

Original Article

AphL antibody ELISA as an alternative to anticardiolipin test for the diagnosis of antiphospholipid syndrome

Brenda B Suh-Lailam¹, Anndorie Cromar¹, K Wayne Davis¹, Anne E Tebo^{1,2}

¹ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT, United States; ²Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT, United States

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Abstract: Background: Persistent levels of antiphospholipid (aPL) antibodies [lupus anticoagulant (LA), anticardiolipin (aCL), anti-beta 2 glycoprotein I (a β ₂GPI) IgG and/or IgM] in association with clinical features of thrombosis and/or pregnancy associated morbidity are indicative of antiphospholipid syndrome (APS). Of the aPL antibodies, aCL is the most sensitive for APS, however, their lack of specificity constitute a laboratory and clinical challenge. IgG/IgM antibodies directed against AphL (a mixture of phospholipids) has been reported to predict APS more reliably than aCL tests. The main objective of this study was to evaluate the performance characteristics of the AphL IgG/IgM ELISA, relative to the aCL and a β ₂GPI tests. Methods: Sixteen (16) clinically confirmed APS and 85 previously tested serum (PTS) samples for aCL and a β ₂GPI IgG/IgM antibodies were evaluated with the AphL IgG/IgM ELISA. Clinical specificity was determined in 100 serum samples (50 healthy and 50 infectious disease controls [parvo- and syphilis-IgG/IgM positive]). Results: The IgG antibody prevalence for aCL and AphL in the APS and PST groups was comparable with marginal differences in clinical specificities. In contrast to the aCL IgM ELISA, the AphL test showed improved clinical specificities (72% aCL vs 94% AphL in the healthy controls; 38% aCL vs 78% AphL in the infectious disease controls) with implications for increased reliability in the diagnosis of APS. The overall agreement of the AphL with the aCL or a β ₂GPI for the IgG tests was 89% and 85% respectively, and that of the AphL IgM to the aCL or a β ₂GPI IgM tests was 72% and 86% respectively. Conclusion: Routine use of the AphL IgG/IgM ELISA may substantially reduce the high number of false positives associated with the aCL test without loss in sensitivity for APS.

Keywords: Anticardiolipin, AphL, antiphospholipid antibodies, method comparison

Introduction

The anti-cardiolipin (aCL) and anti-beta 2 glycoprotein I (a β ₂GPI) IgG and/or IgM immunoassays together with the lupus anticoagulant (LA) test are considered 'criteria' laboratory markers for the diagnosis of definite antiphospholipid syndrome (APS), an autoimmune disorder characterized by pregnancy-related morbidity, arterial and/or venous thrombosis [1-2]. Based on the laboratory recommendations for APS, a confirmed positive result of one immunoassay, i.e. aCL or a β ₂GPI IgG or IgM is sufficient for classifying patients with vascular thrombosis and/or pregnancy related morbidity as having APS [1]. Of the 'criteria' immunoassays for APS, aCL is the most sensitive while a β ₂GPI antibodies are considered highly specific with low sensitivity for APS. Although the increased sensitivity of the aCL ELISA makes it a favorable test in the initial

diagnostic work-up of APS patients, their lack of specificity with associated high degree of false positive results constitute both a laboratory and clinical challenge. Indeed, several clinical studies as well as systematic review of the literature indicate that IgG isotype of either aCL or a β ₂GPI is more strongly associated with APS than that of IgM [3-8]. The inherent difficulty in the standardization of aCL and a β ₂GPI IgM as well as their unreliability in the context of infectious diseases and interfering substances like IgM rheumatoid factor poses significant challenges in the determination of this antibody isotype in APS [3, 9-15].

The aCL IgM antibodies in particular have been shown to occur in infections such as chronic hepatitis C, leprosy, syphilis, Kala-azar, parvovirus B19 among others [10, 12-13, 16]. The presence of these antibodies in different infec-

tious diseases and the recognition that they do not usually correlate with thrombotic events and/or pregnancy-related morbidity in APS makes testing at 2 time points necessary for differentiation of APS-associated from infection-associated aPL antibodies [1]. Based on these observations, there have been suggestions to replace aCL and a β ₂GPI measurements from routine laboratory determinations with more reliable tests for the diagnosis of APS [17, 18]. Indeed alternative tests to aCL IgG/IgM antibodies and other potential diagnostic markers for APS have been described [19-23]. Of these, the AphL IgG/IgM as determined by ELISA has been reported to have improved specificity with optimal sensitivity for the diagnosis of APS [19]. The main objective in this study was to evaluate the performance characteristics of the AphL IgG/IgM ELISA relative to the aCL and a β ₂GPI IgG/IgM antibody tests. Recognizing the inherent challenge of comparing the AphL assays to the sensitive aCL ELISAs, we sought to investigate its performance in 4 four distinct groups to reduce selection bias. These groups included: 16 confirmed APS patients, 85 previously tested samples for aCL and a β ₂GPI IgG/IgM, 50 healthy and 50 infectious disease (syphilis or parvovirus B19 IgG/IgM positive) controls. Our data shows comparable performance of the AphL and aCL IgG assays with significant difference in the clinical specificities for the IgM isotype. Use of the AphL IgG/IgM ELISA may be useful as an alternate assay to aCL IgG/IgM ELISA without loss of diagnostic accuracy for APS.

Materials and methods

To evaluate the AphL IgG/IgM assays, we used two groups of samples that had previously been tested for APS. Sixteen (16) clinically confirmed APS patient sera obtained from the APLA 2010 wet workshop (Courtesy of Dr. Silvia Pierangeli, UTMB Galveston, TX) and 85 serum specimens obtained after completion of clinical testing for aCL and a β ₂GPI IgG/IgM (INOVA Diagnostics, San Diego, USA) at ARUP laboratories (previously tested samples, PTS) were employed. The PTS group was made up of 16 males and 69 females, mean age 45.5 years (\pm 19.0 years). To determine clinical specificity, two groups of control serum samples were investigated. The first group consisted of 50 serum samples from self-reported healthy individuals (HC), 10 males and 40 females with

mean age 38.3 (\pm 12.3 years). The second control group (n=50) was made up of 36 parvovirus IgG/IgM positive patients (mean age \pm standard deviation: 30.3 \pm 15.2 years) and 14 syphilis IgG/IgM positive cases (mean age \pm standard deviation: 39.6 \pm 21.1 years). IgG/IgM antibodies to parvovirus were determined using a commercial ELISA (Biotrin, Dublin, Ireland). Syphilis testing was performed with a *Treponema pallidum* antibody IgG/IgM ELISA (Trep-check, Bramecos Diagnostics Inc., Canada).

All 201 sera were tested with the AphL IgG/IgM ELISA (Louisville APL Diagnostics, Inc., Seabrook, TX) as well as the aCL and a β ₂GPI IgG/IgM commercial ELISAs (INOVA Diagnostics, San Diego, CA) by investigators who were blinded to the original aPL antibody results following manufacturers' recommendations. Test results were interpreted in one of two ways. First, based on the manufacturers' suggested cut-off values [aCL IgG/IgM (15 GPL and 13 MPL), a β ₂GPI IgG/IgM (20 SGU and 20 SMU) and AphL IgG/IgM (15 GPL and 15 MPL)] and secondly adjusted to exclude the equivocal samples according to manufacturers' recommendations [aCL IgG/IgM (20 GPL and 20 MPL), a β ₂GPI IgG/IgM (20 SGU and 20 SMU) and AphL IgG/IgM (27 GPL and 38 MPL)].

To evaluate the analytical performance of the different assays, the prevalence, sensitivity or specificity of each aPL antibody was estimated in the different sample groups using the manufacturers' suggested and adjusted cutoff values. The correlation between the different aPL assays was evaluated using EP evaluator release 8 (Data Innovations LLC, South Burlington, VT).

Results and discussion

In this study we assessed the performance characteristics of the AphL IgG/IgM ELISA relative to current 'criteria' immunoassays (aCL and a β ₂GPI IgG/IgM) for the diagnosis of APS using four distinct groups of samples. The first group consisted of known APS samples that were evaluated in the Wet Workshop of the 13th International Congress on Antiphospholipid Antibodies (APLA 2010), in Galveston, Texas. In addition to these samples, we examined previously tested samples (PTS) for APS using aCL and a β ₂GPI IgG/IgM assays (INOVA Diagnostics, San Diego, USA). As controls, two sets of samples from 50 reported healthy and 50 infectious

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Table 1. Performance Characteristics of the aPL Antibody Assays at Manufacturers' Cutoff and Adjusted Cutoff Values

Assay	Sensitivity* (%)		Specificity (%)			
			Healthy Controls		Infectious Disease Controls	
	Suggested Cut-off	Adjusted Cut-off	Suggested Cut-off	Adjusted Cut-off	Suggested Cut-off	Adjusted Cut-off
aCL IgG	100	100	90	94	80	90
aCL IgM	44	31	72	96	38	64
aCL IgG/IgM	100	100	66	92	38	62
APhL IgG	100	100	98	98	96	98
APhL IgM	25	19	94	98	78	98
APhL IgG/IgM	100	100	92	96	76	96
a β ₂ GPI IgG	81	81	96	N/A	98	N/A
a β ₂ GPI IgM	31	31	98	N/A	88	N/A
a β ₂ GPI IgG/IgM	88	88	96	N/A	88	N/A

*Sensitivity for the different assays was determined only for confirmed APS group. Data for clinical specificity is presented based on the healthy and infectious disease control groups. No recommendation to adjust cut-off values for a β ₂GPI assays is provided by the manufacturer, N/A represents not available.

disease controls (36 parvo- and 16-syphilis-antibody positive sera). In each of these groups, we determined the prevalence of APhL, aCL and a β ₂GPI IgG/IgM antibodies as recommended by the manufacturers. **Figure 1A** shows the antibody prevalence for the APhL, aCL and a β ₂GPI IgG/IgM assays in the study cohort. At the manufacturers' suggested cutoff values, the aPL antibody prevalence was highest in the aCL IgG/IgM assay in all groups evaluated. While the APhL IgG/IgM antibody prevalence in the APS and 'suspected' APS patient group was comparable to that of the aCL assays, its specificities in the healthy and infectious disease control cohorts were comparable to those of the a β ₂GPI tests. The most significant difference in antibody prevalence between these assays was observed in the infectious disease group where the aCL IgG had a prevalence of 20% that was 5-fold greater than the APhL IgG assay and 10-fold more than the a β ₂GPI IgG tests. With regards to the IgM antibody isotype, the prevalence of aCL was 62% compared to 24% and 12% for the APhL and a β ₂GPI tests respectively. In addition, the unadjusted specificities of the IgG aPL assays were significantly higher than those of the IgM analytes in both control groups (**Figure 1A**). Thus, the aCL and APhL assays showed comparable sensitivities but distinct clinical specificities for APS (**Table 1**).

Since the prevalence of the APhL were comparable to those of the aCL for the IgG in the clini-

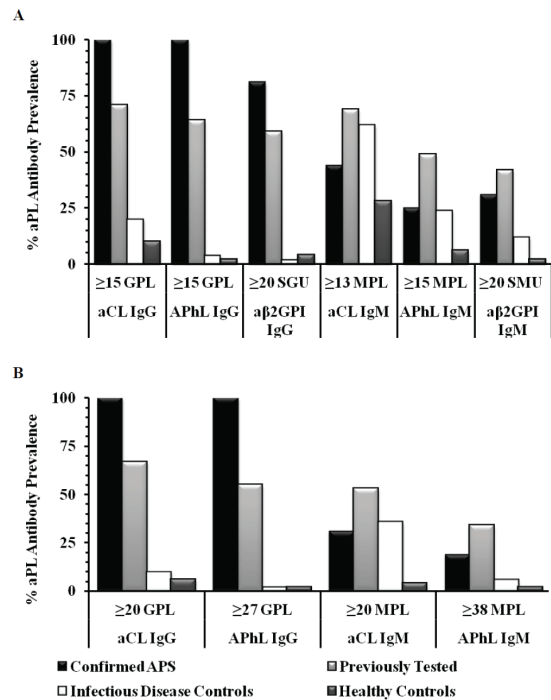


Figure 1. Prevalence of specific aPL antibodies is dependent of cut-off determination. The per cent aCL, APhL or a β ₂GPI IgG/IgM antibody isotype is shown for the confirmed APS, previously tested and controls groups based the manufacturers' (A) suggested and (B) adjusted cut-off values.

cally confirmed APS and PTS group but differed significantly for the IgM isotype, we adjusted the

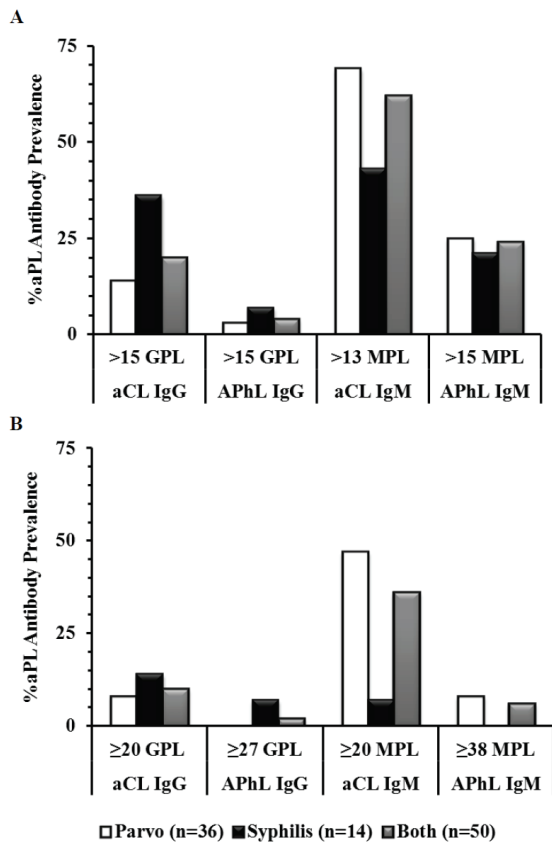


Figure 2. Clinical specificity of aCL and APhL IgG/IgM antibody tests is dependent on assay principle. Differential specificities aCL and APhL IgM assays in the context of parvovirus infection at manufacturers' (A) suggested and (B) adjusted cut-off values.

cut-off values based on manufacturers' recommendations for all assays (except aβ₂GPI tests) and compared the outcomes (Figures 1B, 2A and 2B). Except in the APS confirmed group, the adjustment in cut-off values resulted in a general decrease in the aCL and APhL IgG/IgM antibody prevalence in all groups (Figure 1B). However, compared to the APhL IgM assay which showed significant improvement in clinical specificity (78% versus 98%), the change in specificity for the aCL IgM assay was also significant, but not as high (38% versus 64%). Overall, adjusting the cut-off values for the assays increased the specificities for the aCL IgG/IgM (38% to 62%) and APhL IgG/IgM (76% to 96%) for the infectious disease groups. Further examination of analyte-specific (aCL, APhL and aβ₂GPI) clinical performance based on specific infectious disease serologies showed the aCL assays to be the most affected (Table 1, Figure

2A and 2B). Although significant improvement in specificities was observed with the APhL assays and aCL IgG following adjustment of cut-off values, the effect on the aCL IgM isotype was minor. Our results indicate that the use of the APhL IgG/IgM assays may provide substantial improvement in clinical specificity for APS without a significant loss in sensitivity and supports a recent report that the choice of the cut-off value used can markedly influence the outcome of aPL antibody testing and hence APS diagnosis [23].

Assay selection bias in the evaluation of alternate tests for aCL is any inherent challenge for a study of this nature. It is well recognized that aCL is required for optimal sensitivity in the diagnosis of APS [24]. In addition, for the most confirmed APS cases, the use of cut-off values representing the 99th percentile or 40 GPL or MPL units [1] allow for a relatively homogenous patient population, i.e. those with high risk for disease. Thus, comparison of methods under unselected conditions which mimic routine laboratory practice represents an appealing approach to evaluate the APhL assay. Using specimens which have been tested for aCL and aβ₂GPI IgG/ IgM at our reference laboratory, our concordance analyses show that the agreement between the aCL and APhL for IgG isotype antibody to exceed 80% with a positive agreement of 68% between the IgM assays (data not shown). Furthermore, the overall correlation between the aCL and APhL IgG assays was greater than 80% (Table 2). Due to the differential clinical specificities of the aPL IgM assays, the positive agreements for the aCL and APhL IgM tests differed significantly with comparable concordance between the APhL and aβ₂GPI analytes. Consequently, the APhL assay may probably have similar clinical sensitivity to the aCL ELISA but with better specificity particularly in the context of infectious disease.

Although, we have sought to reduce bias in our investigation by using a diverse number of study groups to evaluate the performance characteristics of the APhL IgG/IgM assays for APS, there are some limitations in our study design. The most obvious weakness of this investigation is the lack of clinical data for patients in our suspected APS disease group. This was unavoidable as our efforts to recruit patient samples without assay selection bias was unsuccessful since most of the patients in our database have

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Table 2. Correlation between Antiphospholipid Antibody Assays Based on Isotype Class

Antibody Isotype	Analytes	% Positive Agreement	% Negative Agreement	% Overall Agreement	Kappa (%) 95% (CI)
IgG	APhL vs aCL	77	98	89	77 (68-86)
	APhL vs aB ₂ GPI	83	85	85	65 (54-76)
	aCL vs aB ₂ GPI	59	92	77	53 (41-65)
IgM	APhL vs aCL	51	97	72	46 (34-57)
	APhL vs aB ₂ GPI	82	87	86	63 (50-75)
	aCL vs aB ₂ GPI	40	98	67	36 (24-49)

Concordance was computed based on manufacturers' recommended cut-off values with aCL and aB₂GPI assays as reference tests. In the case of aCL vs aB₂GPI, aCL was used as the reference test. Percent agreement, kappa and confidence interval (CI) values were generated using the EP evaluator 8.

been diagnosed using INOVA aCL and aβ₂GPI IgG/IgM assays. Thus for this study, we elected to use a limited number of APS confirmed patients as well as an unselected population of samples that have previously been tested for aCL and aβ₂GPI antibodies. A second potential limitation of our study is the use of parvovirus B19 antibody positive patient samples to evaluate the clinical specificities of the aPL assays given that this infection may be a potential trigger of APS and/or SLE [16]. While antibody responses to specific B19 viral proteins and estimation of parvovirus B19 DNA in the specimens have not been performed to rule out acute, persistent or past infectious, we included known parvovirus IgG and/or IgM-positive sera to determine interference on aPL antibody testing especially since both APhL and aCL assays are β₂GPI-dependent. Of the parvo-antibody positive samples, those which were only IgG positive had the least number of aPL antibody prevalence (data not shown). Overall, the APhL and aβ₂GPI IgG/IgM assays had similar positivity rates in the parvovirus antibody positive group (**Figure 2A**) while the aCL IgM had the highest prevalence at the manufacturer's recommended cut-off.

Current laboratory criteria for the diagnosis of APS are dependent on the demonstration of the presence and persistence of 1 or more of LA, aCL, and aβ₂GPI IgG and/or IgM aPL antibodies. Although these guidelines are meant for research purposes, they are the best available tool for the evaluation of APS in clinical practice. The aCL ELISA is most sensitive test for the diagnosis of APS but lacks specificity despite efforts for clinical improvement. With an array of alternative and emerging diagnostic assays for APS, it is highly anticipated that choosing tests with the best medical benefit would outweigh the use of established analytes with suboptimal

clinical value. Our data demonstrate that routine use of alternate tests such as the the APhL IgG/IgM ELISA may substantially reduce the high number of false positives associated with the aCL IgG/IgM tests without loss in diagnostic sensitivity for APS.

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Address correspondence to: Dr. Anne E. Tebo, ARUP Laboratories, 500 Chipeta Way, Salt lake City, UT 84108 Tel: +1 801 583 2787 x3138; Fax: +1 801 584 5207; E-mail: anne.tebo@aruplab.com

References

- [1] Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, Derksen RH, PG DEG, Koike T, Meroni PL, Reber G, Shoenfeld Y, Tincani A, Vlachoyiannopoulos PG and Krilis SA. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006; 4: 295-306.
- [2] Pierangeli SS, de Groot PG, Diott J, Favaloro E, Harris EN, Lakos G, Ortel T, Meroni PL, Otomo K, Pengo V, Tincani A, Wong R and Roubey R.

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- 'Criteria' aPL tests: report of a task force and preconference workshop at the 13th International Congress on Antiphospholipid Antibodies, Galveston, Texas, April 2010. *Lupus* 2011; 20: 182-190.
- [3] Tincani A, Allegrì F, Sanmarco M, Cinquini M, Taglietti M, Balestrieri G, Koike T, Ichikawa K, Meroni P and Boffa MC. Anticardiolipin antibody assay: a methodological analysis for a better consensus in routine determinations—a cooperative project of the European Antiphospholipid Forum. *Thromb Haemost* 2001; 86: 575-583.
- [4] Galli M, Luciani D, Bertolini G and Barbui T. Lupus anticoagulants are stronger risk factors for thrombosis than anticardiolipin antibodies in the antiphospholipid syndrome: a systematic review of the literature. *Blood* 2003; 101: 1827-1832.
- [5] Ruffatti A, Tonello M, Del Ross T, Cavazzana A, Grava C, Noventa F, Tona F, Iliceto S and Pengo V. Antibody profile and clinical course in primary antiphospholipid syndrome with pregnancy morbidity. *Thromb Haemost* 2006; 96: 337-341.
- [6] Opatrny L, David M, Kahn SR, Shrier I and Rey E. Association between antiphospholipid antibodies and recurrent fetal loss in women without autoimmune disease: a metaanalysis. *J Rheumatol* 2006; 33: 2214-2221.
- [7] Giannakopoulos B, Passam F, Ioannou Y and Krilis SA. How we diagnose the antiphospholipid syndrome. *Blood* 2009; 113: 985-994.
- [8] Erkan D, Barbhaya M, George D, Sammaritano L and Lockshin M. Moderate versus high-titer persistently anticardiolipin antibody positive patients: are they clinically different and does high-titer anti-beta 2-glycoprotein-I antibody positivity offer additional predictive information? *Lupus* 2010; 19: 613-619.
- [9] Agopian MS, Boctor FN and Peter JB. False-positive test result for IgM anticardiolipin antibody due to IgM rheumatoid factor. *Arthritis Rheum* 1988; 31: 1212-1213.
- [10] Leroy V, Arvieux J, Jacob MC, Maynard-Muet M, Baud M and Zarski JP. Prevalence and significance of anticardiolipin, anti-beta2 glycoprotein I and anti-prothrombin antibodies in chronic hepatitis C. *Br J Haematol* 1998; 101: 468-474.
- [11] Galperin I, Fortin PR, Subang R, Newkirk MM and Rauch J. A subset of rheumatoid factors crossreacts with cardiolipin in patients positive for IgM rheumatoid factor and anticardiolipin antibodies. *J Rheumatol* 2000; 27: 820-821.
- [12] Santiago M, Martinelli R, Ko A, Reis EA, Fontes RD, Nascimento EG, Pierangeli S, Espinola R and Gharavi A. Anti-beta2 glycoprotein I and anticardiolipin antibodies in leptospirosis, syphilis and Kala-azar. *Clin Exp Rheumatol* 2001; 19: 425-430.
- [13] Zachou K, Liaskos C, Christodoulou DK, Kardasi M, Papadamou G, Gatselis N, Georgiadou SP, Tsianos EV and Dalekos GN. Anti-cardiolipin antibodies in patients with chronic viral hepatitis are independent of beta2-glycoprotein I cofactor or features of antiphospholipid syndrome. *Eur J Clin Invest* 2003; 33: 161-168.
- [14] Pengo V, Biasiolo A, Bison E, Chantarangkul V and Tripodi A. Antiphospholipid antibody ELISAs: survey on the performance of clinical laboratories assessed by using lyophilized affinity-purified IgG with anticardiolipin and anti-beta2-Glycoprotein I activity. *Thromb Res* 2007; 120: 127-133.
- [15] Lakos G, Teodorescu M. IgM, but not IgA rheumatoid factor interferes with anti-cardiolipin and anti-beta2 glycoprotein I measurements: a quantitative analysis. *Lupus* 2011; 20: 614-619.
- [16] Von Landenberg P, Lehmann HW, Knoll A, Dorsch S and Modrow S. Antiphospholipid antibodies in pediatric and adult patients with rheumatic disease are associated with parvovirus B19 infection. *Arthritis Rheum* 2003; 48: 1939-1947.
- [17] de Groot PG, Derksen RH and de Laat B. Twenty-two years of failure to set up undisputed assays to detect patients with the antiphospholipid syndrome. *Semin Thromb Hemost* 2008; 34: 347-355.
- [18] Galli M, Reber G, de Moerloose P and de Groot PG. Invitation to a debate on the serological criteria that define the antiphospholipid syndrome. *J Thromb Haemost* 2008; 6: 399-401.
- [19] Merkel PA, Chang Y, Pierangeli SS, Harris EN and Polisson RP. Comparison between the standard anticardiolipin antibody test and a new phospholipid test in patients with connective tissue diseases. *J Rheumatol* 1999; 26: 591-596.
- [20] Atsumi T, Ieko M, Bertolaccini ML, Ichikawa K, Tsutsumi A, Matsuura E and Koike T. Association of autoantibodies against the phosphatidylserine-prothrombin complex with manifestations of the antiphospholipid syndrome and with the presence of lupus anticoagulant. *Arthritis Rheum* 2000; 43: 1982-1993.
- [21] de Laat B, Pengo V, Pabinger I, Musial J, Voskuyl AE, Bultink IE, Ruffatti A, Rozman B, Kveder T, de Moerloose P, Boehlen F, Rand J, Ulcova-Gallova Z, Mertens K and de Groot PG. The association between circulating antibodies against domain I of beta2-glycoprotein I and thrombosis: an international multicenter study. *J Thromb Haemost* 2009; 7: 1767-1773.
- [22] Atsumi T, Koike T. Antiprothrombin antibody: why do we need more assays? *Lupus* 2010; 19: 436-439.
- [23] Ruffatti A, Olivieri S, Tonello M, Bortolati M, Bison E, Salvan E, Facchinetti M and Pengo V. Influence of different IgG anticardiolipin antibody cut-off values on antiphospholipid syndrome classification. *J Thromb Haemost* 2008; 6: 1693-1696.
- [24] Nash MJ, Camilleri RS, Kunka S, Mackie IJ, Machin SJ and Cohen H. The anticardiolipin assay is required for sensitive screening for antiphospholipid antibodies. *J Thromb Haemost* 2004; 2: 1077-1081.