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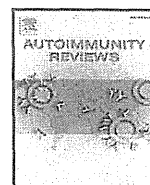
Review

14th International Congress on Antiphospholipid Antibodies Task Force. Report on antiphospholipid syndrome laboratory diagnostics and trends

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ARTICLE INFO

ABSTRACT

Current classification criteria for definite Antiphospholipid Syndrome (APS) require the use of three laboratory assays to detect antiphospholipid antibodies (aCL, anti-β2GPI and LA) in the presence of at least one of the two major clinical manifestations (i.e. thrombosis or pregnancy morbidity) of the syndrome. However, several other autoantibodies shown to be directed to other proteins or their complex with phospholipids have been proposed to be relevant to APS but their clinical utility and their diagnostic value remains elusive.

This report summarizes the findings, conclusions and recommendations of the "APS Task Force 3—Laboratory Diagnostics and Trends" meeting that took place during the 14th International Congress on Antiphospholipid Antibodies (APLA 2013, September 18–21, Rio de Janeiro, RJ, Brazil).

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Contents

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1. Introduction

Current classification criteria for definite Antiphospholipid Syndrome (APS) require the use of three laboratory assays to detect antiphospholipid antibodies (aPL) in the presence of at least one of the two major clinical manifestations (i.e. thrombosis or pregnancy morbidity) of the syndrome [1]. Anticardiolipin antibodies (aCL), anti-β2 glycoprotein I (anti-β2GPI) antibodies and the lupus anticoagulant (LA) are the laboratory tests included in the revised criteria for the classification of the APS.

However, several other autoantibodies shown to be directed to other proteins of the coagulation cascade (i.e. prothrombin and/or phosphatidylserine–prothrombin complexes) or their complex with phospholipids other than cardiolipin, or to some domains of β2GPI, have been proposed to be relevant to APS [2] but their clinical utility and their diagnostic value remain elusive. The clinical relevance of IgA aPL and whether these isotype tests should be part of the routine diagnostic algorithm is also being a subject of hot debate.

A task force of worldwide scientists in the field firstly met, discussed and analysed critical questions related to "criteria" and "non-criteria" aPL tests in an evidence-based manner during the 13th International Congress on Antiphospholipid Antibodies (APLA 2010, April 13–16, Galveston, TX, USA) [3,4]. Members of these task forces continued to work and reunited to evaluate the utility of various laboratory assays.

This report summarizes the findings, conclusions and recommendations of the "APS Task Force 3—Laboratory Diagnostics and Trends" meeting that took place during the 14th International Congress on Antiphospholipid Antibodies (APLA 2013, September 18–21, Rio de Janeiro, RJ, Brazil). This task force comprised a group of clinical laboratory scientists, researchers and clinicians, involved within 7 subgroups (Table 1) according to their expertise. All available data was assigned a level of evidence according to the design of the study [5] (Table 2) and the grading system was applied to evaluate the quality of that available evidence (Table 3) [6,7].

Table 1
Task force 3—laboratory diagnostics and trends.

Subgroup	
I	Harmonization of aCL and anti-β2GPI
II	Lupus anticoagulant
III	IgA aPL tests
IV	Tests for antibodies to negatively charged phospholipids and antibodies to phosphatidylethanolamine (aPE)
V	Tests for antibodies to prothrombin (aPT) and phosphatidylserine/prothrombin (aPS/PT)
VI	Tests to antibodies to domain I
VII	aPL as risk factors

Last but not least, this manuscript is dedicated to the memory of Prof. Silvia Pierangeli (1955–2013), an exceptional friend, a remarkable colleague and one of the main contributors to the study of APS, including the standardization of aPL tests. Prof. Pierangeli embarked on a tireless effort to promote standard test performance through multiple publications and workshops, and by providing proficient advice worldwide. Her efforts culminated in the assembly of experts for this task force to which she devotedly dedicated during the last months of her life.

1.1. Subgroup I—harmonization of aCL and anti-β2GPI

This session was dedicated to the memory of Drs. John A McIntyre and Doug A Triplett.

2. Standardization of antiphospholipid immunoassays

A report from the 'criteria' aPL task force formed at the 13th International Congress on Antiphospholipid Antibodies outlined critical issues relating to the performance of antiphospholipid (aPL) immunoassays and made several recommendations to improve their standardization [3]. Among these recommendations were the need for an international consensus protocol for anticardiolipin (aCL) and anti-βeta2 glycoprotein I (anti-β2GPI) tests (which have subsequently been published) as well as the establishment of international units (IUs) of measurement for anti-β2GPI assays and the development of internationally recognized polyclonal and monoclonal standards for this assay [8,9]. Members of subgroup I were charged with continuing the development of international units and reference materials for anti-β2GPI testing and more broadly with critical examination and discussion of proficiency testing programs, cut-off establishment and the significance of low-positive titers for aPL immunoassays.

3. Development of polyclonal and monoclonal reference material and international units for anti-β2GPI measurement

According to an approved protocol prepared by Drs Silvia Pierangeli, Pier Luigi Meroni and Gabriella Lakos, IgG and IgM polyclonal reference sera (IgG and IgM reference material) were each prepared by pooling serum from well-characterized APS patients with very high anti-β2GPI levels of the desired isotype. Once prepared, IgG and IgM anti-β2GPI fractions were purified from their respective reference material utilizing combinations of affinity and ion-exchange chromatography; then were subsequently pooled, concentrated, sterile filtered and their binding activity and protein concentration measured using ELISA and Bradford protein assays respectively. The anti-β2GPI IU was thus defined using these affinity-purified fractions—where 1 IU is equivalent to the binding

activity of 1 µg/ml of affinity-purified anti-β2GPI. Each reference material was then extensively characterized using the respective affinity-purified anti-β2GPI material as a calibrate material. The IgG reference material was determined to have a value of 270 IgG anti-β2GPI IU and the IgM reference material—a value of 220.3 IgM anti-β2GPI IU.

To determine the suitability of the reference material among different anti-β2GPI immunoassays, several diagnostics companies were invited to evaluate each reference material in a two-step process—first examining unit equivalency and linearity and second, commutability according to an approved protocol following CLSI guidelines (EP14-A2, EP06A and C53-A). Participating companies included INOVA Diagnostics, Bio-Rad, TheraTest Laboratories, Instrumentation Laboratories, Corgenix, Phadia/ThermoFisher, Aesku and Human GmbH. Each reference material was shipped to all companies along with 30 APS patient samples. Analysis of the obtained data revealed wide variation of the IgG reference material in the various arbitrary kit units (115 to 9993.1) but less so for the IgM reference material (35.4 to 98.4), with variation being reduced by conversion of arbitrary kit units to international units. Both the IgG and IgM reference material were found to be commutable among the assays tested.

A similar analysis of a monoclonal IgG anti-β2GPI reference material (a chimeric monoclonal IgG anti-β2GPI producing clone 'HCAL'—INOVA Diagnostics) was performed. Spectrophotometric measurements at 280 nm revealed that the material had a working concentration of 133 µg/ml and cross-validation comparison with polyclonal IgG reference material showed excellent agreement with insignificant bias. The monoclonal reference material was also shown to be commutable utilizing INOVA and Corgenix anti-β2GPI immunoassays.

Further validation studies on both the polyclonal and monoclonal reference material are currently being performed by the Institute for Reference Materials and Measurements (IRMM), an internationally recognized body with respect to certification of reference materials. These on-going efforts will significantly contribute towards the improvement of inter-laboratory and inter-assay agreement for aPL immunoassays. The following experts in the field of standardization initiatives actively participated and are still involved in the project: Dr. Joanna Sheldon; Consultant Immunologist; Chair Harmonization of Autoimmune Serology Testing—Working Group (WG HAT)—International Federation of Clinical Chemistry and Laboratory Medicine. Protein Reference and Immunopathology unit, St. George's Hospital, London UK; Dr. Ingrid Zegers; RM Unit, European Commission—DG JRC (IRMM); Maria Orietta Borghi Division of Rheumatology, Department of Clinical Sciences and Community Health, University of Milan and Experimental Laboratory of Immunorheumatology, Istituto Auxologico Italiano, Milan, Italy; Claudia Grossi Experimental Laboratory of Immunorheumatology, Istituto Auxologico Italiano, Milan, Italy. These on-going efforts will significantly contribute towards the improvement of inter-laboratory and inter-assay agreement for aPL immunoassays.

4. Proficiency testing programs report—College of American Pathology (CAP)

Proficiency testing programs for aPL are offered by a number of organizations, including the College of American Pathologists (CAP). The CAP defines qualitative agreement for the aCL survey as ≥80% positive/negative agreement across all participants, regardless of specific assay method or test kit. Therefore, a review of the participant consensus results within the aCL survey can provide some information regarding standardization of clinical tests and laboratory performance. Between 2007 and 2012, twelve surveys (a total of 36 samples) were conducted and ≥80% participant consensus for IgG and IgM aCL results was achieved for 32/36 and 31/36 samples, respectively. Similarly, the required rate of agreement was observed for 30/36 samples for IgG and 34/36 samples for IgM anti-β2GPI. In contrast, relatively poor agreement was observed for the IgA isotypes, with only 21/36 and 22/36 samples achieving ≥80% participant consensus for IgA aCL and

IgA anti-β2GPI, respectively. It is also important to note that lack of participant consensus was observed for at least one analyte on at least one survey every year, indicating that qualitative agreement between participating labs is an on-going issue.

The reasons why lack of qualitative agreement occurs may vary, depending on the specific specimen and analyte. In some cases, the lack of agreement may be attributable to issues related to poor standardization between the various methodologies or platforms. In other cases, it may be caused by analytical imprecision; this is particularly problematic when the sample contains a low level antibody with a quantitative value close to the positive/negative cut-off. Lastly, the exact procedure used by a laboratory for performing a given method may vary, which can impact the overall performance of the test. It must be kept in mind that there are limitations of data acquired from proficiency testing programs for the purposes of method evaluation and standardization. Although the number of participating labs may be significant, the number of samples evaluated each year is small. In addition, the characteristics of the specimens used for proficiency testing surveys may not accurately reflect true patient matrix. However, as long as these limitations are understood, proficiency testing can still provide valuable information to both participating laboratories and assay manufacturers.

5. Cut-off establishment and the significance of low positive aPL antibody levels

The method of cut-off establishment and the accuracy of the cut-off value are key factors in determining the diagnostic performance characteristics of an assay. Consequently, reaching consensus on the method of cut-off establishment is important from the point of view of harmonization of aPL assays. Fortunately, this is an area, where researchers and laboratory scientists alike have the highest level of agreement. Reference ranges for aCL and anti-β2GPI test results must be established by nonparametric methods since the distribution of these antibody levels in the population is not Gaussian. The recommended cut-off value is the 99th percentile of the reference (normal) population, which is in concordance with previously published guidelines [8,10–12]. Although the instruction manuals of many aPL assays recommend that laboratories establish their own reference ranges, end users (diagnostic laboratories) rarely have the resources to conduct a proper reference-range

Table 2
Level of evidence according to the study design [5].

Level	Study design
I-A	Meta-analysis of randomised controlled trials
I-B	Randomized controlled trial
II-A	Controlled study without randomization
II-B	Quasi-experimental study
III	Descriptive study (comparative, correlation, case–control)
IV	Expert committee report/opinion an/or clinical opinion of respected authority

Table 3
GRADE system—quality of the evidence [7].

Quality	
High	Low probability of further research completely changing the presented conclusions
Moderate	Estimate lies close to the true value, but further research may completely change the conclusions
Low	Estimate and the true value may be substantially different. Further research is likely to change the presented conclusions completely
Very low	The authors do not have any confidence in the estimate

study, thereby laboratories should instead focus on verifying the manufacturer's suggested reference intervals and cut-off values [13].

The presence of aPL antibodies in a patient can precede the occurrence of clinical symptoms, and a patient can be positive for a long period of time without a clinical manifestation ever occurring. It means that even with a properly established cut-off, a group of so called "analytically true positive, clinically false positive" results will be detected, posing a special challenge for interpretation. These results seem to be more prevalent with newer analytical technologies [14], presumably due to better analytical sensitivity, and better resolution of results. Their function and significance is unclear, and may be clarified in long-term prospective studies only. These antibodies can be the result of any, or the combination of the following scenarios: natural autoantibodies; temporary, infection-induced antibodies; real pathogenic antibodies. Because of this very special clinical situation, the term "false positive" may not be applicable to aPL assays, and should be avoided.

The value and clinical significance of low positive aPL values has been the topic of research and publications. According to the current definition, the threshold between low and medium antibody titer is 40 GPL and MPL units for aCL antibodies, or 99th percentile of the values obtained on reference subjects for both aCL and anti-β2GPI antibodies [1]. However, two things need to be considered. First, different clinical symptoms may be associated with various levels of aPL antibodies. For example, there are data pointing to the significance of lower aPL levels in pregnancy complications compared to thrombosis [15]. Second, given the variability of aPL assays, using the same numerical value does not guarantee the same clinical utility. In fact, the definition of medium-positive antibody titers depends on the performance characteristics of the particular assay, the statistical method, and the reference population used to establish cut-off values. The committee overseeing the revised classification criteria mentioned the lack of suitable evidence on this issue, and specifically commented that these values are to be used "until an international consensus is reached" [1]. What exactly is the meaning of a low positive aPL result? Until the new reference materials will be able to harmonize the different tests, the question should be approached from a clinical point of view. The significance of a low positive aPL result depends on the whole risk profile of the patient for a given clinical manifestation. For example, a low positive aPL assay could display a higher risk in a older pregnant woman than in a younger one. Locking in certain numerical values as low or medium aPL antibody levels may pose the risk of misinterpretation: either by overestimating the significance of a "low positive" value, or by underestimating it.

In conclusion the Committee supports the opinion that all risk factors for clinical manifestations should be taken into account. Risk, however, is changing on a continuous scale, as much as aPL levels are measured on a continuous scale; thereby, the most appropriate approach is to consider that higher antibody titer means higher risk.

5.1. Subgroup II—lupus anticoagulant

Testing for a LA is the assay of choice for the detection of clinically relevant aPL. Different studies have shown that the LA is a better predictor of thrombotic complications and adverse pregnancy outcome than aCL or anti-β2GPI antibodies [16,17]. However, there are still a number of uncertainties in the interpretation of the results of LA testing, such as "What is the relevance of a weak LA?" and "Can we trust LA measurements in a patient on oral anticoagulants?" We address these questions in this section.

5.1.1. What is a weak LA?

In the diagnostic laboratory, the normal range for healthy individuals is typically determined by establishing the mean value ±2SD of a minimum of 40 healthy individuals. A measurement that results in a value just above the mean + 2SD can be considered "weak" positive. Samples with these minimally positive results can be difficult for

individual clinical laboratories to detect, as has been documented in several studies [18,19].

5.1.2. What is the predictive value of a weak LA?

At the moment, we do not have data that state that weak positive results are not clinically relevant. In fact, we do not have data to state at what level of detection we should consider a LA to be weak. An additional problem is that there are multiple assays to detect LA, and these assays vary in their sensitivity to the presence of a LA. Although there is a general consensus, that the higher the titer of LA the greater the risk for adverse outcomes, there are no convincing scientific data that support this claim.

• Based on these considerations we conclude that weak LA results should be considered positive when making clinical decisions.

5.1.3. What is the role of the mixing study?

Historically, LA testing has been based on three consecutive assays: screen, mix and confirm. The screen assay identifies a prolongation of clotting assay. The mixing assay excludes the possibility that the prolongation is due to a factor deficiency. The confirm assay finally identifies the inhibitor of coagulation as phospholipid-dependent by neutralizing the prolongation with extra phospholipids. Integrated tests that omit the mixing step have been introduced on the market.

The question whether the mixing step is essential in the detection of a LA has never been answered. Recent studies have shown that low levels of coagulation factors do not result in a false positive LA result. Thus, it is possible to detect a LA in patients on vitamin K antagonists. However, the combination of low clotting factor levels and low levels of the cofactor β2GPI can mask the presence of a LA, resulting in a negative screen. Mixing patient plasma 1:1 with normal plasma will solve this problem, facilitating detection of a weak LA. Thus, performing mixing studies is indicated when there is a suspicion of APS but the screen is negative.

Detection of a LA in patients treated with the new direct oral anticoagulants, such as dabigatran, rivaroxaban or apixaban is difficult. For the factor Xa inhibitors, assays based on the use of snake venoms that directly activate prothrombin can be used [20]; for the thrombin inhibitors, however, such an approach is not possible. In comparison to the vitamin K antagonists, the direct oral anticoagulants have a very short half-life. It is therefore advisable to evaluate for the presence of LA in a sample collected just before taking the drug (i.e., when the drug level is at a "trough"). A dilute thrombin time can determine whether there is still inhibition caused by dabigatran, and a factor Xa assay can determine whether there is still an effect of direct factor Xa inhibitors.

• LA can be measured in plasma of patients on vitamin K antagonists. It might be necessary to dilute the patient plasma 1:1 with normal plasma to increase the sensitivity of the assay. Detection of LA in plasmas containing direct oral anticoagulants is not possible with the regular assays.

The observation that mixing studies are not always necessary for LA testing asks for an adaptation of the guidelines for LA testing. We propose to perform the confirm assay immediately after the screen assay. In patients highly suspected to have APS but in whom the screen assay is negative, the screening test should be repeated in a sample diluted at 1:1 with normal plasma. We propose the following algorithm based on this approach.

1. Screen
   a. Positive result → continue directly with Confirm
   b. Negative result → in a high suspicion patient, repeat Screen in a 1:1 mix
      i. Positive result → continue with Confirm in 1:1 mix
      ii. Negative result → LA not detected

2. Confirm
   a. Positive result → LA detected
   b. Negative result → in a high suspicion patient, repeat Confirm in a 1:1 mix
      i. Positive result → LA detected
      ii. Negative result → LA not detected

5.1.4. Subgroup III–IgA aPL tests

Most studies on aPL have mainly focused on the estimation of the IgG and IgM isotypes, with only a few studies reporting on the pathogenic significance of IgA aPL. In this subgroup we aimed to summarize and analyze the available evidence on the prevalence and the clinical significance of IgA aPL and to evaluate the relationship between IgA aPL positive results and APS diagnosis by reviewing the literature for published data, and reporting and analyzing unpublished data by applying the GRADE system [6].

IgA anticardiolipin antibodies (aCL) have been studied since the early 80s in patients with systemic lupus erythematosus (SLE) and in APS [21–24]. Their prevalence seems extremely variable in different studies, ranging from 0% to nearly 50% in the population included. Data suggest that Afro-American, Afro-Caribbean and Japanese patients are those showing the highest prevalence of IgA aCL [25–27].

Altogether twelve studies show an association between IgA aCL and some clinical features related to APS, specifically thrombosis, pregnancy loss and thrombocytopenia [22–33]. Notably, ten out of twelve cohorts included only patients with SLE or other systemic autoimmune diseases, while one included both SLE and primary APS and one other 472 consecutive unselected patients tested for aPL.

Fifteen studies, eleven conducted in SLE patients, failed to find any relationship between the presence of IgA aCL and clinical signs of APS [21,25,34–46].

The analysis of published IgA aCL data shows their general weakness deriving from observational cross sectional studies that lack of prospective confirmation and controls groups. Usually IgG and/or IgM positivity associated to IgA did not allow understanding of the role of IgA alone. In addition, the great variability of the results suggests that the studies are scarcely comparable in the population included, in the methods used and in the outcome measured. Finally, many studies come from the same group of researchers with the potential for publication bias. As a consequence, after evaluation, the published data was categorized as low level with a weak recommendation to include testing for aCL IgA in the clinical practice.

IgA anti-β2GPI seems to be highly prevalent in SLE patients. Thrombosis, particularly arterial thrombosis [33,47], is frequently found associated with IgA anti-β2GPI, although the simultaneous presence of other isotypes makes often difficult the interpretation of this finding. Only two groups independently described the presence of IgA anti-β2GPI antibodies in patients with pregnancy loss and negative for all the other aPL tests including LA [44,48].

In summary, 1 controlled study [49] and 14 descriptive studies show significant association between anti-β2GPI and clinical features related to APS [32,33,38–40,46,50–56]. Nine out of these studies focused on SLE and other systemic autoimmune diseases, while the remaining included APS, obstetric APS, patients that tested negative or positive in other different aPL assays and consecutive patients undergoing testing for aPL. Four studies did not find a significant association between IgA anti-β2GPI and thrombosis and/or pregnancy loss [45,57–59].

When published data are critically regarded and compared to those of IgA aCL, a better agreement of the results becomes evident. However, again data analysis shows a number of limitations. In general samples are not prospectively examined, there are differences in the methods used, in the population tested, in the number of included patients and in the outcomes expected. Moreover, in most of the cases IgA anti-β2GPI was associated with other aPL. Furthermore, most of the studies came from the same group or related groups of researchers, making the quality of the available evidence average [6].

Only 4 studies focus on anti domain IV–V IgA. Two out of these come from related groups and the cohort examined is partially overlapping. Although the results are encouraging the available data are really too small to allow any practical conclusion [33,47,56,60].

For this task force, data from four unpublished studies were reviewed to evaluate the relationship between IgA aPL positive results and APS diagnosis. Of these studies, two examined the contribution of IgA anti-β2GPI in SLE and/or APS [61,62], one in stroke [63], and another their role in a mouse model of thrombosis [64].

In the APS (PROMISSE cohort; n = 97) and SLE (Hopkins lupus cohort; n = 205) studies, the clinical performance of 4 different IgA anti-β2GPI antibody kits in addition to IgG and IgM isotypes were investigated for correlation and/or risk for specific clinical manifestations [61,62]. Compared to the IgG and IgM anti-β2GPI, the IgA assays had increased variability in performance irrespective of the disease cohorts. The overall agreement between any two assays ranged from 92.2% to 99.6% for IgG, 95.4% to 98.8% for IgM and 77.6% to 92.2% for IgA in both cohorts. While the Kappa coefficients (K) showed moderate to almost-perfect agreement for IgG and IgM (0.54–0.98), the analysis revealed fair to substantial correlations for IgA anti-β2GPI assays (0.24–0.75). Despite these differences, in the SLE cohort, 3 out of the 4 IgA anti-β2GPI assays showed significant correlation with venous thrombosis (p < 0.05) [62]. The frequency of isolated IgA anti-β2GPI antibodies (any kit) was not significantly different between patients with SLE only vs. those with SLE and APS. Isolated IgA anti-β2GPI antibodies showed generally lower titers when compared to those occurring in the presence of IgG and/or IgM anti-β2GPI. Ben Said et al. [63], showed a strong correlation between IgA and IgM anti-β2GPI antibodies in patients with ischemic stroke (n = 41) compared to healthy controls (n = 80). Similar to the previously cited studies by Tebo et al. [61,62], the role of IgA anti-β2GPI antibodies as independent predictors of disease and/or specific clinical manifestations was not determined. Of relevance in the pathogenicity of disease, Willis et al. [64] showed that IgA anti-β2GPI antibodies are capable of inducing thrombogenicity as well as upregulating tissue factor (TF) in an in-vivo experimental model.

Available data led to the following conclusions:

- Positive IgA aCL and IgA anti-β2GPI are usually associated to other aPL, making it difficult to understand the role of IgA alone.
- Isolated positivity for IgA aCL is rare. Its utility can be restricted to those patients with a strong suspicion of APS but negative aPL tests.
- Testing for IgA anti-β2GPI could contribute to the assessment of risk for thrombosis and/or pregnancy morbidity, especially in SLE patients.
- The significance of IgA domain IV–V anti-β2GPI should be further investigated.
- Level of evidence III-Low quality evidence.

5.1.5. Subgroup IV–tests for antibodies to negatively charged phospholipids and antibodies to phosphatidylethanolamine (aPE)

5.1.5.1. Tests for antibodies to negatively charged phospholipids. At the 13th International Congress on Antiphospholipid Antibodies (APLA 2010, 13–16 April 2010, Galveston, Texas, USA), the diagnostic and analytical properties of antibodies directed against negatively charged phospholipids such as IgG and IgM antibodies directed against phosphatidic acid (aPA) phosphatidylinositol (aPI), and phosphatidylserine (aPS) were reviewed extensively in an evidence-based manner [4]. Given the considerable variability in the study designs including patient populations investigated and analytical differences in methodologies and reagents for detecting these antibodies, there were uncertainties in the diagnostic relevance for these tests in APS. Furthermore, the relationship between these tests alone or in combination with other criteria aPL markers were poorly defined. Indeed several studies have shown that aCL broadly cross-react to both aPS and aPA antibodies [21,65,66]. In addition, the largest part of aPL detected by these assays is closely related to the reactivity against β2GPI. Taking into consideration the

inherent analytical and diagnostic challenges of aCL antibodies as well as the performance characteristic of the aPS assays relative to aPA and aPI, it was suggested that aPS antibody testing may be of potential test for further investigation especially in the context or pregnancy related-morbidity [4].

Further analysis of peer-reviewed studies for negatively charged phospholipids including the APhL antibody tests in the evaluation of APS was investigated by a group of experts and presented in a task force at the 14th APLA Congress in Rio de Janeiro, Brazil [67–71]. Overall, the published data showed general weakness in study design, methodologies, and potential for bias. No recent study on aPS, aPI and aPA antibody testing documented significant improvement for the diagnosis of APS since the last meeting in Galveston [67,68]. Of note, one study examined the performance of all these markers using a new platform [67] and another tested for aPS in the context of pregnancy-related morbidity [68]. These authors confirmed previous investigations that IgG aPS antibodies occurred at significantly higher frequency along IgG aCL and anti-β2GPI. Furthermore, the presence of the less frequently found IgG aPI were dependent on IgG antibodies to PS, CL and β2GPI [67].

5.1.5.2. aPhL. With respect to the APhL, a commercially available assay kit (Louisville APL Diagnostic, Inc, Louisville, KY, USA) composed by a mixture of phospholipids [72], the published studies showed overall improved specificities to aCL in the context of infectious diseases [70,71,73–76]. However, the number of studies that examined its performance in the context of autoimmune diseases was quite few [69,77,78].

Unpublished data or data published in an abstract form were also presented. Seif et al. [79] reported that aPhL had the best PPV for thrombosis and pregnancy losses when compared to aCL, anti-β2GPI and the LA. Willis et al. [80] and Sciascia et al. [81] also reported the clinical value of these antibodies in their lupus populations.

5.1.5.3. Antibodies to phosphatidylethanolamine (aPE). Antibodies directed to phosphatidylethanolamine (aPE) were given attention as they have been described in some instances as the sole aPL in patients that have manifestations of APS and no methodically robust studies were available at the time of our previous meeting [4].

5.1.5.3.1. Are aPE important in pregnancy morbidity?. Several studies were presented on the prevalence of aPE in women with history of pregnancy morbidity. Most of these studies showed a higher prevalence of aPE in heterogeneous populations of patients with unexplained early and late pregnancy losses [82–85]. One study analysed the prevalence of aPE in 101 infertile women [86] and one another, their association with hypertension during pregnancy in a cohort of 1155 consecutive women [87].

In 2000, Gris et al. reported aPE to be an independent risk factor for unexplained early fetal loss [88]. These findings were later refuted by Obayashi et al. [89]. While Balada et al. [90] showed an association between aPE and fetal loss, these antibodies were always found in the presence of aCL and/or LA. Two other recent studies failed to show an association between aPE and pregnancy morbidity in SLE [91,92].

A recent study by Velayuthaprabhu et al. [93] showed that passive immunization of aPE in mice slightly increased fetal resorption, but markedly induced thrombosis and hemorrhage in the placenta supporting the pathogenic role of aPE in pregnancy complications.

5.1.5.3.2. Are aPE relevant in thrombosis?. While there are many case reports associating aPE to thrombotic events such as stroke [94], pulmonary embolism [95] and lower limbs arterial thrombosis [96,97], only 3 studies confirmed these findings [98–100], with many others failing to find any associations [90–92,101].

Group conclusions:

- aPI and aPS may identify additional women with recurrent pregnancy loss

- aPhL seem to be more specific than standard aCL discriminating better APS from non-APS. aPhL could be used as a confirmatory test.
- aPhL application as an alternative to aCL assay needs further proof
- Most of the studies do not support an association between aPE and thrombosis or pregnancy morbidity, making the assumption of "*no need to test*" a valid one. However, the level of evidence is even low for this recommendation on and further well designed studies may probably change the presented conclusions dramatically
  Level of evidence III—Very low/low quality evidence

5.1.6. Subgroup V—tests for antibodies to prothrombin (aPT) and phosphatidylserine/prothrombin (aPS/PT)

Antibodies targeting human prothrombin (aPT) and the complex of prothrombin bound phosphatidylserine (aPS/PT) are detected by ELISA and strongly associated to the APS [102]. Although a correlation between the two assays have been reported [103], it seems that aPT and aPS/PT belong to different populations of autoantibodies even though they can both be present in the same patient [104].

Several studies with regard to the relationship between APS-related clinical features and the presence of aPT and/or aPS/PT have been published. A systematic review of the literature published in the last 25 years was recently reported [102]. The available information included more than 7000 patients and controls. Data come from 38 clinical studies analysing the presence of aPT and 10 evaluating aPS/PT and the risk of thrombosis. Most of the reports assessing aPT are retrospective and only few are case–control or prospective studies. Almost all but one is retrospective in those assessing aPS/PT. Patients involved mainly had primary or SLE-associated APS. However, SLE patients without arterial or thrombosis events and asymptomatic individuals were also included. Most of the studies have an evidence level of III and only few papers reached a IIA or IIB evidence level. Studies evaluating aPT showed conflicting results because almost half of them demonstrated that aPT are associated to thrombosis while the others showed no clear association. Antibodies to prothrombin (both aPT and aPS/PT) increased the risk of thrombosis (OR 2.3 [95%CI 1.72–3.5]). aPS/PT seemed to represent a stronger risk factor for thrombosis, both arterial and/or venous than aPT (OR 5.11 [95%CI 4.2–6.3] and OR 1.82 [95%CI 1.44–2.75], respectively). This systematic review concluded that routine measurement of aPS/PT (but not aPT) might be useful in establishing the thrombotic risk of patients with previous thrombosis and/or SLE.

Based on a strong association between aPS/PT and the LA, a recently published study suggests that aPS/PT may be a surrogate test for LA, particularly useful to confirm its presence in case of ambiguous results or to replace it when clotting test cannot be performed because of technical limitations [105].

An important observation reported by several recent studies is that the risk of thrombosis progressively increases with the increase in number of positive aPL tests. A recent retrospective evaluation including 230 patients with SLE reported that the combination of LA, anti-β2GPI and aPS/PT had the best diagnostic accuracy for APS [106]. Triple positivity for LA + anti-β2GPI + aPS/PT was more strongly associated with clinical events (thrombosis and/or pregnancy loss) when compared with double or single positivity (OR 23.2 [95%CI 2.57–46.2] vs. OR 7.3 [95%CI 2.21–25.97], OR 5.7 [95%CI 2.12–17.01] or OR 3.11 [95%CI 1.56–7.8] for single positivity for LA, aPS/PT and anti-β2GPI, respectively).

This subgroup also reviewed the available unpublished evidence on the relationship between antiprothrombin antibodies and APS. We performed a search of all the abstracts that assessed the association between aPT and/or aPS/PT with any of the clinical features or laboratory manifestations of APS and were accepted at the following scientific meetings: International Society on Thrombosis and Haemostasis (ISTH) from 2001 to 2013, European League against Rheumatism (EULAR) from 2010 to 2013 and the American College of Rheumatology (ACR) from 2010 to 2012. Abstracts published after the conferences as full papers were excluded. Unpublished abstracts presented at the

14th International Congress on Antiphospholipid Antibodies taking place during the Task Force meetings were also included.

Twelve abstracts met the inclusion criteria. Four out of the 12 abstracts investigated aPT only, five aPS/PT only and in 3 abstracts, the authors evaluated both aPT and aPS/PT. One abstract referred to the validation of a commercially available test to detect aPS/PT [107]. Two studies demonstrated a correlation between aPT and thrombosis [102, 108], one showed and association between aPT and APS manifestations [109] and one revealed a relationship between aPT and the presence of LA [110]. Regarding aPS/PT, five studies found an association between the antibodies and some of the clinical manifestation of APS, such as pregnancy complications [111–113], thrombosis [102,113] and even severe APS manifestations such as catastrophic APS [114]. Three studies reported a correlation between the presence of aPS/PT and that of LA [110,112,115]. On the other hand, one study did not find correlation between thrombosis and either aPT nor aPS/PT in samples from patients with LA [116] and no association between the presence of aPT and coronary artery disease was reported [117]. Preliminary unpublished data from an in-progress Multicenter Study (aPS/PT IMCS-2012), led by Prof Atsumi and Dr Amengual were presented at the Task Force, showing positive correlation between aPS/PT and clinical APS.

Group conclusions:

Based on data showing that aPT and aPS/PT are different subpopulations of autoantibodies [104,118]:

1) This group does not recommend routine testing for aPT based on the following:
   - Results widely differ between groups suggesting a true difference between laboratories/techniques/assays
   - Most data come from retrospective studies
   - Based on available data, it is not possible to identify the role of aPT alone
   - Lack of multivariate adjustment in most, if not all, studies makes interpretation of the clinical relevance of aPT difficult
   - Level of evidence III—Very low/low quality evidence

2) Regarding testing for aPS/PT, the group concludes that:
   - Testing for aPS/PT can contribute to assess the risk of thrombosis
   - Testing for aPS/PT can contribute to a better identification of patients with APS
   - Multivariate analysis confirm aPS/PT as independent risk factor for thrombosis
   - Results do not substantially differ between groups, suggesting that aPS/PT are truly relevant in APS
   - The association of aPS/PT with LA deserves further study
   - Level of evidence III—Low/Moderate quality evidence

5.1.7. Subgroup VI—test for antibodies to domain I

β2GPI has five homologous domains. The N-terminal domain, designated Domain I or DI, is of particular interest because studies from a number of different groups have suggested that antibodies to this domain (anti-DI antibodies) are particularly important in the pathogenesis of APS. Apart from the serological studies that are discussed in greater depth below, Ioannou et al. showed that administration of recombinant DI could inhibit the induction of thrombosis by human IgG from patients with APS in a mouse model [119]. More recently, eluted fractions rich in anti-DI antibodies obtained from an APS patient were shown to induce a greater increase in tissue factor activity and significantly larger thrombi compared to the anti-DI poor fraction remaining after affinity-purification [120]. In addition, human monoclonal anti-DI IgG, when infused together with LPS to naïve mice, induced clotting and fetal loss, providing a direct demonstration of the pathogenic effects of anti-DI antibodies [121]. The pathogenic potential of anti-DI antibodies is further supported by the good correlation with annexin A5 resistance assay evinced in cohorts of APS subjects as well as adult and paediatric SLE patients [122–124].

It should be stressed that not all anti-β2GPI antibodies in patients with APS bind to DI. However, since the evidence suggests that anti-DI antibodies form a subset of anti-β2GPI that are particularly closely associated with pathogenicity a number of groups have investigated whether anti-DI binding assays might be useful in diagnosis and management of APS. The anti-DI assay could potentially be useful in several ways;

- If it is more sensitive than existing assays, it could aid the diagnosis of APS in patients who are negative in the current assays (aCL, anti-β2GPI and LA tests).
- If it is more specific than the current assays, it may reduce the rate of false positive diagnoses, being potentially used as a second-line test in case of inconsistent results.
- If it shows stronger association with thrombosis or other clinical symptoms than the whole molecule anti-β2GPI assay, it may aid risk stratification and patient management.
- If it is equally sensitive and specific compared to current anti-β2GPI assays but has analytical benefits, for example if the assay is more reproducible than anti-whole β2GPI, it may eventually replace the whole molecule anti-β2GPI assay.

A number of different anti-DI assays have been reported in the literature. The reports differ in the source of DI, the principle of the method, the range of samples tested and the way in which the results are reported. However, all of them were retrospective and most reported solely on IgG isotype anti-DI antibodies.

The earliest anti-DI assay results were reported by an American group at La Jolla, who used a baculovirus system to express whole β2GPI and variants of β2GPI that lacked one or more domains or contained mutations in DI [125]. Using the domain deleted mutants in both direct and inhibition enzyme-linked immunosorbent assays (ELISAs) and surface plasmon resonance experiments, they showed that serum from patients with APS bound more strongly to variants containing DI than to variants lacking it [125,126]. For example, McNeeley et al. reported that 88% of 106 APS patients showed this preference for DI [127]. Subsequently they showed that within DI, these APS sera showed affinity for a particular epitope between residues glycine 40 and arginine 43 (the G40-R43 epitope) [128].

These experiments were designed to discover key epitopes for binding pathogenic IgG upon the whole β2GPI molecule rather than to develop an anti-DI ELISA, and indeed most did not use DI expressed as a single domain. However, the same baculovirus expression system was used by a group in the Netherlands to develop a true anti-DI assay. The crux of this assay is use of a direct ELISA to compare the strength of binding of the same serum sample to DI coated at the same density on hydrophobic and hydrophilic plates. On hydrophobic plates, the G40-R43 epitope is exposed for binding by antibodies in the sample whereas on hydrophilic plates it is not exposed. The hypothesis underlying this assay is that antibodies from patients with APS, because of their preferential binding to the G40-R43 epitope, will bind more strongly to DI on the hydrophobic plates. The result of the assay is expressed as a ratio (Optical Density on hydrophobic plate/Optical Density on hydrophilic plate). If this ratio exceeds 2, the sample is said to test positive in the assay [123]. This reporting method gives the result in a dichotomous yes/no form rather than allowing an estimate of binding strength as a continuous variable. However, this assay has been used in the largest and most meaningful studies so far published on clinical relevance of anti-DI. In 2005 de Laat et al. showed that in a group of 198 patients, (176 with SLE, 16 with lupus-like illness and 6 with primary APS) positivity for anti-DI in this assay was associated with increased risk of thrombosis [129]. A larger, multicentre study in 2009 looked at 442 patients who all tested positive for anti-β2GPI, but only 364 had thrombosis [123]. This study is important because it considers the question of whether testing for anti-DI in addition to anti-β2GPI adds important clinical information. In fact the results showed clearly that those patients who were IgG anti-DI positive were more likely to develop vascular thrombosis (OR 3.5, 95%CI 2.3 to 5.4) or pregnancy morbidity

(OR 2.4, 95% CI 1.4 to 4.3) than those who tested negative for IgG anti-DI in this assay. IgG anti-DI was positive in 55% of patients with APS, a high prevalence which also supports the idea that the test might be useful in clinical practice. Conversely IgM anti-DI positivity was not associated with increased risk of thrombosis or pregnancy morbidity. However, a caveat is that not all groups have obtained the same results and that there may be a difference between adults and children. Thus, using the same assay in 183 children with SLE, Wahezi et al. found that 25.1% were IgG anti-DI positive (compared to none of 22 healthy controls) but that only seven children had thrombosis i.e. there was no strong correlation between anti-DI positivity and thrombosis in this pediatric study [130].

The baculovirus system is not the only way to make recombinant DI. Ioannou et al. described a novel bacterial expression system for DI [131] and used this product to develop a simple direct ELISA that does not require hydrophobic and hydrophilic plates. Testing purified IgG from 22 patients with APS, 20 with SLE (but no APS) and ten healthy controls they showed significantly higher binding for the APS samples than the other groups [132]. By using the bacterial system to make site-directed mutants of DI, they also confirmed that changes in the G40-R43 epitope did alter binding to the APS IgG samples and that the adjacent arginine 39 (R39) residue also played a major role [132]. However, it is important to note that these tests were done using purified IgG rather than serum; as this would not be convenient for a routine clinical assay, the assay has been modified to test serum.

Banzato et al. synthesized DI chemically. When used in a direct ELISA, the results were disappointing as IgG anti-DI levels did not differ between patients with APS and controls [133]. However, when this DI was used to inhibit binding of plasma from patients with APS to whole β2GPI on a plate, the level of inhibition was higher for samples derived from patients with triple-positivity (i.e. positive in all three of the anti-cardiolipin, anti-whole β2GPI and LA tests) than for those derived from double-positive or single positive subjects or healthy controls [133]. Since triple-positivity is known to be associated with increased risk of thrombosis [134], this result supports the idea that anti-DI antibodies play an important pathogenic role. However, since triple-positive patients are already known to have high thrombosis risk using standard assays, the study does not add much to the evidence for extra clinical value of measuring anti-DI.

INOVA Diagnostics, Inc. have developed a prototype anti-β2GPI-DI ELISA that has been used by two groups in published studies, but with contrasting results. Reporting on 67 Italian patients with APS, Andreoli et al. showed that 43/67 tested positive for IgG anti-DI while a low anti-DI frequency was reported in anti-β2GPI positive healthy children born to mothers with systemic autoimmune diseases and children with atopic dermatitis (9/57 and 9/33 respectively) [135]. Conversely, using stored samples from 326 patients with SLE, of whom 164 had a history of thrombosis, Akhter et al. found that only 11/164 thrombosis patients were IgG anti-DI positive [33]. Such discrepancy might arise from the different cut-offs for anti-DI positivity used in these two studies.

In a more recent and as yet unpublished study [136], Andreoli et al. observed 128 selected anti-β2GPI positive subjects. Forty-two were patients with autoimmune conditions such as SLE or undifferentiated connective tissue disease but with no clinical feature characteristic of APS. These 42 subjects displayed a positivity rate for anti-DI comparable to the other 86 subjects, who had all been diagnosed with APS (33/42 (78.6%) and 61/87 (70%) respectively). This implies that the remaining 30% of anti-β2GPI positive patients diagnosed with APS displayed auto-antibodies targeting domains of β2GPI molecule other than DI. Another interesting finding emerging from this work was the identification of DI as the prevalent domain specificity even among APS women with pure obstetric morbidity (20/31 women with pregnancy complications, com-pared to 41/56 in the thrombotic APS group, p = NS). Consistently with what found by de Laat in 2009 [123], the positivity rate for anti-DI anti-bodies was slightly lower among women with obstetric APS compared to subjects with thrombosis (61.3% versus 78.2%) [136].

INOVA Diagnostics, Inc. has also developed an aβ2GPI-DI chemilu-minescence immunoassay (CIA), which uses the BIO-FLASH technology, with a recombinant DI coupled to paramagnetic beads. This novel assay has been evaluated in some studies; none of them has been published to date but the results have been presented at international meetings, as discussed below.

In a paper discussed at the VIII Congress on Autoimmunity held in Granada in 2012, Albesa et al. detected anti-DI antibodies by CIA in 122 out of 144 APS patients, compared to 1/200 healthy controls and 10/72 subjects with infectious diseases, resulting in a sensitivity of 85% and a specificity of 86% [137]. In another abstract presented at the same meeting, Albesa et al. reported that anti-DI titers were signif-icantly higher among 72 patients with thrombotic APS compared to 35 APS subjects with no history of vascular events. 24/72 of throm-botic APS patients and 3/31 of those without thrombosis were found to be anti-DI positive (p = 0.0022), conferring a likelihood ratio for thrombosis of 3.78 for anti-DI compared to 2.17 for anti-β2GPI ELISA test [138].

Concordant data were discussed by Hollestelle et al. at the XXIV Congress of the International Society of Thrombosis and Haemostasis held in Amsterdam in June 2013 [139]. These authors suggested that anti-DI were more strongly associated with APS than antibodies targeting the whole molecule. Indeed, in a cohort of 24 APS patients and 55 controls, anti-DI displayed an OR for APS diagnosis of 6.4 (95% CI 1.7–24.0), in contrast anti-β2GPI antibodies were not signifi-cantly correlated with APS (OR 1.9, 95% CI 0.7–5.5) [139].

However, in a larger cohort of 273 APS patients and 1096 controls (including healthy individuals, patients with infectious diseases and autoimmune conditions), Zohoury et al. reported anti-DI at a cut off value of 20 CU to be less sensitive for APS than antibodies against the whole β2GPI molecule (50.2% versus 72.8%), with anti-DI being on the other hand more specific (99.2% versus 83.7%) [140].

At APLA 2013, Agmon-Levin et al. presented their data from a cohort of 178 APS patients [141]. In line with the results reported by Andreoli et al. [136], they detected anti-β2GPI antibodies in 70% of cases and anti-DI in 49%. As already proposed by Banzato et al. [133], Agmon-Levin et al. suggested that anti-DI antibodies might provide a marker of high-risk aPL profile [141]. Indeed, 89% of anti-DI positive subjects carried a triple aPL positivity, compared with 16% among anti-DI nega-tive patients. Moreover, anti-DI positivity was related to the occurrence of any thrombotic event (91% versus 79%, OR 2.54), at medium levels anti-DI were associated with arterial thrombosis (55% versus 33%, OR 2.5), while high levels of anti-DI were predictive of multiple thrombotic events (62% versus 31%, OR 3.58), arterial thrombosis (60% versus 33%, OR 3.04) and neurologic manifestations (45% versus 27%, OR 1.99).

This is in agreement with the report of Zuilly et al., who at the same meeting presented data from a longitudinal study (median follow-up 35 months) of 92 patients with SLE and aPL, SLE alone or aPL alone. The presence of high levels of anti-DI antibodies, detected by the INOVA anti-DI ELISA, was associated with a 3.6 fold increase in the risk of thrombotic events [142].

Preliminary data support comparability between the ELISA and the CIA. Indeed, when the ELISA and CIA research assays by INOVA Diagnostics, Inc. have been directly compared, the two methods displayed the same specificity although a different sensitivity [143]. A good agreement between the INOVA CIA immunoassay and the ELISA assay of Ioannou et al. has also been observed [144].

In summary, studies from multiple groups using DI from different sources have all shown that IgG anti-DI binding is higher in APS patients than controls, and several groups showed independently that the R39–R43 epitope is important in this binding. The largest studies, by de Laat and colleagues in the Netherlands [123,129], suggest that testing for IgG anti-DI as well as for anti-whole □2GPI would enable clearer identification of the patients at highest risk for developing thrombosis or pregnancy morbidity. Even though these Dutch studies used a method that has not been utilized by any other groups, their findings

have been substantially confirmed in some unpublished studies that exploited different assays for the detection of anti-DI.

However, it has clearly emerged that not all anti-β2GPI detectable in APS patients target DI, with significant subpopulations reacting against other β2GPI epitopes. Thus, testing for antibodies against the whole molecule is still required, as it allows identification of a broader group of patients.

Overall, anti-DI assays are very promising, but several important issues remain to be clarified.

1. The main clinical utility of the anti-DI assay. It can potentially be a diagnostic tool or a risk stratification tool.
2. The scientific community has to reach agreement on the type of the antigen and the principle of the method to be used. Results of various studies can be compared only if analytical harmonization has been reached.
3. Longitudinal, prospective studies need to be carried out to help clarify the clinical utility of the anti-DI assay.

5.1.8. Subgroup VII—aPL as risk factors

5.1.8.1. Designing the perfect study: how best to assess risks associated with aPL.

"Antiphospholipid antibodies (aPL) are associated with an increased risk of arterial and venous thrombosis and pregnancy loss/morbidity."

Many review articles and book chapters on the APS begin with a statement like the one above. While a large body-work supports the statement, quantification of the risks associated with aPL is difficult. Assessment of the risk associated with various aPL profiles and antibody levels, risks associated with aPL in the setting of other risk factors, and the evaluation of risk in individual patients are challenging issues. Many published studies that attempt to address these issues are limited by factors involving study design, the scope of aPL testing performed, and data analysis.

5.1.8.1.1. Study design. Many published studies are limited by retrospective study design, ascertainment bias, and small sample size. Retrospective studies, such as case–control studies, are helpful for studying rare conditions and require less time to conduct than prospective studies. Inherent disadvantages to case–control studies include potential problems with data quality and problems finding an appropriate control group. Ascertainment bias is particularly an issue when physician-investigators at tertiary academic medical centers study a disease in which they have a high level of expertise. In such a situation, the patients available for study may be highly selected, e.g., have more severe disease, and not be representative of what is seen in the general community. Small sample size can limit the value of studies due to wide confidence intervals and the increased risk of error in hypothesis testing.

5.1.8.1.2. aPL testing. Many studies in the field suffer from a limited scope of aPL testing. Rather than testing a full range of aPL tests (LA, IgG, IgM, and IgA aCL, IgG, IgM, and IgA anti-β2GPI), only certain tests were performed. The classification of definite APS (based on international consensus criteria) [1] requires positivity for only one test (LA, IgG or IgM aCL, IgG or IgM anti-β2GPI). Thus a study looking only at IgG and IgM aCL would miss an APS patient with sole LA positivity. Additionally, there is growing evidence that positivity in multiple aPL assays is associated with greater risk than positivity in a single test. Another limitation of many studies is that aPL testing was performed at only a single time point. Persistence of test positivity is important and is part of the APS classification criteria. Although advances have been made, problems with aPL assay standardization and intra- and inter-laboratory variability remain. Lastly, studies differ in the length of time between clinical events and aPL testing which may confound results.

5.1.8.1.3. aPL test data analysis. LA testing is designed to be interpreted in a dichotomous fashion, i.e., results expressed as present (positive) or not present (negative). In contrast, ELISAs and other immunoassays for aCL and anti-β2GPI are quantitative and can be analyzed as dichotomous variables (positive or negative based on a cut-off value) or as quantitative or continuous variables. The literature demonstrates that levels of aCL and anti-β2GPI are positively correlated with the risk of thrombosis and other clinical manifestations of APS. Failure to consider quantitative levels of these aPL may confound data interpretation. For example, if aCL/anti-β2GPI are considered as dichotomous variables using a relatively low cut-off value, many positive subjects may have a relatively low antibody level that is not associated with significant clinical risk.

Several factors that need to be considered in designing and analyzing ELISAs and other immunoassays will be briefly reviewed.

1. Analytical sensitivity (lower limit of detection (LLD)): This is the lowest amount of an analyte that can be detected in an assay, i.e., the lowest signal that is clearly discernable from background noise. It is a technical characteristic of the assay and is independent of the normal controls or patient data. Analytical sensitivity should not be confused with diagnostic sensitivity (the percentage of patients with a disease that have a positive test).
2. Clinical "cut-off" values: This is the level of a test that is considered "positive" or different from a normal or control group. The cut-off value can be determined in a number of ways. While some methods assume the values of the control group are normally distributed, other methods do not. When the distribution of most autoantibodies in the normal population is not normal, then, non-parametric methods are preferred. One method commonly used in aPL assays is the 99th percentile of the normal population.
3. Levels of antibodies associated with risk: These levels are determined in clinical studies and may differ from the "cut-off" value based on a normal population.

Problems with data analysis and interpretation can arise depending on the relationship among these numbers. Two examples are described below.

- The "cut-off" level falls below the LLD. In some cases, the 99th percentile of the normal population falls below the LLD. In this situation, a patient specimen with a low value could be interpreted as positive although the value is below the LLD and should be considered negative.
- The level of antibodies associated with risk is significantly greater than the "cut-off" value. The literature suggests that IgG or IgM aCL levels equal to or greater than 40 GPL/MPL are associated with risk of thrombosis, whereas lower levels may not be. In contrast, the "cut-off" levels of positivity for most aCL assays are significantly below 40 GPL/MPL. Thus, individuals with a test value above the "cut-off" but below 40 GPL/MPL have a positive test but may not at an increased risk of thrombosis.

Taking these concerns into consideration, an ideal study to assess aPL-associated risk would have the following characteristics: prospective, population-based (to eliminate ascertainment bias); large sample size (to increase statistical power and decrease the risk of error); long-term; clinical manifestations (thromboses, cardiovascular events, pregnancy outcomes) assessed objectively at regular intervals; data on co-morbidities, other risk factors, and medications; blood specimens drawn at inception and at regular intervals; specimens collected, processed, and stored appropriately for aPL testing; comprehensive, state-of-the-art panel of aPL tests; robust data analyses.

There are a number of hurdles that need to be surpassed in order to perform such studies. Large, prospective, population-based studies are expensive. It is unlikely that the APS Task Forces acting alone will have the resources to conduct such studies. The most cost-effective and productive approach will be collaboration with existing large,

prospective study cohorts with stored specimens. The APS Task Forces have the expertise to design a comprehensive aPL testing panel. Tests could be performed within the APS Task Forces, by APS ACTION, a network of international physicians and scientists working in the field of APS (www.apsaction.org), or in collaboration with large commercial laboratories.

In summary, the long-term goal is to be able to interpret aPL testing in terms of risk for individual patients in the clinical setting. Some features of high-risk aPL profiles are known, e.g., high titer, persistence, "triple positivity," although precise quantification of that risk remains difficult. It is hoped that large, prospective studies as described above will be performed and answer these important questions.

5.1.8.2. Scoring systems in APS.
Risk prediction models have great potential to support clinical decision-making and are increasingly incorporated into clinical practice. Many prediction models have been developed for cardiovascular disease—the Framingham risk score, SCORE, QRISK, and the Reynolds risk score—to mention just a few [145,146].

Three score systems have been formulated to quantify the risk of thrombosis/obstetric events in APS, aiming to help physicians to stratify patients according to risk [147–149] (Table 4).

The first model [147] retrospectively studied 3088 consecutive patients who were referred within a 24-month period to coagulation laboratory for suspected thrombophilia, suspected obstetric APS, unexplained prolonged clotting time, and screening in co-existent autoimmune disease. All the patients were tested for LA, aCL and anti-β2GPI. A risk model for APS diagnosis based on aPL positivity, their titer and the methods used for LA investigation was set-up. Estimates for the probability of APS diagnosis were derived from logistic regression equations and the resulting chart showed that multiple aPL positivity, particularly the triple association of LA, aCL and anti-β2GPI, increases the risk of APS. Among the aPL, LA was more strongly associated with the diagnosis of APS, particularly if detected by a particular test, namely the hexagonal phospholipid neutralization test (PTT-LA/STACLOT) and the dilute Russell's viper venom time.

More recently, Otomo et al. [148] designed the "antiphospholipid score" (aPL-S) with the purpose of quantifying the risk based on the aPL profile. This study comprised two independent sets of patients with autoimmune diseases. In the first set of patients (n = 233), the aPL profiles were analyzed, using five clotting assays for LA and six ELISAs (IgG/IgM aCL, IgG/IgM anti-β2GPI, and IgG/IgM aPS/PT). An algorithm was created to generate the aPL-S based on multiple aPL

assays, with each assay being assigned a different score weighted on the relative risk of having clinical manifestations of APS.

The association of the aPL-S with a history of thrombosis/pregnancy morbidity was assessed. The prevalence of APS manifestations increased in accordance with increasing aPL-S. The authors concluded that the aPL-S was a potential marker of the "probability" of APS and a valuable tool for predicting thrombosis in the setting of autoimmunity. aPL-S was also independently validated in a separate cohort of 211 consecutive SLE patients, proving that its correlation with a history of thrombosis or pregnancy loss [150].

Recently, an alternative score derived from the combination of independent risk factors for thrombosis and pregnancy loss in a large cohort of well-characterized SLE patients was formulated [149]. This score takes into account not only the aPL profile (criteria [1] and non-criteria aPL [4]) but also includes the conventional cardiovascular risk factors and the autoimmune antibodies profile into the equation. The Global APS score or GAPSS was developed and validated in a cohort of SLE patients who were randomly divided into two sets by a computer-generated randomized list. Data on clinical manifestations, conventional cardiovascular risk factors, aPL profile, ANA, ENA and anti-dsDNA were collected and included in the analysis. GAPSS was developed in the first set of patients (n = 106), assigning the risk factors identified by multivariate analysis weighted points proportional to the β-regression-coefficient values. Validation in a second set of patients (n = 105) showed statistically higher values of GAPSS in patients with a clinical history of thrombosis and/or pregnancy loss compared to those without events (GAPSS 9.5 ± 5.6 [range of 0–20] and 3.9 ± 4.1 [range of 0–17], p < 0.001).

When applied in a prospective cohort of SLE patients, an increase in the GAPSS during the follow up (mean 32.94 ± 12.06 months) was associated with a higher risk of vascular events (RR 12.30 [95%CI 1.43–106.13], p = 0.004). In detail, an increase of more than 3 GAPSS points seemed to have the best risk accuracy for vascular events (HR 48 [95%CI 6.90–333.85], p = 0.0001) [151].

Interestingly, in a cohort of Primary APS, it was shown that higher values of GAPSS are seen in APS patients who experienced thrombosis when compared to those with previous pregnancy loss alone. In addition, APS patients who experienced recurrent thrombotic events showed higher GAPSS when compared to those without recurrences [152].

In summary, GAPSS is a score model based on six clinical factors that has been proven to represent the "probability" or likelihood of having thrombosis or pregnancy loss in SLE. The advantage of GAPSS, when compared to the previous proposed scores, includes the inclusion of conventional cardiovascular risk factors in the setting up of the model.

The use of GAPSS may provide important information regarding thrombosis or pregnancy loss risk for each SLE patients, switching from the concept of aPL as diagnostic antibodies to aPL as risk factors for clinical events.

However, its application should be independently validated in a prospective fashion, including not only primary APS, but also aPL positive patients without clinical symptoms suggestive of APS or other autoimmune disease.

6. Conclusions

This report summarises the findings, conclusions and recommendations of the "APS Task Force 3—Laboratory Diagnostics and Trends" meeting that took place during the 14th International Congress on Antiphospholipid Antibodies (APLA 2013, September 18–21, Rio de Janeiro, RJ, Brazil). Along with other already published recommendations [153–155], we are expected to update this report at the next International Congress (September 2016 in Istanbul, Turkey—www.apsistanbul2016.org).

Table 4
Main characteristics of the score systems formulated to quantify the risk of thrombosis/obstetric events in APS.

	Risk scale	aPL-S	GAPSS
Population	aPL + ve	AD	SLE
Reference	[147]	[148]	[149]
APS risk assessment	Yes	Yes	Yes
Thrombotic risk assessment	No	Yes	Yes
PM risk assessment	No	Yes	Yes
aPL			
LA	Yes[a]	Yes[b]	Yes[c]
aCL	Yes	Yes	Yes
anti-β2GPI	Yes	Yes	Yes
aPS/PT	No	Yes	Yes
Cardiovascular Risk Factors	No	No	Yes[d]
Approach	Semi-quantitative	Quantitative	Quantitative

LA, Lupus anticoagulant; aCL, anticardiolipin antibodies; anti-β2GPI, anti-β2-glycoprotein I antibodies; aPS/PT anti-phosphatidylserine/prothrombin complex antibodies.
aPL + ve, antiphospholipid antibodies positive; AD, autoimmune diseases; SLE, systemic lupus erythematosus.
[a] Values were assigned for each test used to detect LA (APTT/StaClot LA kit, dRVVT, Kaolin Clotting Time, Silica clotting time).
[b] Values were assigned for each test used to detect LA (APTT/StaClot LA kit, dRVVT, Kaolin Clotting Time).
[c] Values were assigned for LA positivity, regardless of the test used.
[d] Hypertension and hyperlipidemia.

Take-home message

- The development of international units and polyclonal and monoclonal reference materials for anti-β2GPI testing is under way. These ongoing efforts will significantly contribute towards the much-needed improvement of inter-laboratory and inter-assay agreement for aPL immunoassays.
- A weak LA results should be considered positive when making clinical decisions.
- While the LA can be measured in plasma of patients on vitamin K antagonists under certain consitions, detection of LA in plasmas containing direct oral anticoagulants is not possible with the regular assays.
- Positive IgA aCL and IgA anti-β2GPI are usually associated to other aPL. Its utility can be restricted to those patients with a strong suspicion of APS but negative aPL tests.
- While testing for aPS/PT can contribute to assess the risk of thrombosis, routine testing for aPT is not recommended.
- The main clinical utility of the anti-DI assay as a diagnostic tool or a risk stratification tool is being investigated comprehensively.
- aPL should not only be considered as diagnostic markers but also as risk factors for clinical events.

Acknowledgments

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Frequency of Th17 CD4+ T Cells in Early Rheumatoid Arthritis: A Marker of Anti-CCP Seropositivity

To examine the frequency and phenotype of Th17 cells in the peripheral blood of early RA (eRA) patients, Arroyo-Villa I, et al. (PLoS One 2012;7:e42189) isolated CD4+ T cells from the peripheral blood of 33 eRA patients, 20 established RA patients and 53 healthy controls (HC), and from the synovial fluid of 20 established RA patients (RASF), by ficoll-hypaque gradient and magnetical negative selection. After polyclonal stimulation, the frequency of Th17 and Th1 cells was determined by flow cytometry and concentrations of IL-17, IFN-γ, TNF-α and IL-10 were measured by ELISA in cell-free supernatants. When all eRA patients were analyzed together, a significantly lower percentage of circulating Th17 cells and a lower CD4-derived IL-17 secretion were observed in comparison with HC. However, after stratifying by anti-CCP antibody status, circulating Th17 cells were decreased in anti-CCP(+) but not in anti-CCP(-)-eRA. All Th17 cells were CD45RO+CD45RA- and CCR6+. Dual Th17/Th1 cells were also exclusively decreased in anti-CCP(+)-eRA. Circulating Th17 and Th17/Th1 cells were negatively correlated with anti-CCP titres. When anti-CCP(+)-eRA patients were retested one year after initiating treatment with oral methotrexate, their circulating Th17 frequency was no longer different from HC. Of note, the percentage of circulating Th1 cells and the secretion of CD4-derived IFN-γ, TNF-α and IL-10 were not different between eRA patients and HC. In established RA patients, circulating Th17 and T17/Th1 cell frequencies were comparable to HC. In RASF, both Th17 and Th1 cells were increased when compared with blood of eRA patients, established RA patients and HC. Decreased circulating Th17 levels in eRA seem to be a marker of anti-CCP seropositivity, and return to levels observed in healthy controls after treatment with methotrexate.

Testing for Antiphospholipid antibodies with Solid Phase Assays: guidance from the SSC of the ISTH

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Introduction

According to classification criteria for antiphospholipid syndrome (APS), laboratory diagnosis is based on the detection of antiphospholipid antibodies (aPL) by immunological assays with cardiolipin (aCL) and/or $\beta_2$glycoprotein I (aβ_2GPI) as antigen and/or lupus anticoagulants (LA) [1]. There is a large variety of assays assessing aPL and despite consensus guidelines, some issues remain unanswered [2]. Factors that contribute to result variability include pre-, post- and analytical conditions, calibration and assay-specific issues [3]. Recommendations published in 2009 by this Subcommittee for the detection of LA have proven useful in standardization of this assay [4]. Likewise, recommendations for detection of aCL and aβ_2GPI antibodies by immunoassays are needed.

Clarification of the recommendations summarized in Table 1

Patient selection

Testing for aPL should focus on patients who are likely to have APS [4] (Table 1).

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Blood collection

Serum or plasma can be used [5] as dilution by citrate is negligible. The manufacturer should indicate the sample type recommended with the kit. Plasma samples should be platelet poor and prepared by double centrifugation, according to LA guidelines [4].

Choice of assays

The aCL and aβ_2GPI are most commonly detected by ELISA. Recently, solid phase assays with various detection systems have been introduced [6–8].

The classification criteria for APS include IgG and IgM [1]. Debate is ongoing about the value of IgM but is beyond the scope of this paper. The presence of antibodies of the same isotype reinforces the clinical probability of APS [4]. Equally, the role of IgA and 'non-criteria' aPL (e.g. anti-phosphatidylcholine and anti-prothrombin) [1,9] should be further investigated. Recent studies demonstrated that aβ_2GPI against domain I correlate with thrombosis and obstetric complications [10], but additional clinical studies and commercially-available assays are needed before this test can be recommended.

Although there is debate on the role of aCL in the diagnosis of APS, methodologically correct aCL assays have diagnostic value with similar sensitivities/specificities to aβ_2GPI [7,11]. β_2GPI, when included as reagent, increases the aCL assay specificity [12]. As aβ_2GPI assays use β_2GPI as antigen, a high correlation may be observed between aCL and aβ2GPI measured by some automated systems as well as ELISA [7,11].

Performance characteristics

Technical specifications should be strictly followed for the chosen assay. As the characteristics used in commercially

Table 1 Recommendations for the optimal laboratory detection of aPL by solid phase assays

1. Patient selection
   Generalized search for aPL is discouraged to prevent incidental findings
   Testing should focus on younger patients (< 50 years) with unprovoked venous/arterial thromboembolism, thrombosis at unusual sites or thrombotic/pregnancy complications associated with autoimmune disease
2. Blood collection
   Serum or citrated (0.109m sodium-citrate) platelet-poor (< 10 000 platelets per μL) plasma
   Assay specifications should be validated if a different sample type to the one indicated by the manufacturer is used
   Samples stored at 2–8 °C and tested within 2–3 days or at −20 °C or below for longer storage. Avoid freeze-thawing cycles
3. Choice of assays
   Perform a β_2GPI-dependent aCL and an aβ_2GPI assay
   Human β2GPI should be used as β2GPI source
4. Performance characteristics
   Between-run imprecision should be < 20% (ELISA) and < 10% for automated systems
   Internal quality control material (included in the kit or non-kit material) that is negative and another with potency around cut-off should be included in every run
   At least one non-kit negative and positive control (commercial or patient material) material should be included in every run
   A run should be rejected if one control sample is out of the allowed range
   Participation in an external quality control scheme is strongly recommended
   Detection limits should be determined in a negative sample with the same matrix as the patient samples
   Samples with values above the analytical measuring range of the test should be diluted and re-tested or reported as 'higher than the upper measurement range value'
   Values below detection limits should be reported as 'lower than the lower limit of detection'
   The imprecision of the assay should be considered for interpretation of results around cut-off
   Whenever feasible assays should be evaluated for clinical performance in detecting thrombotic/pregnancy complications
5. Interferences
   RF can produce falsely elevated IgM aCL and aβ_2GPI
   Avoid icteric, hemolytic and lipemic samples
   Heterophile antibodies, human anti-animal antibodies and high levels of (monoclonal) immunoglobulins may produce false-positive results
6. Duplicate vs. single testing
   Manual ELISA: duplicate testing for calibrators, controls and patient samples
   Automated platforms: evaluate the imprecision; if < 10% singlet testing for patient samples and controls may be considered; duplicate testing for calibrators
7. Standards and calibration
   Whenever feasible traceability towards a primary standard should be defined
   Secondary calibrators may be used in daily practice
   A multi-point calibration curve (at least six points and covering the whole range) should be included in every run for ELISA
8. Results expression
   No international units available
   Results expressed according to the calibration of the assay
   Low and high results reported as < detection limits or > measurement range
9. Cut-off values
   Use local cut-off determined/validated for the local reagent/instrument combination
   Perform testing on at least 120 plasmas or sera and calculate the 99th percentile
   Or validate the manufacturer's cut-off on a limited number of at least 20 locally-collected healthy donors; manufacturer's cut-offs can be transferred if the statistical method is indicated and the donor population is comparable with the local population
   Whenever feasible clinical laboratories should check local cut-off values through a clinical approach regarding the association with thrombotic/pregnancy complications in the local population
10. Results interpretation and reporting
    Results of aPL should be interpreted in view of the clinical context
    State whether results are positive or negative according to method and laboratory-specific cut-off
    Consider the performance characteristics of the assay
    Confirm positive diagnosis after 12 weeks and consider only persistently positive results as clinically relevant
    Perform all three assays (LA, aCL and aβ_2GPI) on the same blood sampling to increase diagnostic utility
    Perform integrated interpretation of LA, aCL and aβ_2GPI
    Report analytical results and an interpretative comment

available assays cannot be changed, assay performance should be validated based on clinical performance.

Internal quality control samples should be included in each run. Controls independent from the kit add value and allow assessment of batch-to-batch variation [5,13].

Precision is an important requirement, especially at values around the cut-off. Between-run imprecision (i.e. coefficient of variation, CV) of manually-performed ELISAs should be < 20%, preferably < 15% [5,13]. For automated systems, < 10% is recommended [14].

As no gold standard exists, new assays should be validated locally based on analytical and clinical criteria. Whenever feasible, the association between assay results and thrombotic/pregnancy complications should be evaluated [6–8].

Interferences

Manufacturers should indicate in the package insert the level of IgM rheumatoid factor (RF) as well as the concentrations of hemoglobin/bilirubin/triglycerides and other interfering factors that may bias results [12–15].

Duplicate vs. single testing

Although a decision on single or duplicate testing depends on performance characteristics, it is recommended that duplicate testing should be carried out, especially when inter- and intra-run imprecision determined for a quality control sample is > 10%.

Standards and calibration

Lack of uniformity in reference material for calibration remains a problem. Manufacturers use a variety of calibrators, often using secondary standards and working calibrators. Whenever feasible, these calibrators should be traceable to a primary standard [16].

Calibration curves should be rejected if the correlation coefficient (test signal vs target values) is < 0.90 or if the curve does not fulfill manufacturers' recommendations. In contrast to ELISA, for newer automated platforms calibration curves are not required for every run [14, 17], when the same reagent lot is used.

Results expression

The test signal is converted into antibody units derived from the calibration curve. aCL assays calibrated against the Harris standards are expressed in GPL or MPL; aβ_2GPI are expressed in arbitrary units. Development of an international standard is in progress and will facilitate the uniformity in reporting aCL and aβ2GPI results [18].

Cut-off values calculation

Only medium and high antibody levels are included as diagnostic criteria for APS [1]. The nonparametric 99th percentile cut-off appears to be more specific than the > 40 GPL value [19]. Therefore, it is recommended that in-house cut-off values should be determined by the 99th percentile based on a population of healthy volunteers [4,17,20]. If this is not feasible, manufacturers' cut-offs may be acceptable if local measurements on 20 or more healthy subjects yield similar results [20]. The committee warns against indiscriminate use of cut-offs

determined elsewhere even for the same method/equipment.

Results interpretation

Solid-phase assays should be interpreted together with LA results to fully assess their clinical significance [1,4]. Recent studies showed that the risk of thrombosis in APS patients increases with the number of positive tests [1,11,21]. Positive results need to be confirmed after 12 weeks as transient antibodies have been described in infectious diseases and are not of clinical significance [1,4].

Given the variability in assay methods, reporting of semi-quantitative results is difficult to define. Each test result above cut-off should be regarded as positive and reported quantitatively. However, inter-laboratory and inter-method variability does not allow comparison between numerical values. Imprecision of the method should be considered, especially for results around cut-off.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interests.

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SPECIAL ARTICLE

Antiphospholipid scoring: significance in diagnosis and prognosis

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Recently our group introduced the "antiphospholipid score" (aPL-S), a quantitative marker that represents aPL profile. We have validated its efficacy for the diagnosis of antiphospholipid syndrome (APS) and predictive value for thrombosis. The study comprised two independent sets of patients with autoimmune diseases. In the first set of patients ($n = 233$), the aPL-S was established by analyzing aPL profiles. In the second set of patients ($n = 411$), the predictive value of the aPL-S for thrombosis was evaluated. To define aPL-S, we calculated the relative risks (approximated by odds ratios (ORs)) of having APS manifestations (thrombosis and/or pregnancy morbidity) for each of the aPL tests and devised an original formula in which aPL-S was determined by OR: aPL-S $= 5 \times \exp ([OR] -5)/4$. The receiver operating characteristic (ROC) curve showed a hyperbolic pattern and the area under the ROC curve value was 0.752 (0.686 for revised Sapporo criteria), implying that aPL-S is a potential quantitative marker for APS diagnosis. The OR for thrombosis in patients with a high aPL-S (≥ 30) was 5.27 (95% confidence interval (95% CI) 2.32–11.95, $p < 0.0001$). By multivariate analysis, an aPL-S of ≥ 30 appeared to be an independent risk factor for thrombosis (hazard ratio 3.144 (95% CI 1.383–7.150), $p = 0.006$). The aPL-S is a useful quantitative index for diagnosing APS and may be a predictive marker for thrombosis in autoimmune diseases. *Lupus* (2014) 23, 1269–1272.

Key words: Antiphospholipid syndrome; thrombosis; anticardiolipin antibodies; lupus; anticoagulant

Introduction

In consideration of the heterogeneity of antiphospholipid antibodies (aPL), multiple assays have been performed, not only for research purposes but also as routine clinical practice for the diagnosis of antiphospholipid syndrome (APS). Recently our group introduced the "antiphospholipid score" (aPL-S),[1] a quantitative marker that represents aPL profile. We demonstrated that the profile of aPL can be successfully quantitated as aPL-S and that aPL-S correlated with the history of thrombosis or pregnancy morbidity. In this study, we retrospectively analyzed two cohorts of autoimmune patients who attended the Rheumatology Clinic of Hokkaido University Hospital. The first group comprised 233 consecutive patients examined in 2006. The second set of patients included 296 patients who visited our clinic from 2002 to 2003 and were followed up for more than two years (median 72

months). A complete aPL testing profile was performed in our laboratory. For lupus anticoagulant (LA) mixing tests of three clotting tests (activated partial thromboplastin time (APTT), dilute Russell's viper venom time (dRVVT) and kaolin clotting time (KCT)) and phospholipid neutralizing assays of APTT/dRVVT were carried out according to the previous guidelines recommended by the Subcommittee on Lupus Anticoagulant/ Antiphospholipid Antibody of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis.[2] Anticardiolipin antibodies (aCL) (immunoglobulin (Ig)G and IgM), anti-beta2glycoprotein I antibodies (aβ_2GPI) (IgG and IgM) and phosphatidyl-dependent antiprothrombin antibodies (aPS/PT) (IgG and IgM) were assayed by enzyme-linked immunosorbent assay (ELISA) as described previously.[3–5]

Methods

To define aPL-S, we first calculated the relative risks (approximated by odds ratios (ORs)) of

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