-DI IgG negative			
			Total		without T		Total		with T	
	LAC	anti-CL IgG/IgM	anti-β2GPI IgG/IgM	N (%) ^a	N (%) ^b	N (%) ^c	N (%) ^b	N (%) ^c	N (%) ^c	N (%) ^c
Goup1	+	+	+	134 (16)	100 (75)	87 (87)	13 (13)	34 (25)	24 (71)	10 (29)
Goup2	-	+	+	34 (4)	15 (44)	9 (60)	6 (40)	19 (56)	7 (37)	12 (63)
Goup3	+	+	-	14 (2)	0	0	0	14 (100)	7 (50)	7 (50)
Goup4	+	-	+	21 (2)	2 (10)	1 (50)	1 (50)	19 (90)	7 (37)	12 (63)
Goup5	+	-	-	105 (12)	0	0	0	105 (100)	76 (72)	29 (28)
Goup6	-	+	-	26 (3)	1 (4)	1	0	25 (96)	11 (44)	14 (56)
Goup7	-	-	+	15 (2)	1 (7)	1 (100)	0	14 (93)	10 (71)	4 (29)
Goup8	-	-	-	502 (59)	2 (0.4)	2 (100)	0	500 (99.6)	219 (44)	281 (56)
Total				851 (100)	121 (14)	101 (83)	20 (17)	730 (86)	361 (49)	369 (51)

In the pregnancy morbidity subpopulation	aPL profile		Anti-DI IgG positive				Anti-DI IgG negative			
			Total		without P		Total		with P	
	LAC	anti-CL IgG/IgM	anti-β2GPI IgG/IgM	N (%) ^a	N (%) ^b	N (%) ^c	N (%) ^b	N (%) ^c	N (%) ^c	N (%) ^c
Goup1	+	+	+	56 (12)	42 (75)	29 (69)	13 (31)	14 (25)	5 (36)	9 (64)
Goup2	-	+	+	35 (7)	17 (49)	11 (65)	6 (35)	18 (51)	8 (44)	10 (56)
Goup3	+	+	-	9 (2)	0	0	0	9 (100)	2 (22)	7 (78)
Goup4	+	-	+	13 (3)	1 (8)	0	1 (100)	12 (92)	1 (8)	11 (92)
Goup5	+	-	-	57 (12)	0	0	0	57 (100)	38 (67)	19 (33)
Goup6	-	+	-	16 (3)	0	0	0	16 (100)	5 (31)	11 (69)
Goup7	-	-	+	8 (2)	0	0	0	8 (100)	4 (50)	4 (50)
Goup8	-	-	-	287 (60)	1 (0.3)	1 (100)	0	286 (99.7)	50 (17)	236 (83)
Total				481 (100)	61 (13)	41 (67)	20 (33)	420 (87)	113 (27)	307 (73)

Abbreviations: aPL, antiphospholipid antibody; β2GPI, β2glycoprotein I; CL, cardiolipin; DI, domain I; IgG (M), immunoglobulin G (M); LAC, lupus anticoagulant; N, number of patients in each group; P, pregnancy morbidity; T, Thrombosis. ^aThe percentage of each group antibody profile. ^bThe percentage of anti-DI IgG positive/negative in each group antibody profile. ^cThe percentage of individuals with/without clinical events.

FIGURE 1. Distribution of the titer of anti-DI IgG according to distinct antibody profiles for anti-CL and anti- β 2GPI measured by HemosIL AcuStar®, BioPlex®2200, Phadia®, and QUANTA Lite® ELISA in the thrombosis subpopulation (A) and pregnancy morbidity subpopulation (B). Investigated aPL profiles include triple positive (LAC +, anti-CL IgG/IgM +, anti- β 2GPI IgG/IgM +), solid phase double positive (LAC –, anti-CL IgG/IgM +, anti- β 2GPI IgG/IgM +), and combined single positive (isolated LAC +, and isolated anti-CL IgG/IgM +, and isolated anti- β 2GPI IgG/IgM +). Patients without any aPL reactivity were defined as negative controls. Titer of anti-DI IgG are expressed as the median with interquartile ranges within each profile; dashed lines indicate the cut-off value of anti-DI IgG (20 CU). * $P < .05$, ** $P < .01$, *** $P < .0001$, ns: not significant. Abbreviations: aPL, antiphospholipid antibody; DI, domain I; IgG, immunoglobulin G; CU, chemiluminescence units

Table 7 Matrix of correlation between anti-DI IgG and anti-β2GPI IgG or anti-CL IgG in the indicated subpopulation

A. In the thrombosis subpopulation					B. In the pregnancy morbidity subpopulation				
Agreement of pos/neg (Kappa value)	HemosIL AcuStar®	BioPlex®2200	Phadia®	QUANTA Lite® ELISA	Agreement of pos/neg (Kappa value)	HemosIL AcuStar®	BioPlex®2200	Phadia®	QUANTA Lite® ELISA
anti-DI IgG vs. anti-β2GPI IgG	0.694***	0.774***	0.783***	0.731***	anti-DI IgG vs. anti-β2GPI IgG	0.692***	0.758***	0.776***	0.656***
anti-DI IgG vs. anti-CL IgG	0.839***	0.776***	0.748***	0.750***	anti-DI IgG vs. anti-CL IgG	0.861***	0.755***	0.797***	0.760***
Correlation of titers (Spearman's rho)	HemosIL AcuStar®	BioPlex®2200	Phadia®	QUANTA Lite® ELISA	Correlation of titers (Spearman's rho)	HemosIL AcuStar®	BioPlex®2200	Phadia®	QUANTA Lite® ELISA
anti-DI IgG vs. anti-β2GPI IgG	0.808***	0.842***	0.679***	0.581***	anti-DI IgG vs. anti-β2GPI IgG	0.797***	0.838***	0.647***	0.563***
anti-DI IgG vs. anti-CL IgG	0.756***	0.800***	0.645***	0.734***	anti-DI IgG vs. anti-CL IgG	0.729***	0.783***	0.662***	0.747***

Abbreviations: aPL, antiphospholipid antibody; β2GPI, β2glycoprotein I; CL, cardiolipin; DI, domain I; IgG, immunoglobulin G; LAC, lupus anticoagulant
****P* < .0001

reactivity (Fisher's exact test, $P < .0001$). A good agreement was found between triple positivity and anti-DI IgG positivity (Kappa value = 0.746, $P < .0001$). In this triple positive group, 87.0% (87/100) of anti-DI IgG positive individuals had a history of thrombosis, compared with 70.6% (24/34) of anti-DI IgG negative patients (Pearson chi-square, $P = .028$).

Concerning the pregnancy morbidity subpopulation (Table 6), positive values of anti-DI IgG were found in 42 out of 56 individuals with triple positivity (75.0%), compared with 48.6% (17/35) in the solid-phase double-positive group (Pearson chi-square, $P = .013$). As seen in the thrombosis subpopulation, the combined single-positive group ($0/81 = 0\%$) and aPL negative patients ($1/287 = 0.3\%$) hardly showed anti-DI IgG reactivity (Fisher's exact test, $P < 0.0001$). Albeit lower than for the thrombosis subpopulation, also in the pregnancy morbidity subpopulation a good agreement was demonstrated between triple positivity and anti-DI IgG (Kappa value = 0.679, $P < .0001$). In this triple-positive group, 69.0% (29/42) of anti-DI IgG positive individuals had a history of pregnancy morbidity, compared with 35.7% (5/14) of anti-DI IgG negative patients (Fisher's exact test, $P = .027$). In general, similar conclusions can be drawn when anti- β 2GPI and anti-CL were measured by the other assays (Tables S2-S5 in supporting information).

Interestingly, both for thrombosis and pregnancy morbidity, the ORs of anti-DI IgG were higher than the ORs of triple positivity except when anti-CL and anti- β 2GPI were measured by the QUANTA Lite® ELISA platform for thrombosis. To verify if anti-DI positivity has an added value in risk stratification compared to triple positivity, ORs of combined triple positivity and DI positivity were calculated for thrombosis and pregnancy morbidity. As to thrombosis, combined positivity resulted in higher OR compared to the separate OR for both triple positivity and DI reactivity. For pregnancy morbidity, ORs of anti-DI plus triple positivity were higher compared to ORs of triple positivity, but hardly improved compared to OR of anti-DI positivity (Table 2A).

Furthermore, as to the thrombosis subpopulation, within the triple-positive group for anti- β 2GPI and anti-CL measured by Acustar®, anti-DI IgG titers varied greatly but no significant difference was observed between patients with thrombosis ($n = 111$) and unaffected individuals ($n = 23$), with a median (quartile[Q]25-Q75) anti-DI IgG titer of, respectively, 169.0 CU (25.7-761.3) and 63.7CU (3.7-625.7) (Mann-Whiney U test, $P = .084$). Similarly, concerning the pregnancy morbidity subpopulation, anti-DI IgG titers differed greatly but no significant difference between patients with pregnancy morbidity ($n = 34$) and unaffected individuals ($n = 22$) could be demonstrated with a median (Q25-Q75) anti-DI IgG titer of 200.2 CU (49.6-462.1) and 65.0 CU (3.7-654.3), respectively (Mann-Whiney U test, $P = .179$). Similar results were obtained with the three

other assays, although for the BioPlex®2200 (thrombosis and pregnancy morbidity) and Phadia® (thrombosis) results reached statistical significance (Table S6 in supporting information).

Correlation between detection of anti-DI IgG and anti-β2GPI IgG and anti-CL IgG

Concordance was evaluated by comparing the results of the detection of IgG anti-DI antibodies with anti-β2GPI IgG and anti-CL IgG measured by four solid-phase assays (Table 7). A good agreement was established between IgG anti-DI antibodies and IgG anti-CL as well as with IgG anti-β2GPI antibodies (Kappa value = 0.656-0.861) in the thrombosis subpopulation (Table 7A) and the pregnancy morbidity subpopulation (Table 7B). The IgG anti-DI antibody titers were likewise significantly correlated with IgG anti-CL titers and IgG anti-β2GPI titers (Spearman's rho = 0.563-0.842) in the thrombosis subpopulation (Table 7A) and the pregnancy morbidity subpopulation (Table 7B; all $P < .0001$).

DISCUSSION

Solid phase aPL assays included in the laboratory criteria suffer from a lack of standardization, limiting their utility in clinical practice. Variability results not only from methodological shortcomings but also from the heterogeneity of aPLs.^{5,7} More and more literature evidence suggests that detection of a subset of aPLs reactive against DI of β2GPI is a promising classification or risk stratification tool in APS.^{26,27} However, a recent review concluded inconsistent results about the added value of the detection of anti-DI IgG compared to conventional aPL tests, most probably due to differences in study design, study population, and the methodology to detect the anti-DI antibodies.²⁸

In this multicenter study, all samples were measured for aPL reactivity by one technician in the same lab to avoid variability coming from differences in working conditions. In most studies evaluating the added value of anti-DI IgG, the only commercially available assay (CIA of QUANTA Flash®) is compared with anti-β2GPI and anti-CL of the same manufacturer.²⁹⁻³⁵ Importantly, we hypothesized that the added value of the anti-DI IgG assay measured by QUANTA Flash® depends on the platform used to detect anti-β2GPI and anti-CL IgG. This hypothesis comes from our previously published results demonstrating a variable exposure of the G40-R43 epitope on domain I of β2GPI coated in the different commercially available anti-CL and anti-β2GPI assays.^{8,36,37} In this

multicenter study, we therefore determined the added value of anti-DI testing to the current APS classification criteria, when anti-CL and anti- β 2GPI were measured by our different commercially available assays.

As literature is inconclusive whether the same aPL subset induces thrombosis and pregnancy morbidity, separate analyses were performed in a thrombosis and pregnancy morbidity subpopulation, including proper control populations. Both for the thrombosis and the pregnancy morbidity subpopulation, we found that the detection of anti-DI IgG was less sensitive but more specific compared to the laboratory criteria aPL tests, resulting in a higher OR for thrombosis and pregnancy morbidity compared to LAC, anti- β 2GPI, or anti-CL IgG, except for the QUANTA Lite® ELISA. Interestingly, upon restriction to patients positive for anti- β 2GPI IgG, anti-DI IgG positivity still resulted in significant ORs for clinical complications, except for the QUANTA Lite® ELISA (Table 2B); that could be explained by the lower number of anti- β 2GPI IgG detected by this assay (Table S1). These findings are consistent with previous studies.^{19,20,29,30,32} Based on these results, apart from those obtained by the QUANTA Lite® ELISA, we expected anti-DI IgG to have an additional value on top of the current laboratory criteria, or to be a candidate to replace the anti- β 2GPI IgG detection. Contrary to our expectation, the addition of anti-DI or replacement of anti- β 2GPI IgG by anti-DI hardly improved the ORs for thrombosis or pregnancy morbidity. The absence of an added value of anti-DI to the current aPL criteria panel was also found in previous studies.^{34,35}

This at-first-sight contradiction can be explained by the results presented in Table 3 and Table S1. Independent of the assay used, only few individuals (n = 11) negative for the criteria aPL panel (with one or more platforms) become positive when anti-DI IgG is added to the criteria aPLs panel or used as a substitute for anti- β 2GPI IgG and/or anti-CL IgG, even though most of them experienced clinical events (n = 7 with thrombosis, n = 2 with pregnancy morbidity). Of note, despite anti-DI IgG positivity, the titers of anti-DI IgG in these individuals proved to be low (from 21.1 to 73.2 CU).

Interestingly, upon removal of anti-DI IgG positive patients, anti-CL IgG and anti- β 2GPI IgG were no longer significantly correlated with thrombosis and pregnancy morbidity, except for anti-CL IgG measured by Phadia® and by QUANTA Lite® ELISA (OR for thrombosis of 2.3 [95% CI 1.1-4.8] and 2.4 [95% CI 1.0-5.5], respectively; Table 5). Although the OR for thrombosis of anti-CL IgG measured by Phadia® and by QUANTA Lite® ELISA was still significant, the lower limit was adjacent to 1. These findings are in agreement with previous studies demonstrating that especially anti-DI antibodies are pathogenic while antibodies targeting other domains such as domain 4/5 are innocent or even protective antibodies.^{20,23,38,39}

We previously demonstrated that the large variability observed in commercially available anti- β 2GPI assays results at least in part from a variable exposure of the pathogenic G40-R43 DI epitope.⁸ Importantly, our recent data show that, as for anti- β 2GPI IgG assays, the pathogenic DI epitope is not exposed correctly in the commercially available anti-DI CIA assay.²⁸ Furthermore, both as categorical variables (positive/negative) and as quantitative variables (titer), anti-DI IgG strongly correlated with anti-CL IgG and anti- β 2GPI IgG on the same AcuStar® CIA. The high correlation between anti-DI IgG and anti- β 2GPI IgG suggests a high overlap between both assays performed on the automated CIA platform. Previous studies also observed a high agreement (69%-92%) between anti-DI IgG and anti- β 2GPI IgG when measured using the CIA methodology.^{29,34,35,40} These results may explain the absence of an added value of measuring anti-DI using this assay. In this study, and also for the other platforms, a high agreement was found between anti-DI and the anti-CL IgG and anti- β 2GPI IgG and no added value of measuring anti-DI could be demonstrated (Table 7).

Of note, apart from the anti-DI CIA assay used in this study, other methods are available to detect anti-DI antibodies.²⁸ Previously a home-made two-step ELISA strongly indicated that testing for IgG anti-DI enables identification of the patients at highest risk for developing thrombosis or pregnancy morbidity.^{19,20} In contrast to the results obtained by CIA assays, only 50% of the anti- β 2GPI IgG antibodies tested by this home-made two-step ELISA were demonstrated to be reactive against DI. Importantly, using this two-step ELISA assay, correct exposure of the G40-R43 epitope was already confirmed.²⁰ The added value of measuring anti-DI reactivity using this assay remains to be determined.

Interestingly, LAC remained significantly correlated with thrombosis and pregnancy morbidity upon removal of anti-DI IgG positive patients. Further illustrated by the only fair to moderate agreement between IgG anti-DI and functional LAC in the thrombosis subpopulation (Kappa value = 0.398) and the pregnancy morbidity subpopulation (Kappa value = 0.320), these data illustrate that the CIA anti-DI IgG assay only detects part of the pathogenic aPLs. The presence of pathogenic anti-phosphatidylserine/prothrombin (anti-PS/PT) antibodies able to induce LAC positivity may explain the remaining correlation between LAC and thrombosis. Indeed, previous studies have demonstrated correlations of anti-PS/PT with clinical symptoms in APS patients and positivity proved to be associated with LAC.^{41,42}

Evidence is growing that the determination of combined antibody positivity helps to categorize patients according to their risk profile. Especially triple-positive patients (positive for LAC and anti-CL and anti- β 2GPI IgG/IgM) show a strong association with

thrombotic and obstetric manifestations.^{43,44} Clinical studies confirm that triple positivity in APS patients and asymptomatic aPL carriers indicates a high risk of recurrence of thrombosis or development of a first thrombotic event, respectively.^{44,45} Although identification of triple positives is dependent on the solid-phase assay used,⁴⁶ the percentage of anti-DI IgG positives in individuals with distinct antibody profiles were comparable for the four tested solid-phase assays. In this study, a very good agreement was found between triple positivity and anti-DI IgG positivity, irrespective of the solid-phase assay used (Table S5), which is consistent with previous studies.^{30-32,35,40} Moreover, independent of the platform used to measure anti-CL and anti- β 2GPI IgG/IgM, we observed that anti-DI IgG antibodies are mainly present in triple positive individuals, also showing significantly higher titers compared to patients with other aPL profiles. More importantly, within the triple positive group, a higher percentage of clinically affected cases was present in the anti-DI IgG positive compared to the anti-DI IgG negative group. Furthermore, the higher ORs of combined DI and triple positivity for both thrombosis and pregnancy morbidity compared to only triple positivity, support the idea that detection of anti-DI IgG is interesting to identify patients at risk.

We acknowledge that our study has some limitations. First, as in both the thrombotic and obstetric patient groups, a small number of patients (8.9% and 7.4%, respectively) also suffers from the other clinical manifestation, the correlation with the specific clinical manifestation may be affected by the comorbidity of the other manifestation. Also, the different groups in our study population did not match according to age and gender, but were representative to population groups tested in daily practice. Another limitation of our study is the retrospective design. Interestingly, in the AID and normal control group, a number of the triple positives were also positive for anti-DI reactivity (depending on the platform 11/22 [50%]-9/14 [64%] for AID; 1/3 [33%]-2/4 [50%] for the normal controls). Prospective studies are necessary to verify the risk of patients with these characteristics to develop clinical manifestations of APS, with possible implications for their treatment.

This study showed again that the choice of the commercial assays used to detect the presence of antiphospholipid antibodies influences the classification of APS. It is impossible to determine which commercial assay is the best because we lack a gold standard. However, a good agreement with a domain I specific assay seems to be essential because antibodies against DI have shown to be pathogenic in animal models of APS.

CONCLUSION

Despite the higher OR of anti-DI antibody detection for clinical manifestations of APS, our study was unable to demonstrate an added value of measuring anti-DI IgG on top of the laboratory criteria, independent of the platform used to measure anti-CL and anti- β 2GPI. We put the hypothesis forward that the reduced exposure of the pathogenic DI epitope in this automated assay possibly explains the absence of an added value. Therefore, it may be interesting to re-evaluate the added value of anti-DI using the in-house anti-DI assay previously developed, for which correct exposure of the pathogenic epitope was already demonstrated. The high correlation between anti-DI IgG and triple positivity indicates that anti-DI IgG positivity confirms the patients at higher risk for clinical events in APS. Importantly, combined DI and triple positivity confirms a higher risk for both thrombosis and pregnancy morbidity compared to only triple positivity. As LAC positivity remains significantly correlated with thrombosis and pregnancy morbidity upon removal of anti-DI positive patients, the anti-DI IgG assay only detects part of the pathogenic aPLs.

ADDENDUM

K. M. J. Devreese, B. de Laat and H. Kelchtermans designed the study. K. M. J. Devreese organized the sample collection at the different centers. K. M. J. Devreese, G. W. Moore, J-C Gris, S. Zuily and J. Musiał collected samples and identified sample characteristics. Samples were analyzed under the supervision of K. M. J. Devreese. D.Yin, K. M. J. Devreese, B. de Laat, H. Kelchtermans and W. Chayouâ interpreted data, performed statistical analyses and wrote the manuscript. P. G. de Groot, G. W. Moore, J-C Gris, S. Zuily and J. Musiał critically reviewed the manuscript.

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DISCLOSURE OF CONFLICTS OF INTERESTS

All authors state that they have no conflict of interest.

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SUPPLEMENTAL DATA

SUPPLEMENTARY TABLE 1. Number of patients testing positive (+) or negative (-) for anti-DI IgG in relation to positivity for LAC or anti-β2GPI IgG or at least one of the criteria aPL panel measured by other three solid phase assays

	Manufacturer	Thrombosis subpopulation (N = 851)		With T (N = 462)		Without T (N = 389)		Pregnancy morbidity subpopulation (N = 481)		With P (N = 154)		Without P (N = 327)	
		Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	
Anti-DI IgG	BioPlex®2200	+	-	+	-	+	-	+	-	+	-	+	-
		115	6	96	5	19	1	59	2	40	1	19	1
		47	683	26	335	21	348	29	391	9	104	20	287
Total		162	689	122	340	40	349	88	393	49	105	39	288
Anti-DI IgG	Phadia®		Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG
		+	-	+	-	+	-	+	-	+	-	+	-
		101	20	85	16	16	4	54	7	38	3	16	4
Total	QUANTA Lite® ELISA	35	695	24	337	11	358	16	404	7	106	9	298
		136	715	109	353	27	362	70	411	45	109	25	302
			Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG	Anti-CL IgG
Anti-DI IgG	BioPlex®2200	+	-	+	-	+	-	+	-	+	-	+	-
		95	26	81	20	14	6	47	14	33	8	14	6
		26	704	18	343	8	361	11	409	5	108	6	301
Total		121	730	99	363	22	367	58	423	38	116	20	307
Anti-DI IgG	Phadia®		Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG
		+	-	+	-	+	-	+	-	+	-	+	-
		116	5	96	5	20	0	60	1	40	1	20	0
Total		49	681	25	336	24	345	30	390	9	104	21	286
	165	686	121	341	44	345	90	391	49	105	41	286	
		Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG
Anti-DI IgG	BioPlex®2200	+	-	+	-	+	-	+	-	+	-	+	-
		104	17	86	15	18	2	52	9	34	7	18	2
		30	700	18	343	12	357	16	404	5	108	11	296

SUPPLEMENTARY TABLE 1. Continued

Manufacturer		Thrombosis subpopulation (N = 851)		With T (N = 462)	Without T (N = 389)	Pregnancy morbidity subpopulation (N = 481)	With P (N = 154)	Without P (N = 327)					
Total		134	717	104	358	30	359	68	413	39	115	29	298
Anti-DI IgG	QUANTA Lite® ELISA		Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG	Anti-β2GPI IgG
	+	+	-	+	-	+	+	+	-	+	-	+	-
	81	40	69	32	12	8	33	28	21	20	12	8	306
-	10	720	8	353	2	367	2	418	1	112	1	306	314
Total		91	760	77	385	14	375	35	446	22	132	13	314
	BioPlex®2200		Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *
Anti-DI IgG	+	118	3	98	3	20	0	61	0	41	0	20	0
-	205	525	128	233	77	292	119	301	56	57	63	244	244
Total		323	528	226	236	97	292	180	301	97	57	83	244
	Phadia®		Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *
Anti-DI IgG	+	117	4	97	4	20	0	60	1	40	1	20	0
-	253	477	149	212	104	265	151	269	67	46	84	223	223
Total		370	481	246	216	124	265	211	270	107	47	104	223
	QUANTA Lite® ELISA		Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *	Criteria aPL panel *
Anti-DI IgG	+	113	8	95	6	18	2	58	3	40	1	18	2
-	223	507	138	223	85	284	133	287	67	46	66	241	241
Total		336	515	233	229	103	286	191	290	107	47	84	243

Abbreviations: aPL, antiphospholipid antibody; β2GPI, β2glycoprotein I; DI, domain I; IgG (M), immunoglobulin G (M); LAC, lupus anticoagulant; N, number of patients; P, pregnancy morbidity; T, thrombosis *Criteria aPL panel positivity: patients testing positive for at least one of the criteria aPL panel (i.e. LAC, anti-CL IgG/M and/or anti-β2GPI IgG/M)

SUPPLEMENTARY TABLE 2. Number of cases with distinct antibody profiles (anti-CL IgG/IgM and anti-β2GPI IgG/IgM measured by BioPlex®2200)

In the thrombosis subpopulation	aPL profile			Anti-DI IgG positive				Anti-DI IgG negative			
	LAC	anti-CL IgG/IgM	anti-β2GPI IgG/IgM	Total		with T	without T	Total		with T	without T
				N (%) [*]	N (%)			N (%) [*]	N (%)		
Goup1	+	+	+	146 (17)	88 (87)		13 (13)	45 (31)	30 (67)		15 (33)
Goup2	-	+	+	37 (4)	15 (41)		6 (40)	22 (59)	8 (36)		14 (64)
Goup3	+	+	-	3 (0.4)	0	0	0	3 (100)	1 (33)		2 (67)
Goup4	+	-	+	5 (1)	1 (20)	0	1 (100)	4 (80)	1 (25)		3 (75)
Goup5	+	-	-	120 (14)	0	0	0	120 (100)	82 (68)		38 (32)
Goup6	-	+	-	6 (1)	1 (17)	1 (100)	0	5 (83)	2 (40)		3 (60)
Goup7	-	-	+	6 (1)	0	0	0	6 (100)	4 (67)		2 (33)
Goup8	-	-	-	528 (62)	3 (1)	3 (100)	0	525 (99)	233 (44)		292 (56)
Total				851 (100)	121 (14)	101 (83)	20 (17)	730 (86)	361 (49)		369 (51)

In the pregnancy morbidity subpopulation	aPL profile			Anti-DI IgG positive				Anti-DI IgG negative			
	LAC	anti-CL IgG/IgM	anti-β2GPI IgG/IgM	Total		with P	without P	Total		with P	without P
				N (%) [#]	N (%)			N (%) [#]	N (%)		
Goup1	+	+	+	62 (13)	42 (68)	29 (69)	13 (31)	20 (32)	6 (30)		14 (70)
Goup2	-	+	+	39 (8)	17 (44)	11 (65)	6 (35)	22 (56)	9 (41)		13 (59)
Goup3	+	+	-	2 (0.4)	0	0	0	2 (100)	1 (50)		1 (50)
Goup4	+	-	+	3 (1)	1 (33)	0	1 (100)	2 (67)	0		2 (100)
Goup5	+	-	-	68 (14)	0	0	0	68 (100)	39 (57)		29 (43)
Goup6	-	+	-	2 (0.4)	0	0	0	2 (100)	0		2 (100)
Goup7	-	-	+	4 (1)	1 (25)	1 (100)	0	3 (75)	1 (33)		2 (67)
Goup8	-	-	-	301 (63)	0	0	0	301 (100)	57 (19)		244 (81)
Total				481 (100)	61 (13)	41 (67)	20 (33)	420 (87)	113 (27)		307 (73)

Abbreviations: aPL, antiphospholipid antibody; β2GPI, β2glycoprotein I; CL, cardiolipin; DI, domain I; IgG (M), immunoglobulin G (M); LAC, lupus anticoagulant; N, number of patients in each group; P, pregnancy morbidity; T, Thrombosis. #: the percentage of each group antibody profile; &: the percentage of anti-DI IgG positive/negative in each group antibody profile. *: the percentage of individuals with/without clinical events

SUPPLEMENTARY TABLE 3. Number of cases with distinct antibody profiles (anti-CL IgG/IgM and anti-β2GPI IgG/IgM measured by Phadia®)

In the thrombosis subpopulation	aPL profile			Anti-DI IgG positive			Anti-DI IgG negative		
	LAC	anti-CL IgG/IgM	anti-β2GPI IgG/IgM	Total		without T	Total		without T
				N (%)&	N (%)		N (%)&	N (%)	
Goup1	+	+	+	90 (72)	78 (87)	12 (13)	35 (28)	23 (66)	12 (34)
Goup2	-	+	+	12 (33)	8 (67)	4 (33)	24 (67)	12 (50)	12 (50)
Goup3	+	+	-	3 (17)	3 (100)	0	15 (83)	12 (80)	3 (20)
Goup4	+	-	+	6 (43)	5 (83)	1 (17)	8 (57)	2 (25)	6 (75)
Goup5	+	-	-	3 (3)	2 (67)	1 (33)	114 (97)	77 (68)	37 (32)
Goup6	-	+	-	2 (4)	1 (50)	1 (50)	50 (96)	20 (40)	30 (60)
Goup7	-	-	+	1 (13)	0	1 (100)	7 (88)	3 (43)	4 (57)
Goup8	-	-	-	4 (1)	4 (100)	0	477 (99)	212 (44)	265 (56)
Total				121 (14)	101 (83)	20 (17)	730 (86)	361 (49)	369 (51)

In the pregnancy morbidity subpopulation	aPL profile			Anti-DI IgG positive			Anti-DI IgG negative		
	LAC	anti-CL IgG/IgM	anti-β2GPI IgG/IgM	Total		without P	Total		without P
				N (%)&	N (%)		N (%)&	N (%)	
Goup1	+	+	+	37 (73)	25 (68)	12 (32)	14 (27)	5 (36)	9 (64)
Goup2	-	+	+	14 (42)	10 (71)	4 (29)	19 (58)	10 (53)	9 47(1)
Goup3	+	+	-	2 (22)	2 (100)	0	7 (78)	4 (57)	3 (43)
Goup4	+	-	+	1 (14)	0	1 (100)	6 (86)	1 (17)	5 (83)
Goup5	+	-	-	3 (4)	2 (67)	1 (33)	65 (96)	36 (55)	29 (45)
Goup6	-	+	-	2 (6)	1 (50)	1 (50)	32 (94)	7 (22)	25 (78)
Goup7	-	-	+	1 (11)	0	1 (100)	8 (89)	4 (50)	4 (50)
Goup8	-	-	-	1 (0.4)	1 (100)	0	269 (100)	46 (17)	223 (83)
Total				61 (13)	41 (67)	20 (33)	420 (87)	113 (27)	307 (73)

Abbreviations: aPL, antiphospholipid antibody; β2GPI, β2glycoprotein I; CL, cardiolipin; DI, domain I; IgG (M), immunoglobulin G (M); LAC, lupus anticoagulant; N, number of patients in each group; P, pregnancy morbidity; T, Thrombosis. #: the percentage of each group antibody profile; &: the percentage of anti-DI IgG positive/negative in each group antibody profile.. *: the percentage of individuals with/without clinical events

SUPPLEMENTARY TABLE 4. Number of cases with distinct antibody profiles (anti-CL IgG/IgM and anti-β2GPI IgG/IgM measured by QUANTA Lite ELISA®)

aPL profile				Anti-DI IgG positive				Anti-DI IgG negative			
				Total	with T	without T	Total	with T	without T	Total	without T
In the thrombosis subpopulation	N (%)#	LAC	anti-CL IgG/IgM	anti-β2GPI IgG/IgM	N (%)&	N (%)	N (%)&	N (%)	N (%)	N (%)&	N (%)
Goup1	106 (12)	+	+	+	82 (77)	72 (88)	24 (23)	17 (71)	7 (29)		
Goup2	25 (3)	-	+	+	8 (32)	5 (62)	17 (68)	6 (35)	11 (65)		
Goup3	22 (3)	+	+	-	6 (27)	5 (83)	16 (73)	9 (56)	7 (44)		
Goup4	13 (2)	+	-	+	2 (15)	2 (100)	11 (85)	8 (73)	3 (27)		
Goup5	133 (16)	+	-	-	12 (9)	9 (75)	121 (91)	80 (66)	41 (34)		
Goup6	28 (3)	-	+	-	3 (11)	2 (67)	25 (89)	15 (60)	10 (40)		
Goup7	9 (1)	-	-	+	0	0	9 (100)	3 (33)	6 (67)		
Goup8	515 (61)	-	-	-	8 (2)	6 (75)	507 (98)	223 (44)	284 (56)		
Total	851 (100)				121 (14)	101 (83)	730 (86)	361 (49)	369 (51)		

aPL profile				Anti-DI IgG positive				Anti-DI IgG negative			
				Total	with P	without P	Total	with P	without P	Total	without P
In the pregnancy morbidity subpopulation	N (%)#	LAC	anti-CL IgG/IgM	anti-β2GPI IgG/IgM	N (%)&	N (%)	N (%)&	N (%)	N (%)	N (%)&	N (%)
Goup1	36 (7)	+	+	+	29 (81)	19 (66)	7 (19)	1 (14)	6 (86)		
Goup2	28 (6)	-	+	+	10 (36)	7 (70)	18 (64)	9 (50)	9 (50)		
Goup3	13 (3)	+	+	-	6 (46)	5 (83)	7 (54)	1 (14)	6 (86)		
Goup4	6 (1)	+	-	+	0	0	6 (100)	3 (50)	3 (50)		
Goup5	80 (17)	+	-	-	8 (10)	5 (62)	72 (90)	41 (57)	31 (43)		
Goup6	20 (4)	-	+	-	4 (20)	3 (75)	16 (80)	9 (56)	7 (44)		
Goup7	8 (2)	-	-	+	1 (13)	1 (100)	7 (88)	3 (43)	4 (57)		
Goup8	290 (60)	-	-	-	3 (1)	1 (33)	287 (99)	46 (16)	241 (84)		
Total	481 (100)				61 (13)	41 (67)	420 (87)	113 (27)	307 (73)		

Abbreviations: aPL, antiphospholipid antibody; β2GPI, β2glycoprotein I; CL, cardiolipin; DI, domain I; IgG (M), immunoglobulin G (M); LAC, lupus anticoagulant; N, number of patients in each group; P, pregnancy morbidity; T, Thrombosis. #: the percentage of each group antibody profile; &: the percentage of anti-DI IgG positive/negative in each group antibody profile. *: the percentage of individuals with/without clinical events

SUPPLEMENTARY TABLE 5. Agreement between anti-DI IgG positivity and triple positivity for four platforms in the different subpopulations

Agreement1	HemosIL AcuStar®	BioPlex® 2200	Phadia®	QUANTA Lite® ELISA
In the thrombosis subpopulation	0.746***	0.712***	0.686***	0.680***
In the pregnancy morbidity subpopulation	0.679***	0.636***	0.616***	0.556***

¹ Kappa values are shown*** $P < 0.0001$ **SUPPLEMENTARY TABLE 6.** Comparison of anti-DI IgG titers between triple positive patients with and without clinical events

Manufacturers for anti-CL and anti-β2GPI	In the thrombosis population			In the pregnancy morbidity population		
	With T	Without T	P value	With P	Without P	P value
HemosIL AcuStar®	169.0 CU (25.7-761.3)	63.7 CU (3.7-625.7)	0.084	200.2 CU (49.6-462.1)	65.0 CU (3.7-654.3)	0.179
BioPlex® 2200	156.1 CU (18.9-635.2)	11.8 CU (3.7-349.4)	0.014	191.2 CU (47.9-433.6)	13.8 CU (3.7-364.2)	0.024
Phadia®	202.5 CU (30.1-769.6)	25.1 CU (3.7-560.3)	0.044	200.2 CU (47.9-588.2)	66.4 CU (4.6-682.8)	0.238
QUANTA Lite ELISA®	229.0 CU (39.8-833.4)	66.4 CU (3.7-799.6)	0.236	308.2 CU (160.2-881.7)	87.4 CU (3.7-829.3)	0.285

Abbreviations: β2GPI, β2glycoprotein I; CL, cardiolipin; CU, chemiluminescence units; DI, domain I; P, pregnancy morbidity; T, thrombosis

Titer of anti-DI IgG are expressed as the median (quartile(Q)25- Q75), Mann-Whiney U test was used to compare difference of two groups, the significant p values are showed in bold

8

Antiprothrombin antibodies induce platelet activation: a possible explanation for anti-FXa therapy failure in patients with antiphospholipid syndrome?

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ABSTRACT

Background: Arterial and venous thrombosis are both common in antiphospholipid syndrome (APS). Recent studies have shown that anti-FXa therapy in APS patients leads to a greater number of patients with arterial thrombosis than with warfarin. We hypothesise that this may be due to the lowering of prothrombin levels by warfarin.

Objectives: To investigate whether antiprothrombin antibodies induce platelet aggregation and to identify the platelet receptors involved. A second aim was to investigate the effect of reduced prothrombin levels on antiprothrombin antibody-induced platelet aggregation.

Methods: Enzyme-linked immunosorbent assays (ELISAs) were performed to measure binding of antiprothrombin antibodies to prothrombin fragment 1+2 and prothrombin. Platelet aggregation assays in washed platelets were performed. FcγRIIA was immunoprecipitated and tyrosine-phosphorylated FcγRIIA was measured by western blot.

Results: The antiprothrombin antibodies 28F4 and 3B1 had lupus anticoagulant (LAC) activity and caused platelet aggregation in the presence of Ca^{2+} and prothrombin. Antiprothrombin antibodies without LAC activity did not activate platelets. Inhibition of Syk, Src and FcγRIIA blocked platelet aggregation. Fab and F(ab')₂ fragments of 28F4 were unable to induce platelet aggregation. Immunoprecipitations showed that whole 28F4 IgG induced tyrosine phosphorylation of FcγRIIA. Platelet aggregation was significantly reduced when prothrombin levels were reduced from 1 μM to 0.2 μM.

Conclusions: Antiprothrombin antibodies with LAC activity are able to activate platelets via FcγRIIA. Decreased prothrombin levels resulted in less antiprothrombin antibody-mediated platelet aggregation. This may explain the lower incidence of arterial thrombosis in patients treated with warfarin than with anti-FXa therapy.

INTRODUCTION

Antiphospholipid syndrome (APS) is characterized by thrombosis and/or pregnancy complications due to the persistent presence of antiphospholipid (aPL) antibodies [1]. Laboratory criteria for classification of APS include detection of lupus anticoagulant (LAC), anti- β 2-glycoprotein I (a β 2GPI) and anticardiolipin (aCL) IgG/M antibodies [1]. Clinical criteria for classification of thrombotic APS include venous, arterial and small vessel thrombosis. The updated European League Against Rheumatism (EULAR) recommendations on managing thrombotic APS patients recommend long-term vitamin K antagonists as the standard of care [2].

Direct oral anticoagulants (DOACs) like rivaroxaban, a direct FXa inhibitor, are currently prescribed to patients with venous thromboembolism and atrial fibrillation. The advantage of DOACs is that they are given at a fixed dose and do not need laboratory monitoring. Interestingly, two trials reported predominantly arterial thrombosis in patients treated with rivaroxaban with a high rate of stroke [3,4]. No cases of stroke were reported in patients treated with warfarin [3,4]. Recent professional guidance statements have been issued regarding the use of DOACs in APS patients and recommended against DOACs in triple positive APS patients (defined by the presence of LAC, a β 2GPI and aCL antibodies) and in arterial thrombosis [5].

Blood platelets play a key role in arterial thrombosis [6]. Antibodies against β 2GPI are known to activate platelets via glycoprotein (GP) Iba and apolipoprotein E receptor 2 [7,8]. Triple positive APS patients have the highest risk of thrombotic complications and these patients usually have both a β 2GPI antibodies and anti-phosphatidylserine/prothrombin (aPS/PT) antibodies [9]. A systematic review identified aPS/PT antibodies as a risk factor for arterial thrombosis [10]. Still, experimental data on aPS/PT antibodies and platelet aggregation are lacking.

The overall aim was to investigate whether antiprothrombin antibodies stimulate platelet activation and to identify the underlying mechanism. This included investigation of the effect of reduced prothrombin levels on platelet aggregation as warfarin reduces the synthesis of prothrombin, while DOACs do not [11]. Decreased levels of prothrombin may explain why warfarin-treated APS patients are protected against arterial thrombosis while those treated with rivaroxaban are not.

MATERIAL AND METHODS

Reagents

Purified human prothrombin was from Synapse Research Institute (Maastricht, The Netherlands) and rivaroxaban (Xarelto) was from Bayer Schering Pharma AG (Berlin, Germany). Anti-FcγRIIA mAb IV.3 was purified in the laboratory from a hybridoma [12]. The antiprothrombin monoclonal antibody (mAb) 28F4 is a mouse IgG antibody as described previously [13,14]. Antiprothrombin fragment 1+2 mAbs 3B1, 6A3, 11H2 and 8H11 were developed and produced according to standard procedures [15]. LAC was measured by activated Partial Thromboplastin Time (aPTT; Diagnostica Stago, Asnières sur Seine, France), dilute Russell Viper Venom Time (dRVVT; Diagnostica Stago), and Ecarin Clotting Time (ECT; Diagnostic Reagents, Thame, UK). The Pierce Classic IP Kit from Thermo Fisher Scientific (Waltham, MA, USA) was used for immunoprecipitation. High-binding ELISA plates from Costar (New York, NY, USA) were used for the ELISAs. Sheep anti-human prothrombin IgG HRP labeled antibody was from Affinity Biologicals, Inc. (Ancaster, ON, Canada), prothrombin fragment 1+2 was from Haematologic Technologies, Inc. (Essex Junction, VT, USA). All other reagents are previously described or are from Sigma-Aldrich (Poole, UK) [16].

Preparation of washed platelets

Blood was drawn by venipuncture into 4% sodium citrate from consenting, self-reported healthy volunteers. Platelet rich plasma (PRP) was obtained by centrifugation at 200 g for 20 minutes at room temperature. Washed platelets were obtained by further centrifugation of PRP at 1,000 g for 10 minutes in the presence of 0.2 µg/mL prostacyclin and resuspended in modified-Tyrode's-HEPES buffer (134 mM NaCl, 0.34 mM Na₂HPO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM HEPES, 5mM glucose, 1 mM MgCl₂; pH 7.3) as previously described [16]. Platelets were used at 2×10⁸/mL and 4×10⁸/mL for aggregation studies and immunoprecipitation, respectively.

Fab and F(ab')₂ antibody fragments

Fab fragments from 28F4 were generated by 4 hour incubation of 5 mg/mL antibody with immobilized Ficin in the presence of 25 mM cysteine (Pierce, ThermoFisher) at 37°C. F(ab')₂ fragments from 28F4 were generated by 24 hour incubation of 5 mg/mL antibody with immobilized Ficin in the presence of 4 mM cysteine (Pierce) at 37°C. Preparations were then applied to an immobilized protein A column (Pierce) followed by dialysis to remove any remaining cysteine. The purity of the Fab and F(ab')₂ fragments was verified by SDS-PAGE.

Lupus anticoagulant testing

Normal pooled plasma was incubated with 50 µg/ml mAb for 10 min at 37°C prior to LAC testing. LAC was performed according to the SSC guidelines and manufacturer's instructions for ECT were followed [17,18].

ELISA

The mAb 28F4 (5 µg/ml) was coated onto an ELISA plate overnight at 4°C. Blocking buffer consisted of 5% Bovine Serum Albumin (BSA) and 5 mM CaCl₂ in Tris Buffered Saline (TBS), (pH 7.6). Washing buffer was 5 mM CaCl₂ and 0.1% Tween in TBS, pH 7.6. Increasing concentrations of antigen (prothrombin fragment 1+2, purified prothrombin or platelet poor plasma) was incubated for 1 hour at room temperature. Sheep anti-human prothrombin IgG HRP-labeled antibody (1:1000) was used as secondary antibody followed by staining with tetra methyl benzidine (TMB) (ThermoFisher). The reaction was stopped with 2M H₂SO₄ and absorbance was measured at 405 nm.

Platelet aggregation assay

Platelet aggregation was assessed by light transmission aggregometry (LTA) in a PAP-8E (Bio/Data Corporation, Horsham, PA, USA) aggregometer for up to 30 minutes. Washed platelets were incubated with aPT antibodies for 5 min with an antiprothrombin antibody (50 µg/mL) followed by CaCl₂ (10 mM) and purified human prothrombin (1 µM). In some studies, platelets were preincubated with eptifibatide (9 µM), vorapaxar (1 µM) + BMS-986120 (1 µM), indomethacin (10 µM), ticagrelor (10 µM), PRT-060318 (5 µM), dasatinib (10 µM), mAb IV.3 (10 µg/ml) or rivaroxaban (200, 400, 800, or 1000 nM) for 10 min.

Immunoprecipitation and western blotting

Washed platelets were pretreated with 9 µM eptifibatide to block integrin αIIbβ3. Cross-linking of IV.3 IgG (10 µg/ml) with 10 µg/ml anti-Fc (Biolegend, San Diego, CA, USA) served as a positive control. Platelets were stimulated at 37°C with stirring at 1200 rpm on a PAP-8E aggregometer. Reactions were terminated by addition of ice-cold lysis buffer (0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP-40, 5% glycerol, pH 7.4). Lysates were incubated overnight with mAb IV.3 to form immune complexes. Precipitated proteins were separated by reducing SDS-PAGE, electro-transferred and Western blotted with the anti-phosphotyrosine antibody, clone 4G10.

Ethics

Ethical approval for collecting blood healthy volunteers was granted by Birmingham University Internal Ethical Review (ERN_11-0175).

Statistical analysis

All data are presented as mean $\pm$ standard deviation (SD). A p-value <0.05 was considered statistically significant. Statistical analysis was performed using Wilcoxon signed rank test, Kruskal-Wallis test with Dunn's Multiple Comparison Test and Mann-Whitney U test as stated. All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc. La Jolla, Ca).

RESULTS

Lupus anticoagulant activity

The five antiprothrombin mAbs (28F4, 3B1, 6A3, 11H2 and 8H11) were raised against F1+2, the activation peptide of prothrombin. Of these mAbs, only 28F4 and 3B1 caused LAC activity in the dilute Russell Viper Venom Time (dRVVT) and activated Partial Thromboplastin Time (aPTT) tests. The other three mAbs (11H2, 6A3 and 8H11) had no effect on the clotting time (Supplemental Table 1). The Ecarin Clotting Time (ECT) reagent converts prothrombin into thrombin, independently of phospholipids. The mAbs 28F4 and 3B1 did not prolong the ECT and did not inhibit thrombin activity when tested with a fluorescent substrate. However, mAb 28F4 did bind to prothrombin fragment 1+2, purified prothrombin and native prothrombin in plasma in an Enzyme-linked immunosorbent assay (Supplemental Figure 1).

Platelet aggregation

Adding mAbs 28F4 and 3B1, but not 11H2, 6A3 and 8H11, resulted in aggregation of washed platelets in the presence of prothrombin and Ca^{2+} (Figure 1A), indicating a correlation between LAC-activity and platelet aggregation. mAb 28F4 induced aggregation in washed platelets in the presence but not absence of Ca^{2+} (Supplemental Figure 2) or prothrombin (not shown). 28F4 was the most potent at inducing platelet aggregation and LAC-activity. Preincubation of washed platelets with 1 $\mu\text{g}/\text{ml}$ 28F4 resulted in partial aggregation. Full platelet aggregation was achieved with 10 $\mu\text{g}/\text{ml}$ 28F4. Addition of 200 - 1000 nM rivaroxaban to washed platelets did not influence the aggregation profile.

Platelet signalling

Inhibitors were used to investigate the mechanism involved in antiprothrombin-induced platelet aggregation to low (10 $\mu\text{g}/\text{ml}$) and high (50 $\mu\text{g}/\text{ml}$) concentrations of mAb 28F4. Pretreatment of platelets with the $\alpha\text{IIb}\beta_3$ antagonist eptifibatide inhibited platelet aggregation to both concentrations of mAb 28F4 (Table 1). Indomethacin and

ticagrelor, which block cyclooxygenase and the P2Y₁₂ receptor, respectively, attenuated but did not block platelet aggregation. Platelet aggregation induced by mAb 28F4 was not altered in the combined presence of the PAR-1 and PAR-4 antagonists, vorapaxar and BMS-986120, respectively. The same Low-dose of mAb 28F4 (10 µg/ml) and high dose of mAb 28F4 (50 µg/ml) was used to assess the effect of platelet antagonists. Platelet aggregation was measured as increase of light transmission. Results are shown as mean $\pm$ SD (n=3) concentrations of the two antagonists blocked thrombin mediated platelet aggregation (data not shown). The Syk and Src inhibitors, PRT-060318 and dasatinib, respectively, blocked aggregation to both concentrations of mAb 28F4, which indicates involvement of signalling via receptors containing an immunoreceptor tyrosine-based activation motif (ITAM). Three ITAM-containing receptors of human platelets are known including: C-type lectin-like receptor 2 (CLEC-2), glycoprotein VI (GPVI) and Fc γ RIIA (Fc receptor for IgG).

FIGURE 1. The mAbs 28F4 and 3B1 induce platelet aggregation in the presence of Ca²⁺ and prothrombin. **A)** Representative traces of light transmission aggregometry using washed platelets at a concentration of 2×10^9 /ml. Aliquots of platelet suspension were stimulated with 50 µg/ml of mAbs 28F4, 3B1, 6A3, 11H2, or 8H11 in the presence of 10 mM Ca²⁺ and 1 µM prothrombin (n=3). **B)** Quantification of platelet aggregation induced by increasing doses of mAb 28F4. Platelet aggregation was measured as increase of light transmission. Results are shown as mean $\pm$ SD (n=3). Statistical significance was analyzed using the Kruskal-Wallis test. *P<0.05. NS, not significant.

TABLE 1. ITAM signaling is involved in antiprothrombin induced platelet aggregation

	28F4 (10 µg/ml) % aggregation ± SD	28F4 (50 µg/ml) % aggregation ± SD
Vehicle	65.4 ± 10.2	70.0 ± 8.1
Eptifibatide (9 µM)	4.5 ± 1.4	18.0 ± 9.2
Indomethacin (10 µM)	22.3 ± 11.2	38.9 ± 18.2
PRT-06318 (5 µM)	2.5 ± 2.8	3.9 ± 1.8
Ticagrelor (10 µM)	11.0 ± 6.0	19.6 ± 15.7
Dasatinib (10 µM)	2.7 ± 1.7	4.2 ± 2.6
Vorapaxar (1 µM) + BMS-986120 (1 µM)	65.8 ± 2.3	74.6 ± 4.1

Involvement of FcγRIIA

Of the three platelet ITAM-containing receptors, the FcγRIIA receptor was felt to be the most likely to be involved in the response to the antiprothrombin antibodies as it is a low affinity receptor for the Fc domain. Tyrosine phosphorylation of FcγRIIA was induced by mAb 28F4 as measured by immunoprecipitation and western blotting (Figure 2). Crosslinked IV.3 (anti-FcγRIIA antibody) was used as a positive control. As expected, rhodocytin and CRP, known stimulators of CLEC-2 and GPVI respectively, did not result in tyrosine phosphorylation of FcγRIIA. Non-crosslinked mAb IV.3 inhibited 28F4 and 3B1-induced platelet aggregation (Figure 3 and not shown). Fab and F(ab')₂ antibody fragments of mAb 28F4 were unable to induce platelet aggregation (not shown) confirming the critical role of the Fc domain.

The link between prothrombin levels and platelet aggregation

We investigated whether prothrombin levels affected mAb 28F4-induced platelet aggregation as a possible explanation for anti-FXa therapy failure in patients with APS. Platelet aggregation was assessed in washed platelets in the presence of 50 µg/ml 28F4 with two concentrations of prothrombin. Aggregation was found to be attenuated by 59% when prothrombin levels were reduced from 1 µM to 0.2 µM, suggesting a direct link between prothrombin levels and platelet aggregation (Figure 4).

FIGURE 2. FcγRIIA is tyrosine-phosphorylated in the presence of mAb 28F4. **A.** Representative blot of five experiments. **B.** Mean data of signal for p-FcγRIIA. The signal obtained from cross-linked mAb IV.3 was normalized against total FcγRIIA and set as a reference (1 AU). Differences were analyzed with the Kruskal-Wallis test. Results are shown as mean $\pm$ SD with $n=5$. * $P<0.05$. NS, not significant.

FIGURE 3. Antiprothrombin induced platelet aggregation can be blocked by mAb IV.3 (anti-FcγRIIA) antibody. Aliquots of platelet suspension were preincubated with mAb IV.3 (10 $\mu\text{g/ml}$) followed by stimulation using mAbs 28F4 (50 $\mu\text{g/ml}$) or 3B1 (50 $\mu\text{g/ml}$). Platelet aggregation was measured as increase of light transmission. Results are shown as mean $\pm$ SD ($n=5$). Statistical significance was analyzed using the Mann-Whitney U test (* $p<0.05$).

FIGURE 4. Decreased prothrombin levels results in attenuated platelet aggregation. A) Representative traces of light transmission aggregometry using washed platelets at a concentration of 2×10^8 /ml. Aliquots of platelet suspension were preincubated with 0.2 μ M prothrombin or 1 μ M prothrombin, followed by stimulation using 50 μ g/ml mAb 28F4. **B)** Quantification of platelet aggregation in the presence of two concentrations of prothrombin. Platelet aggregation was measured as increase of light transmission. Results are shown as mean $\pm$ SD (n=5). Statistical significance was analyzed using the Mann-Whitney U test (* $p < 0.05$).

DISCUSSION

In this study, we show that (i) antiprothrombin antibodies with LAC activity, but not those without LAC activity, are able to activate platelets via the Fc γ RIIA receptor and (ii) decreased prothrombin levels result in less antiprothrombin antibody-mediated platelet aggregation. These results suggest an important role of antiprothrombin antibodies with LAC activity in platelet activation. We speculate that the LAC activity is required for binding of the prothrombin/antiprothrombin complex to the platelet surface leading to binding of the Fc portion of the antiprothrombin antibodies to Fc γ RIIA and platelet activation.

Antiprothrombin antibodies with LAC activity are associated with APC resistance [19]. We were able to demonstrate platelet aggregation caused by antiprothrombin antibodies with LAC activity, indicating that antiprothrombin antibodies with LAC activity are potentially pathogenic. Antiprothrombin antibodies can be detected by coating prothrombin on irradiated ELISA plates or using phosphatidylserine (PS)-prothrombin complex as antigen [10]. The presence of aPS/PT antibodies is considered to be a stronger predictor of thrombosis than antiprothrombin antibodies [10]. However, we demonstrated binding of mAb 28F4 to prothrombin in the absence of PS, by ELISA. A

recent study showed that at least two subpopulations of aPS/PT antibodies exist; aPS/PT antibodies that bind to the “open” conformation of prothrombin and aPS/PT antibodies able to react with “closed” prothrombin [20]. There were, however, no different pathological effects found between the two subpopulations of aPS/PT antibodies. In contrast, we were able to distinguish between antiprothrombin antibodies that were able to induce platelet aggregation and those that did not by measuring LAC activity. From the five antiprothrombin antibodies investigated, only mAbs 28F4 and 3B1 were able to induce platelet aggregation and LAC activity. Although mAb 28F4 is able to bind closed prothrombin, thrombin activity was not inhibited. Which could be explained by the affinity of the investigated mAbs towards the activation peptide of prothrombin, fragment 1+2, and not thrombin. These data indicate that LAC activity determines the ability of antiprothrombin antibodies to induce platelet aggregation.

Data on the role of antiprothrombin antibodies in platelet activation are limited, probably because platelet aggregation studies are performed in citrated plasma and prothrombin needs Ca^{2+} to bind to platelets [21]. Although platelet function assays were not performed, one study showed the potency of mAb 28F4 to induce platelet activation by measuring thromboxane B_2 levels, phospholipid related platelet procoagulant activity, and thrombin generation [19]. The platelet-dependent effect of mAb 28F4 was found to be reduced in the presence of an integrin $\alpha\text{IIb}\beta 3$ inhibitor, but not with IV.3 [19]. The inability of showing involvement of the $\text{Fc}\gamma\text{RIIA}$ receptor might be due to the low concentration of mAb IV.3 used in their study [19]. We have showed involvement of the $\text{Fc}\gamma\text{RIIA}$ receptor in mAb 28F4 induced platelet activation in three ways; (i) inhibited platelet aggregation by IV.3, (ii) tyrosine phosphorylation of the $\text{Fc}\gamma\text{RIIA}$ receptor, and (iii) the inability of Fab and F(ab')_2 fragments of mAb 28F4 to aggregate platelets. In this respect, the mechanism of platelet activation by aPT antibodies mimics another auto-immune disease; heparin-induced thrombocytopenia (HIT) and thrombosis [22]. The presence of Ca^{2+} proved to be essential for platelet aggregation induced by antiprothrombin antibodies, suggesting Ca^{2+} dependent binding of antiprothrombin-prothrombin immune complexes to negatively charged phospholipids expressed by platelets. Here we show a major role for $\text{Fc}\gamma\text{RIIA}$ in antiprothrombin-induced platelet aggregation.

The TRAPS trial included APS patients with exclusively triple positive aPL (LAC, aCL and $\alpha\beta 2\text{GPI}$ antibodies) in which rivaroxaban was compared with warfarin therapy [3]. Although aPS/PT antibodies were not measured in the trial, aPS/PT antibodies have shown to correlate well with triple positivity in other studies [20,23]. We found that platelet aggregation was attenuated when prothrombin levels were reduced. The link between prothrombin levels and platelet aggregation may explain the failure of anti-

FXa therapy in patients with APS as rivaroxaban does not affect prothrombin levels in plasma, while vitamin K antagonists (e.g. warfarin) reduce active prothrombin (antigen) levels.

There are several limitations in our study, we used mAbs that recognize the prothrombin F1+2 fragment, but we did not further specify the site of recognition. In addition, we did not confirm our findings with patient antibodies and did not evaluate antiprothrombin-induced platelet activation in patients receiving vitamin K antagonists or rivaroxaban therapy. Additional data are needed on the specification of antiprothrombin antibodies and their effect on platelets.

Here, we have shown that only antiprothrombin antibodies with LAC activity are able to activate platelets via the FcγRIIA receptor. Decreased prothrombin levels resulted in attenuated platelet aggregation which might be an explanation for the low prevalence of arterial thrombosis in APS patients treated with warfarin compared to rivaroxaban therapy.

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AUTHORSHIP CONTRIBUTIONS

W.C., P.G.dG., P.L.R.N., and S.P.W. designed the study. W.C., P.L.R.N., C.K., and L.G-Q. performed the measurements. LAC was measured under supervision of J.C.M.M. and K.M.J.D. W.C., P.L.R.N., S.P.W., B.dL., and P.G.dG. interpreted data, performed statistical analyses and wrote the manuscript. J.C.M.M., C.K., L.G-Q., and K.M.J.D. critically reviewed the manuscript.

DISCLOSURE OF CONFLICTS OF INTEREST

W.C. and B.dL. are employees of Synapse Research Institute, part of Diagnostica Stago SAS. P.G.dG is an advisor of Synapse Research Institute. SPW holds a BHF Chair (03/003). The other authors state that they have no relevant conflict of interest.

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SUPPLEMENTAL DATA

SUPPLEMENTAL TABLE 1. mAbs 28F4 and 3B1 induce LAC activity.

	dRVVT (ratio)	aPTT (ratio)
NPP	1.0	1.0
28F4	2.05	2.05
3B1	1.56	1.30
6A3	1.08	1.03
11H2	1.03	1.02
8H11	1.06	1.02

Normal pooled plasma (NPP) was incubated with 50 µg/ml monoclonal antibody (28F4, 3B1, 6A3, 11H2 or 8H11) for 10 min at 37°C prior to LAC testing. LAC was performed according to the SSC guidelines [17]. Cut-off ratio for dRVVT and aPTT was 1.2, positive LAC results are shown in bold.

Supplemental Figure 1. The mAb 28F4 binds to closed prothrombin. 28F4 (5 µg/ml) was incubated onto an ELISA plate with increasing antigen concentration; prothrombin fragment 1+2, (purified) prothrombin or platelet poor plasma in the presence of Ca²⁺ for 1 hour. Results are shown as mean ±SD (n=3). OD, optical density.

Supplemental Figure 2. Ca^{2+} is essential for 28F4-induced platelet aggregation. Representative traces of light transmission aggregometry using washed platelets at a concentration of $2 \times 10^8/\text{ml}$. Aliquots of platelet suspension were stimulated with $50 \mu\text{g}/\text{ml}$ of mAbs 28F4 in the presence of $10 \text{ mM } \text{Ca}^{2+}$ and $1 \mu\text{M}$ prothrombin or in the absence of Ca^{2+} . ($n=2$)

9

General discussion, summary and future perspectives

GENERAL DISCUSSION, SUMMARY AND FUTURE PERSPECTIVES

The antiphospholipid syndrome (APS) is defined by thrombosis and/or specific pregnancy morbidity with the persistent presence of lupus anticoagulant (LAC), anti-cardiolipin (aCL) IgG/M and/or anti- β 2glycoprotein I (a β 2GPI) IgG/M antibodies [1]. Detection of antiphospholipid (aPL) antibodies is therefore of great importance for the diagnosis of APS. aCL and a β 2GPI antibodies are traditionally detected using an enzyme linked immunosorbent assay (ELISA). However, technology has paved the way for new methods for the detection of aCL and a β 2GPI antibodies [2–6]. Detection of non-criteria aPL antibodies, e.g. aCL IgA, a β 2GPI IgA, anti-phosphatidylserine/prothrombin (aPS/PT) IgG/M have been proposed in the updated classification criteria [7–13]. However, their relevance in the current aPL panel is unknown due to a lack of well-designed studies with large patient cohorts. Moreover, the role of the different aPL antibodies in clinical manifestations of APS and their interplay is not fully understood.

Antiphospholipid antibodies and their importance in the laboratory diagnosis of APS

Since the 1960's, correlations have been established between aPL antibodies and clinical manifestations of APS (thrombosis and/or pregnancy morbidity) [14]. In **chapter 2, 3 and 4** we confirmed that aCL IgG/M and a β 2GPI IgG/M antibodies correlate with thrombotic events and/or pregnancy morbidity. ELISAs detecting aCL and a β 2GPI antibodies are poorly standardized and external quality control programs have shown that aPL assays produce variable results [15,16]. In **chapter 2** we report low agreement of a new generation ELISA and automated systems detecting aCL and a β 2GPI IgG/M antibodies, which could affect diagnosis of APS. Remarkably, the diagnostic accuracy was comparable, which was also illustrated in other studies [5,6,17–19].

Combined positivity for LAC, aCL and a β 2GPI antibodies (triple positivity) is a high-risk profile of which the identification is suggested to be less affected by inter-method variation [20]. However, as described in **chapter 3**, identification of triple positivity is dependent on the solid phase assay used to detect aCL and a β 2GPI antibodies. Interestingly, computed ORs for thrombosis were comparable. Since the laboratory diagnosis of APS relies on the detection of aPL antibodies, LAC testing and immunoassays should be reliable. Unfortunately, aPL antibody immunoassays obtained from different manufacturers are accompanied by a large inter-assay and inter-

laboratory variation [16,21–24]. With five different aPL assays in the current criteria, many possible combinations (aPL profiles) are possible. However, knowledge about the clinical value of all possible aPL profiles is limited.

There is an ongoing discussion on the role of IgM aPL antibodies in the laboratory diagnosis of thrombotic APS [4,25–30]. A review of literature found more correlations for aCL and a β 2GPI IgG antibodies than for the IgM isotype [25]. However, due to unavailability of paired IgG and IgM results, the added value of IgM aPL antibodies within the current aPL panel could not be established [1,25]. In **chapter 4**, the added value of aCL and a β 2GPI IgM antibodies was investigated within the current aPL panel [1]. In agreement with literature, we found a higher association for IgG aPL antibodies with thrombosis and pregnancy morbidity than the IgM isotype [25]. IgM positivity was not independently associated with thrombosis, but was independently associated with pregnancy morbidity. The complement system has proven to play an important role in aPL antibody induced pregnancy morbidity [31]. Complement C3 activation has shown to be required for IgG aPL antibody induced fetal loss in mice [32]. IgM aPL antibodies might also induce fetal loss via a similar mechanism. However, data on complement activation by IgM aPL antibodies is lacking [33].

Non-criteria aPL (e.g. aCL IgA, a β 2GPI IgA, aPS/PT IgG and aPS/PT IgM antibodies) have been suggested to improve the laboratory diagnosis of APS. **Chapter 5**, describes the added value of detecting aCL and a β 2GPI IgA antibodies in APS. In agreement with multiple studies, aCL and a β 2GPI IgA antibodies correlated with thrombosis and/or pregnancy morbidity [8–11,34–39]. However, we were unable to demonstrate an added value for aCL and a β 2GPI IgA antibodies testing on top of the current aPL panel. Other groups have suggested that aCL and a β 2GPI IgA solid phase assays should be included in the classification criteria of APS as they could demonstrate a significant association of so-called “isolated” aCL and a β 2GPI IgA antibodies with clinical manifestations of APS [8–13]. However, many of these studies did not include LAC testing, hampering assessment of truly isolated IgA positivity [9,11–13].

Antibodies against domain I of β 2GPI (anti-DI) have been proven to be pathogenic in multiple studies [40–49]. In **chapter 6**, the performance characteristics of different anti-DI IgG assays is summarized and the significance of anti-DI antibodies in APS is discussed. We found a large variation in odds ratios (ORs) between studies, probably due to differences in patient and control population, but also as a result of differences in the detection method. In **chapter 7** we were able to demonstrate a significant correlation of anti-DI positivity with previous thrombosis or pregnancy morbidity. However, addition of anti-DI IgG into the current aPL panel did not improve the association with clinical

manifestations of APS. As suggested in our review, the clinical association could be the result of the solid phase assay used to detect anti-DI IgG antibodies [50]. A recent review, confirmed this hypothesis and demonstrated that the chemiluminescence assay (CIA) used in **chapter 7** to detect anti-DI does not expose the essential epitope containing Gly40-Arg43 on the antigen correctly [51].

In **chapter 8** we demonstrated platelet aggregation induced by anti-prothrombin antibodies via the platelet FcγRIIA receptor. Interestingly, only the anti-prothrombin antibodies that also caused LAC activity could induce platelet aggregation. This would indicate that anti-prothrombin antibodies with LAC activity are a distinct group of anti-prothrombin antibodies. Decreased prothrombin levels resulted in less anti-prothrombin antibody-mediated platelet aggregation. The link between prothrombin levels and platelet aggregation may explain the results of the clinical trials describing the failure of anti-FXa therapy (e.g. rivaroxaban) in patients with APS [52,53]. Because anti-FXa therapy does not affect prothrombin levels in plasma, anti-prothrombin antibodies can still induce platelet aggregation. On the contrary, vitamin K antagonists (e.g. warfarin) reduce active prothrombin (antigen) levels, thereby also reducing platelet aggregation mediated by anti-prothrombin antibodies

Other reasons to test for anti-phospholipid antibodies

Testing for aPL antibodies is mandatory to fulfill the laboratory criterion in the diagnosis of APS. Also, detection of aPL antibodies could be useful to explain the occurrence of thrombotic or obstetric events. *In vitro* experiments have shown that the presence of aPL antibodies can induce a pro-thrombogenic and inflammatory state [54–59]. *In vivo*, aPL antibodies have shown to induce thrombosis and pregnancy morbidity in animal models [49,60–63]. Another more important reason to detect aPL antibodies is assessing the risk of a future thrombotic event as clinical studies have shown that APS patients have a higher risk of a thrombotic recurrence compared to thrombotic patients without aPL antibodies [64,65]. Patients with thrombotic APS are therefore often prescribed long-term anticoagulation [66,67]. To avoid overdiagnosis, testing for aPL antibodies is only useful in patients with a significant probability of having APS. The presence of aPL antibodies can help to explain the occurrence of thrombotic or obstetric event and predict the risk of a recurrent clinical event.

Besides thrombosis and pregnancy morbidity, aPL antibodies have also been associated with non-criteria manifestations such as thrombocytopenia, nephropathy, cardiac valve disease, cognitive dysfunction and skin ulcers [68]. Detection of aPL antibodies is also

one of the items for the classification of systemic lupus erythematosus (SLE). Also when a patient has an unexplained prolonged activated partial thromboplastin time (aPTT), aPL detection (LAC) could explain this [69].

To more harmonized anti-phospholipid antibody testing

The detection of LAC is associated with considerable inter-laboratory variability and high rates of false negative and false positive results [16,70,71]. However, the aim here is to elaborate on solid phase assays detecting aPL antibodies.

Why we should consider centralized laboratory testing

Detection of aPL antibodies by ELISAs are known to be associated with large inter-assay and inter-laboratory variation [21,22]. External quality programs have shown variation in antibody titers and classification of the same sample using aCL and a β 2GPI ELISAs [22]. Pre-, post- and analytical conditions contribute to the variability in test results for aPL antibodies [21]. The contributing factors are both biological and methodical; the heterogeneity of the autoimmune antibodies, inadequate standardization of assays, differences in local working conditions, discussions or limited knowledge on the relevance of the antibodies, difficulties in correct interpretation of the results, lack of large prospective evaluation studies and the determination of the pathological cut-off level. Moreover, the lack of a link between antibody potency and recurrent thrombosis also contributes to the observed variation [4,21]. In the last 25 years, guidelines and proposals have been published in an attempt to standardize aPL antibody assays [4]. Moreover, complete standardization of aPL antibody assays is far from reached. With a lack of well standardized solid phase assays, centralized laboratory testing for aPL antibodies by solid phase assays could be an effective and adequate (interim) solution.

The ideal solid phase assay

Technology has advanced since the first description of a radioimmunoassay for the detection of aCL antibodies in 1983 [72]. Shortly after, an ELISA for the detection of aCL antibodies was described [73]. Nowadays automated assays have become available that use different solid phases (e.g. magnetic particles, microbeads, membranes, and coated polystyrene cups) with various detection methods (e.g. fluorescence, chemiluminescence and multiplex systems) [2–6]. Automated systems can reduce the inter-laboratory and intra-laboratory variation, but inter-assay variation still exists [23,74]. Despite the large inter-assay variation, diagnostic performance of solid phase assays detecting aCL and a β 2GPI antibodies is comparable [23]. Antibodies directed against Gly40-Arg43, located in DI of β 2GPI have shown to be pathogenic [40,75]. In agreement, we found that the solid phase assay, that was most sensitive in detecting

a monoclonal antibody against Gly40-Arg43, reached the highest odds ratio (OR) for thrombosis [23]. In addition, sensitivity to the cryptic epitope Gly40-Arg43 ensures correct exposure of β 2GPI [76]. Therefore, a suitable assay should be preferably fully automated and sensitive to (pathogenic) antibodies against Gly40-Arg43.

The ideal reference laboratory

The aPL antibody performing laboratory should be a center of expertise within APS and able to evaluate the obtained results from solid phase assays in correlation with LAC results. Academic hospitals are located across the country, have an expertise in rare syndromes and have mostly fully equipped laboratories. Centralized testing opens the door to new possibilities like initiating a database and biobank for further research into APS. Conducting research is one of the core tasks of academic hospitals, making them excellent candidates for reference laboratories.

Wide range of solid phase assays

A variety of aPL antibody assays are available for the laboratories [66]. Some clinical laboratories do not measure all recommended aPL antibodies, while other laboratories also measure non-recommended aPL antibodies (e.g. aPS/PT IgG, aPS/PT IgM, aCL IgA and a β 2GPI IgA) for the diagnosis of APS [77]. With a reference lab, this issue can be resolved by offering testing for all required assays (aCL IgG, a β 2GPI IgG, aCL IgM and a β 2GPI IgM). In addition, aPL antibody assays that have shown to be valuable in risk stratification like aPS/PT IgG and anti-DI a β 2GPI IgG can also be offered [51].

Clinical cut-off values

An issue influencing interpretation of the results, is that most laboratories are unable to calculate in-house cut-off values from a sufficient number of healthy volunteers [78,79]. Alternatively, manufacturer's cut-off values may be transferred after validation using 20 healthy volunteers [2]. However, it is unsure whether the manufacturer calculated their cut-off value in an appropriate manner [3,78]. Specialized laboratories might be able to calculate cut-off values in a multicenter and clinical approach, which may improve diagnosis of APS [78].

Paving a way for standardization

Centralized laboratory testing might be a (interim) solution to reduce analytical and post analytical errors. However, we still have to acknowledge that variation between solid phase assays will still exist. Therefore, the designated laboratories should participate in quality control programs to improve harmonization.

Efforts have been made for standardization by international reference materials including Harris standards (pool of patient material) and Koike standards (monoclonal antibodies) [21]. However, both standards have their disadvantage. The Harris standards are limited in production and Koike standards are directed against a single epitope, thus decreasing the sensitivity of the assay in which it is used [21]. Initiatives have been taken to ensure availability of international standards for the aCL and a β 2GPI assay [80].

Centralized testing can pave the way for more harmonized aPL antibody testing by concentration of data from patient samples that might provide new insights in APS. Of note, logistical problems might arise in centralized testing.

Testing for antiphospholipid antibodies in an academic, general and rural hospital

Every patient should have access to the best healthcare available. However, in some cases, hospitals are limited by knowledge about rare diseases and/or the possibility to perform specific diagnostic assays. Here, we aim to discuss which laboratory tests should be performed in case a patient is suspected of APS and admitted to an academic hospital, general hospital or rural hospital in a developing country.

Academic hospital

Academic hospitals are equipped with specialized facilities and provide specialized care. If a patient is suspected of having APS, an academic hospital must be able to perform extensive testing. LAC, aCL IgG/M and a β 2GPI IgG/M testing are recommended for the classification of APS in patients with thrombosis or pregnancy morbidity. Although additional aPL antibodies can be tested, we need to bear in mind that immunoassays are associated with false negative and positive results [4].

aCL and a β 2GPI IgM antibodies in thrombotic APS

Studies that investigated the added value of IgM aPL in large patient cohorts are limited [25,81]. In our multicenter study, the detection of aCL and a β 2GPI IgM antibodies was found to be of little value in thrombotic APS [81]. Prospective data on the role of aCL and a β 2GPI IgM antibodies within the current aPL panel is needed to exclude IgM aPL from the classification criteria for APS.

Antiphospholipid antibody profiles

Multiple studies have shown that it is more important to evaluate all aPL antibody results (aPL antibody profile) instead of single serological markers for APS [64,65,82–84]. Evaluation of complete aPL antibody profiles can be used to evaluate the “reliability” of diagnosis and to evaluate the risk of a recurrent event [82].

Reliability of diagnosis

The clinical relevance of single and double positivity for aCL and a β 2GPI IgG/M is uncertain [82]. One of the reasons might be interferences with solid phase assays that induce false positive results. Evaluation of the complete aPL antibody profile can be used to evaluate the “reliability” of diagnosis. Figure 1 illustrates different aPL profiles from patients with negative LAC results and three possible interferences. Rheumatoid factor can give false positive aCL and/or a β 2GPI IgM results [1,85]. If diagnosis of APS is solely dependent on IgM aPL, rheumatoid factor testing might be considered for the correct interpretation of the aPL antibody results [1,85]. Positivity for aCL but not for a β 2GPI antibodies might be the result of an infectious disease (e.g. Syphilis and HIV) [85]. However, a β 2GPI antibodies have shown to be present in patients with infectious diseases (e.g. leprosy) [73,86]. Interestingly, epitopes on domain IV or V have shown to be preferentially recognized by IgG antibodies in sera from antiphospholipid-positive individuals with leprosy [50,86]. While anti-DI antibodies, and not anti-D4/D5 antibodies are considered pathogenic [49]. Further evaluation of epitope specificity in aPL antibody positive patients with negative LAC results could distinguish between pathological and non-pathological aPL antibodies. Common infectious diseases should be ruled out if a patient is suspected of having APS with isolated aCL positivity. Finally, disagreement of the isotype should be labeled as inappropriate and re-testing should be considered [82].

Risk stratification

Once a patient is diagnosed with APS, it might be interesting to evaluate the aPL antibody profile for thrombotic risk stratification. Triple positivity is considered as a high-risk profile for thrombosis [87,88]. However, triple positivity does not take the isotype for which it is positive into an account [82]. Positivity for LAC, aCL IgG and a β 2GPI IgG is considered a triple positive aPL profile. Likewise, positive results for LAC, aCL IgM and a β 2GPI IgM and positive results for LAC, aCL IgG, a β 2GPI IgG, aCL IgM and a β 2GPI IgM are considered triple positive.

FIGURE 1. Reliability of aCL and aβ2GPI IgG/M results in LAC negative patients suspected of APS. Rheumatoid factor is associated with false positive IgM results and should be considered testing if IgG aPL antibodies are negative. Positivity for aCL antibodies with negative aβ2GPI could be an indication for infectious diseases. Re-testing should be considered if there is a disagreement of isotype.

In our study, we have shown that the thrombotic association of triple positivity is predominately caused by IgG aPL antibodies [24]. Patients with positive LAC, aCL IgM and aβ2GPI IgM (i.e. triple positive) results are strongly associated with a previous thrombosis [24]. However, if IgG aPL are absent, the clinical significance is low [24]. Positivity for LAC, aCL IgG and aβ2GPI IgG is strongly correlated with thrombosis even if IgM aPL antibodies are negative. The presence of all criteria aPL antibodies (LAC, aCL IgG/M and aβ2GPI IgG/M) results in the strongest association with thrombosis [24]. Therefore, all criteria aPL antibodies results should be considered to evaluate the clinical significance of an aPL profile (Figure 2).

Additional testing for risk stratification

Additional aPL antibody testing might be performed in triple positive patients within an academic setting. Anti-DI aPL antibodies might be detected for risk stratification as combined anti-DI and triple positivity resulted in a higher OR than only triple positivity [51]. Also, the presence of aPS/PT IgG has shown to increase ORs in triple positive patients positive for LAC, aCL IgG and aβ2GPI IgG [24].

Other laboratory markers for thrombosis could be considered for thrombotic risk stratification in definite APS patients. Activated protein C resistance together with LAC positivity has shown to be strongly correlated with thrombosis [89]. Activated protein C resistance can also be determined with a thrombin generation-based test and has shown to be associated with thrombosis in LAC positive patients [90]. Using thrombin generation, LAC titers can be quantified and could be useful for thrombotic risk stratification together with soluble P-selectin and factor VII results [91]. However, data on the value of each single laboratory marker within thrombotic risk stratification of APS patients is lacking. A well-designed prospective multicenter study is needed for the evaluation of additional aPL antibody assays and established laboratory markers in thrombotic risk stratification of APS patients.

General hospital

In case that centralization of testing is not available, general hospital laboratories should provide LAC, aCL IgG/M and a β 2GPI IgG/M assays for the detection of APS. Due to the high variability among immunoassays detecting aCL and a β 2GPI antibodies, it seems important that in daily practice, aCL IgG/M and a β 2GPI IgG/M antibodies are measured with the same platform. A medical laboratory should take this into an account if not all assays are available in-house and additional aPL testing is needed elsewhere.

Automated algorithm for the interpretation of lupus anticoagulant

Nowadays, LAC assays have integrated a screening and confirmation step into one single assay, which may increase the accuracy and inter-laboratory agreement [92]. However, the mixing procedure should still be performed [92]. Difficulties in performing the mixing step, interpretation of results and calculation of cut-off values may lead to false positive or false negative results [3,4,21,92]. The introduction of an automated algorithm for the interpretation of LAC might be a practical solution [93]. Research has shown that such an algorithm has a high sensitivity and specificity for LAC detection and reduced hands-on time with 60% [93]. An automated algorithm for the interpretation of LAC might also take established interfering factors into consideration including anticoagulation, coagulation factor deficiencies, reduced fibrinogen and elevated C-reactive protein (CRP) levels [94]. For instance, isolated positivity for the aPTT-based LAC assay might require testing of plasma CRP levels as research has shown that CRP levels >50 mg/L can mimic the presence of LAC in all three steps of the aPTT but not in the dilute Russell Viper Venom Time (dRVVT)-LAC assay [95]. Isolated positivity for the aPTT-LAC assay with high CRP levels should therefore be interpreted with care and retesting at a later moment can be considered. A universal automated algorithm could help general hospital laboratories with the interpretation of LAC results.

FIGURE 2. Significance of aPL antibody profiles in thrombosis.

Risk stratification

General hospital laboratories should evaluate the profile of patients with thrombotic APS. One could consider extensive testing similar to an academic setting. However, a distinction between APS patients that are triple positive and patients without triple positivity might be sufficient. Triple positivity is generally accepted to be a high-risk profile for thrombosis. Triple positive patients with additional aPL antibodies could have a higher risk for recurrent thrombosis. However, treatment of triple-positive patients with additional aPL antibodies is most likely not different from patients with triple positivity alone [66,67,83]. Therefore, it would be more convenient to limit risk stratification of patients with thrombotic APS to triple positivity or non-triple positivity and avoid additional aPL antibody testing for risk stratification in general hospitals.

Rural hospitals in developing countries

Rural hospitals in developing countries have limited resources and only selected laboratory assays available. It is therefore not evident to perform all assays suggested by the Sydney criteria [1]. From all types of aPL antibodies in the current criteria, LAC is considered the strongest predictor of thrombosis and pregnancy morbidity [96–98]. However, LAC is labor intensive and complicated to interpret which makes it difficult to perform the assay in a rural hospital with limited resources.

Point of care-lupus anticoagulant

Point of care (POC) diagnostics might be a solution in a low resource setting as rural hospitals in developing countries. Unfortunately, no POC assay exists for the detection of LAC. International guidelines recommend the use of a dRVVT and aPTT based assay in LAC detection [1,99,100]. With the existence of POC assays measuring prothrombin time (PT) and aPTT, a POC-LAC assay seems to be technically feasible [101]. However, more research is needed to evaluate the feasibility of a POC-LAC that is in agreement with the current guidelines [1,100]. In developing countries, infectious diseases are very common and considered to cause the development of aPL antibodies. International guidelines should be followed for the detection of LAC, also in a POC testing [1,99,100]. An automated algorithm for clear LAC results is preferred in this setting.

Solid phase assays in rural hospitals

Detection of aCL and a β 2GPI antibodies by ELISA is labor intensive and requires trained personnel. Automated systems are less labor intensive and easy to perform. However, such systems and reagents are too expensive for rural laboratories in developing

countries. Exclusion of aCL IgG/M and a β 2GPI IgG/M testing might result in decreased sensitivity for APS diagnosis. Another limitation is the inability of risk stratification without aCL and a β 2GPI results.

Lupus anticoagulant versus aCL and a β 2GPI antibodies

In developing countries, the prevalence of infectious diseases is high [102]. Viral infectious diseases like HIV and syphilis can give rise to false positive aPL antibody results [103]. Still, LAC has shown to be strongly associated with stroke in a population from urban and rural Tanzania [104]. The presence of aPL antibodies detected by solid phase assays (aCL, a β 2GPI and aPS/PT) was found to be of only little value as a biomarker for stroke [104]. Therefore, the development of a POC-LAC assay could be a useful biomarker for (arterial) thrombosis in rural areas. Due to the limited data available on prevention of a recurrent stroke, several therapy strategies are possible [66,105–107]. The European League Against Rheumatism (EULAR) recommend the use of VKA over low-dose aspirin (LDA) in APS patients with a first arterial thrombosis [67]. However, in APS patients with a first stroke, therapy with either VKA or clopidogrel is also justified [66]. Antiplatelet therapy could be a good alternative for the prevention of recurrent stroke in APS patients as VKA therapy requires regular monitoring and is therefore not preferred in rural areas of developing countries. Still, more data is needed to evaluate the importance of a POC-LAC assay and its implication on treatment strategies in rural hospitals.

CONCLUSION

This thesis describes the importance of detecting aPL antibodies by immunoassays in the laboratory diagnosis of APS. The newer generation aPL antibody assays are still associated with high inter-assay variation, even in triple positive patients. Isolated aCL and a β 2GPI IgM antibody results should be interpreted with care in thrombotic APS. Detection of aCL and a β 2GPI IgA antibodies did not add value to the laboratory diagnosis of APS within the current aPL panel. The presence of anti-DI antibodies may help in identifying high-risk patients. Well-designed prospective multicenter studies are needed to evaluate aPL antibody profiles and the risk of recurrent thrombosis in APS patients. Anti-prothrombin antibodies with LAC activity are able to induce platelet aggregation via the Fc γ RIIA receptor. However, more data is needed to determine the role of anti-prothrombin antibodies in the failure of anti-FXa therapy in patients with APS.

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A

Appendices

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NEDERLANDSE SAMENVATTING

Het antifosfolipiden syndroom (APS) is een auto-immuunziekte en wordt gekenmerkt door trombose en/of zwangerschapscomplicaties met de aanwezigheid van antifosfolipiden (aPL) antilichamen. Trombose en zwangerschapscomplicaties komen veelal voor zonder een indicatie voor APS. De diagnose APS is daarom voornamelijk afhankelijk van diagnostische testen. Laboratorium criteria voor de diagnose APS bestaan uit de detectie van; 1) lupus anticoagulans (LAC), 2) anti-cardiolipine (aCL) IgG/M antilichamen en 3) anti- β 2glycoproteïne I (a β 2GPI) IgG/M antilichamen. Dit proefschrift beoogt de optimalisatie van de diagnose en risicostatificatie van patiënten met APS. Hierbij is de klinische relevantie van verschillende immunologische- en stollingstesten binnen APS onderzocht.

In **hoofdstuk 2** is aangetoond dat de detectie van aCL en a β 2GPI antilichamen afhankelijk is van de immunologische test die gebruikt wordt. Desalniettemin is de associatie van deze antilichamen met trombose en zwangerschapsmorbiditeit vergelijkbaar tussen immunologische testen. De verkregen resultaten benadrukken dat bij het stellen van de diagnose APS, er rekening gehouden moet worden met de variatie die de immunologische testen teweegbrengen. De aanwezigheid van LAC, aCL en a β 2GPI antilichamen (triple positiviteit) in APS-patiënten is een hoog risicoprofiel voor recidief (terugkomende) trombose. In **hoofdstuk 3** is aangetoond dat ook de identificatie van deze hoog-risico patiënten afhankelijk is van de immunologische test die gebruikt wordt. Daarnaast blijkt positiviteit voor aCL en a β 2GPI IgG uitermate belangrijk te zijn voor de sterke associatie van triple positiviteit met trombose, terwijl positiviteit voor aCL en a β 2GPI IgM maar een geringe bijdrage leveren aan de associatie van triple positiviteit met trombose.

De diagnostische waarde van aCL en a β 2GPI IgM antilichamen in APS staat al kort na de introductie van de herziene criteria voor het vaststellen van APS ter discussie. **Hoofdstuk 4** omschrijft de klinische relevantie van aCL en a β 2GPI IgM binnen APS. De detectie van aCL en a β 2GPI IgM antilichamen is overbodig bevonden voor het opsporen van trombotische APS indien er al getest wordt voor LAC, aCL IgG en a β 2GPI IgG antilichamen. De detectie van aCL en a β 2GPI IgM antilichamen was wel van belang bij het opsporen van obstetrische APS. Deze resultaten suggereren een andere strategie voor het testen van aPL antilichamen bij verdenking van trombotische en obstetrische APS.

Naast LAC, aCL IgG/M en a β 2GPI IgG/M antilichamen is ook voorgesteld om andere aPL antilichamen te includeren in de classificatie criteria van APS, waaronder aCL en a β 2GPI

IgA antilichamen. In **hoofdstuk 5** is de correlatie van aCL en a β 2GPI IgA antilichamen met trombose en zwangerschapscomplicaties bevestigd. Desalniettemin zijn patiënten met aCL en/of a β 2GPI IgA antilichamen, maar negatief voor LAC, aCL IgG/M en/of a β 2GPI IgG/M, zeldzaam. Hieruit kan geconcludeerd worden dat positiviteit voor aCL en a β 2GPI IgA antilichamen geen toegevoegde waarde heeft naast de huidige laboratorium criteria voor APS.

Hoofdstuk 6 biedt relevante achtergrondinformatie over het belang van het testen van antilichamen die gericht zijn tegen het eerste domein van β 2GPI (anti-DI). Daarbij werden de pathogeniciteit en de klinische significantie van anti-DI antilichamen onderzocht. In **hoofdstuk 7** is aangetoond dat anti-DI antilichamen gecorreleerd zijn met trombose en zwangerschapscomplicaties, maar dat het meten van deze antilichamen geen toegevoegde waarde heeft naast de huidige laboratorium criteria voor de diagnose van APS.

Aggregatie van bloedplaatjes speelt een cruciale rol in het ontwikkelen van arteriële trombose. Toch is er maar weinig bekend over het effect van aPL antilichamen op plaatjesaggregatie. In **hoofdstuk 8** is aangetoond dat de aanwezigheid van antiprotrombine antilichamen met LAC activiteit kan leiden tot aggregatie van bloedplaatjes via de Fc γ RIIA receptor. Vitamine K antagonisten (VKAs) zijn antistollingsmiddelen die de productie van vitamine K-afhankelijke stollingsfactoren, waaronder protrombine, remmen. Het directe orale anticoagulans (DOAC) rivaroxaban is een directe remmer van geactiveerde stollingsfactor Xa. Klinisch onderzoek heeft aangetoond dat in vergelijking met het gebruik van VKAs, het gebruik van DOACs het risico kan verhogen op arteriële trombose in triple positieve APS patiënten. In hoofdstuk 8 is ook aangetoond dat het verlagen van de protrombine concentratie zorgt voor verminderde bloedplaatjes aggregatie. Daarentegen wordt plaatjes aggregatie door antiprotrombine antilichamen niet beïnvloed door rivaroxaban. Aggregatie van bloedplaatjes door antiprotrombine antilichamen en het effect van antistolling hierop, kan een mogelijke verklaring zijn voor het falen van DOAC-therapie in triple positieve APS patiënten.

In **hoofdstuk 9** worden de belangrijkste bevindingen van dit proefschrift besproken in het licht van de huidige literatuur. Hierin wordt beargumenteerd wat de klinische relevantie is van aPL antilichamen die gemeten worden door middel van immunologische testen en hoe deze testen beter gestandaardiseerd kunnen worden. Daarnaast is beargumenteerd welke laboratoriumtesten nodig zijn bij het vaststellen van APS in een academisch ziekenhuis, een algemeen ziekenhuis, alsook een (afgelegen) ziekenhuis in ontwikkelingslanden met beperkte middelen.

IMPACT

The antiphospholipid syndrome (APS) is a rare autoimmune disorder, characterized by thrombosis and/or pregnancy morbidity with the persistent presence of antiphospholipid (aPL) antibodies (1). Clinical manifestations of APS (thrombosis and pregnancy morbidity) occur frequently without any indication of APS (1). This makes the detection of aPL antibodies by laboratory assays crucial in the diagnosis of APS. Laboratory assays used for the diagnosis of APS include the detection of lupus anticoagulant (LAC), anti-cardiolipin (aCL) IgG/M antibodies and anti- β 2glycoprotein I (a β 2GPI) IgG/M antibodies (1). We aimed to optimize the diagnosis and risk stratification of patients with APS. The clinical relevance of several immunoassays and non-criteria aPL antibodies (e.g. aCL IgA, a β 2GPI IgA and anti-domain I β 2GPI IgG) within APS was investigated.

Key findings

We found that the detection of aCL and a β 2GPI IgG/M antibodies differs between immunoassays without affecting the association with clinical manifestations of APS. In addition, the identification of high thrombotic risk triple-positive APS patients (i.e. LAC, aCL and a β 2GPI positive) is dependent on the aCL and a β 2GPI antibody detection assay used.

The role of aCL and a β 2GPI IgM antibodies in thrombotic APS is debated by experts in the field, since conclusive data on the role of aCL and a β 2GPI IgM within APS is lacking in current literature (2). Our data support the usage of two separate decision trees, depending on the clinical presentation. We have suggested to test aCL and a β 2GPI IgM in women suspected of obstetric APS, but not in patients suspected of thrombotic APS (Figure 1).

Although not included in the laboratory criteria for the diagnosis of APS, antiprothrombin antibodies are common in patients with APS (3). Antiprothrombin antibodies have shown to be correlated with arterial thrombosis, in which platelets play a key role. However, data on antiprothrombin antibodies and platelet activation are lacking. We found that antiprothrombin antibodies with LAC activity are able to induce platelet aggregation via the Fc γ RIIA receptor.

Recent studies have shown that therapy with direct oral anticoagulants (rivaroxaban) in patients with APS leads to a high incidence of arterial thrombosis compared with vitamin K antagonist (VKA) therapy (4,5). We observed that decreased prothrombin

FIGURE 1 Decision tree for the detection and risk stratification of (A) thrombotic and (B) obstetric antiphospholipid syndrome

levels resulted in less antiprothrombin antibody-mediated platelet aggregation. We therefore hypothesize that the reduction in antiprothrombin levels by VKA attributes to the lower incidence of arterial thrombosis in patients treated with rivaroxaban.

Diagnosis of APS should be independent of the immunoassay used to detect aPL antibodies

We have shown that detection of aPL antibodies varies among immunoassays, which can affect the diagnosis of APS. Medical specialists should be aware of the low agreement between immunoassays and limit testing for aPL antibodies in patients with a significant probability of having APS, to avoid overdiagnosis (1,6). Also, retesting of aPL antibodies in patients with definite APS in a different laboratory and with another immunoassay can result in dilemmas when unexpected aPL results are obtained. Negative aPL results will not support the diagnosis APS and lifelong anticoagulation therapy becomes debatable.

Manufacturers of immunoassays should make more effort in standardizing the detecting of aPL antibodies. Development of an international standard together with the efforts of scientists and medical specialists to provide new insights in the pathogenesis of APS may contribute to a better laboratory diagnosis of APS.

Personalized aPL antibody testing

We have proposed two separate decision trees for thrombosis versus pregnancy morbidity. Exclusion of IgM aPL antibody testing for the diagnosis of thrombotic APS could lead to less false positives. Moreover, risk stratification using IgM aPL antibodies was only found to be useful in thrombotic APS and not obstetric APS. Altogether, these results will lead to improved diagnosis of APS and consequent treatment of patients with APS.

Detection of antiprothrombin antibodies that induce platelet aggregation enables DOAC use in patients with APS

We identified a specific subgroup of antiprothrombin antibodies, able to induce platelet aggregation. We hypothesize that these antiprothrombin antibodies are responsible for the high incidence of arterial thrombosis in patients with APS receiving DOAC therapy. More data are needed to confirm this hypothesis. However, future studies should focus on a diagnostic assay to distinguish between antiprothrombin antibodies that are able to induce platelet aggregation and those that do not induce aggregation of platelets. In the absence of antiprothrombin antibodies that induce platelet aggregation, DOAC

use might be feasible. Although not established in thrombotic APS due to the lack of definite evidence, the use of DOACs over VKAs has many advantageous, including no need for frequent laboratory monitoring, what can be a problem in APS (7,8).

CONCLUSION

The research presented in this thesis provides insight into the diagnostic performance of immunoassays detecting aPL antibodies. The immunoassays used in these studies are commercially available and often used in medical laboratories. Therefore, our findings can be directly related to current practice. Next, we have suggested different decision trees for the laboratory diagnosis of thrombotic and obstetric APS which should be further evaluated in well-designed large prospective multicenter studies. In addition, we provided the first steps of evidence towards DOAC use in APS patients which is a more practical and user-friendly alternative treatment.

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CURRICULUM VITAE

Walid Chayouâ was born on 14 July 1993 in Heerlen, The Netherlands. In 2005 he started secondary school at Sintermeertencollege in Heerlen and received his HAVO degree in 2010 with the specializations Nature & Health and Nature & Technology. Subsequently, he studied Biology and Medical Laboratory Research at Zuyd university of Applied Sciences in Heerlen. After earning his bachelor's degree, Walid enrolled in the Biomedical Sciences master program at Maastricht University in 2014. He performed his master internship at the Department of Biochemistry at Maastricht University, where he studied the protective effect of aspirin and rivaroxaban upon endothelial denudation of the mouse carotid artery. After successfully completing his internship, he obtained his master's degree in 2016. In the same year, Walid started his PhD research at Synapse Research Institute, affiliated to Maastricht University, under the supervision of Prof. Dr. H. ten Cate, Prof. Dr. K.M.J. Devreese, Prof. Dr. P.G. de Groot and Dr. B. de Laat. During his PhD, Walid investigated the clinical importance of detecting antiphospholipid antibodies by immunoassays in the antiphospholipid syndrome. In 2019, he received the Harry Struiker-Boudier Award for Talented Academics (HS-BAFTA) fellowship to investigate the role of antiprothrombin antibodies in platelet activation at the University of Birmingham (UK). As a PhD student, he presented his research at various international conferences. After obtaining his PhD degree he will start his resident training in clinical chemistry at the Medisch Spectrum Twente (MST) in Enschede, The Netherlands.

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1. Kelchtermans H, **Chayoua W**, de Laat B. The Significance of Antibodies Against Domain I of Beta-2 Glycoprotein I in Antiphospholipid Syndrome. *Seminars in thrombosis and hemostasis* (2018) 44: 458-65.
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3. Chayoua W, Do the role of isotypes and antibody profile of antiphospholipid antibodies differ for thrombosis and pregnancy morbidity? *SSC*, Melbourne, Australia (July 2019).
4. Chayoua W, Multicenter study on solid phase assays: role of IgA antiphospholipid antibodies in APS. *SSC*, Melbourne, Australia (July 2019).
5. Chayoua W, The Added Value of Anti-cardiolipin and Anti-b2glycoprotein I IgM Antibodies in the Antiphospholipid Syndrome, a European multicenter study. *International Society for Laboratory Hematology (ISLH)*, Vancouver, Canada (May 2019).
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8. Chayoua W, Role of IgM in APS: The Solid Phase Assay Multicenter Study. *SSC*, Dublin, Ireland (July 2018).
9. Chayoua W, The identification of high risk APS patients for thrombosis with triple antiphospholipid antibody positivity is platform dependent. *American Society of Hematology (ASH)*, Atlanta, USA (December 2017).
10. Chayoua W, First Results out of the Multicenter Study on the Role of IgM in APS. *SSC*, Berlin, Germany (July 2017).

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Awards

1. The Harry Struiker-Boudier Award for Talented Academics (HS-BAFTA) fellowship, CARIM (2019).
2. Award for Scientific Excellence, NVTH (2019).
3. Trainee Travel award, ISLH (2019).
4. American Society of hematology (ASH) abstract achievement award (2018).
5. American Society of hematology (ASH) abstract achievement award (2017).

LIST OF ABBREVIATIONS

ABS	adult bovine serum
aCL	anti-cardiolipin
AID	autoimmune disease
APC	activated protein C
aPL	antiphospholipid antibodies
APS	antiphospholipid syndrome
aPS/PT	anti-phosphatidylserine/prothrombin
aPT	anti-prothrombin
aPTT	activated partial thromboplastic time
Arg	arginine
AT	arterial thrombosis
AU	arbitrary unit
aβ2GPI	anti- β 2glycoprotein I
β2GPI	β 2glycoprotein I
BSA	bovine serum albumin
CCP	complement control protein
CI	confidence interval
CIA	chemiluminescence immunoassay
CL	cardiolipin
CLEC-2	C-type lectin-like receptor 2
CRP	C-reactive protein
DI	domain I
DOAC	direct oral anticoagulants
dRVVT	dilute Russell's viper venom time
DV	domain V
ECAT	external quality control of diagnostic assays and tests
ECT	Ecarin clotting time
ELISA	enzyme-linked immunosorbent assay
EULAR	European league against rheumatism
F	(coagulation) factor
FBS	fetal bovine serum
Gly	glycine

GP	glycoprotein
HIT	heparin induced thrombocytopenia
Ig	Immunoglobulin
ISTH	international society on thrombosis and haemostasis
ITAM	immunoreceptor tyrosine-based activation motif
LAC/LA	lupus anticoagulant
LDA	low-dose aspirin
LMWH	low-molecular-weight heparin
LOD	lower limit of detection
LTA	light transmission aggregometry
mAb	monoclonal antibody
NFκB	nuclear factor kappa B
NPV	negative predictive value
OD	optical density
OR	odds ratio
PAPS	primary antiphospholipid syndrome
PBS	phosphate buffered saline
PL	phospholipid
POC	point-of-care
PPV	positive predictive value
PRP	platelet-rich plasma
PT	prothrombin time
ROC	receiver operating characteristic
SAPS	secondary antiphospholipid syndrome
SD	standard deviation
SLE	systemic lupus erythematosus
SSC	scientific and standardization committee
TBS	tris-buffered saline
TIA	transient ischemic attack
TLR4	toll-like receptor 4
TMB	3,3',5,5'-tetramethylbenzidine
VDRL	venereal disease research laboratory
VKA	vitamin K-antagonists
VT	venous thrombosis