Lupus (2016) 0, 1–11

http://lup.sagepub.com

PAPER

Evaluation of phosphatidylserine-dependent antiprothrombin antibody testing for the diagnosis of antiphospholipid syndrome: results of an international multicentre study

O Amengual[1], R Forastiero[2], M Sugiura-Ogasawara[3], K Otomo[1], K Oku[1], C Favas[4], J Delgado Alves[4], P Žigon[5], A Ambrožič[5], M Tomšič[5], I Ruiz-Arruza[6], G Ruiz-Irastorza[6], ML Bertolaccini[7], GL Norman[8], Z Shums[8], J Arai[9], A Murashima[10], AE Tebo[11], M Gerosa[12], PL Meroni[12], I Rodriguez-Pintó[13], R Cervera[13], J Swadzba[14], J Musial[14] and T Atsumi[1]

[1]Division of Rheumatology, Endocrinology and Nephrology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; [2]Instituto de Medicina Traslacional, Trasplante y Bioingeniería (IMeTTyB), Universidad Favaloro-CONICET, Departamento de Fisiologia, Hemostasia y Trombosis, Buenos Aires, Argentina; [3]Department of Obstetrics and Gynecology, Nagoya City University, Graduated School of Medical Sciences, Nagoya, Japan; [4]Department of Medicine IV, Fernando Fonseca Hospital, Amadora and CEDOC/NOVA Medical School, Lisbon, Portugal; [5]Department of Rheumatology, University Medical Centre Ljubljana, Ljubljana, Slovenia; [6]Autoimmune Diseases Research Unit, Department of Internal Medicine, Biocruces Health Research Institute, Hospital Universitario Cruces, University of The Basque Country, Spain; [7]Academic Department of Vascular Surgery, Cardiovascular Division, King's College London, UK; [8]Inova Diagnostics Inc., San Diego, CA, USA; [9]Medical and Biological Laboratories, Co. Ltd, Nagano, Japan; [10]Center of Maternal-Fetal, Neonatal and Reproductive Medicine, National Center for Child Health and Development, Tokyo, Japan; [11]Department of Pathology, University of Utah and ARUP Laboratories, Salt Lake City, UT, USA; [12]Department of Clinical Sciences and Community Health, Istituto Auxologico Italiano, University of Milan, Milan, Italy; [13]Department of Autoimmune Diseases, Hospital Clínic, University of Barcelona, Barcelona, Catalonia, Spain; and [14]Department of Medicine Jagiellonian University, Cracow, Poland

Objective: A task force of scientists at the International Congress on Antiphospholipid Antibodies recognized that phosphatidylserine-dependent antiprothrombin antibodies (aPS/PT) might contribute to a better identification of antiphospholipid syndrome (APS). Accordingly, initial and replication retrospective, cross-sectional multicentre studies were conducted to ascertain the value of aPS/PT for APS diagnosis. **Methods:** In the initial study (eight centres, seven countries), clinical/laboratory data were retrospectively collected. Serum/plasma samples were tested for IgG aPS/PT at Inova Diagnostics (Inova) using two ELISA kits. A replication study (five centres, five countries) was carried out afterwards. **Results:** In the initial study ($n = 247$), a moderate agreement between the IgG aPS/PT Inova and MBL ELISA kits was observed ($k = 0.598$). IgG aPS/PT were more prevalent in APS patients (51%) than in those without (9%), OR 10.8, 95% CI (4.0–29.3), $p < 0.0001$. Sensitivity, specificity, positive (LR+) and negative (LR–) likelihood ratio of IgG aPS/PT for APS diagnosis were 51%, 91%, 5.9 and 0.5, respectively. In the replication study ($n = 214$), a moderate/substantial agreement between the IgG aPS/PT results obtained with both ELISA kits was observed ($k = 0.630$). IgG aPS/PT were more prevalent in APS patients (47%) than in those without (12%), OR 6.4, 95% CI (2.6–16), $p < 0.0001$. Sensitivity, specificity, LR + and LR– for APS diagnosis were 47%, 88%, 3.9 and 0.6, respectively. **Conclusions:** IgG aPS/PT detection is an easily performed laboratory parameter that might contribute to a better and more complete identification of patients with APS. *Lupus* (2016) **0,** 1–11.

Key words: Antiphospholipid antibodies; thrombosis; lupus anticoagulant; systemic lupus erythematosus

Introduction

Antiphospholipid antibodies (aPL) are a heterogeneous group of antibodies detected in patients with antiphospholipid syndrome (APS). The latest classification criteria for definite APS (Sydney-revised Sapporo criteria) require the presence of at least

Correspondence to: Olga Amengual, Division of Rheumatology, Endocrinology and Nephrology, Hokkaido University Graduate School of Medicine, N15 W7, Kita-ku, Sapporo 060-8638, Japan.
Email: olgaam@med.hokudai.ac.jp

Received 7 February 2016; accepted 24 June 2016

10.1177/0961203316660203

one clinical manifestation and one positive laboratory criterion.[1] Anticardiolipin antibodies (aCL), anti-$\beta_2$glycoprotein I antibodies (anti-β_2GPI) and lupus anticoagulant (LA) are the laboratory tests included in the laboratory criteria for APS classification.

A number of issues concerning the definition of aPL positive are under discussion. Current APS classification criteria identify a homogenous group of APS patients, but exclude patients with clinical manifestations highly suggestive of APS and 'non-criteria' aPL, i.e. autoantibodies targeting other plasma proteins or phospholipid-bound proteins complexes, recognized as 'seronegative APS'.[2]

In the last decade, antibodies against prothrombin emerged as a potential maker for APS. The presence of antibodies solely targeting human prothrombin (aPT-A) by enzyme-linked immunosorbent assay (ELISA) has been recognized since 1995.[3] Antiprothrombin antibodies bind not only to prothrombin coated on gamma-irradiated or activated polyvinyl chloride ELISA plates, but also recognize prothrombin exposed to immobilized phosphatidylserine, namely phosphatidylserine-dependent antiprothrombin antibodies (aPS/PT).[4–6]

Although both aPT-A or aPS/PT are associated with APS-related clinical features and can both be present in the same patient, they belong to different populations of autoantibodies.[5,7]

Several studies have been published with regard to the relationship between the presence of aPT-A and APS-related clinical features with conflicting conclusions.[8–20] A recent systematic review suggested that both antibodies against prothrombin, aPT-A and aPS/PT, are risk factors for thrombosis, but that aPS/PT represent a stronger risk factor for arterial and/or venous thrombosis when compared to aPT-A.[21] In two prospective studies, the presence of aPT-A has been reported as a predictor of thromboembolic events in patients with aPL, mainly in those patients positive for LA.[22,23]

Many reports have shown the clinical utility of aPS/PT in the diagnosis of APS.[5,24–27] In a large cohort of Japanese patients with systemic autoimmune diseases, the presence of aPS/PT significantly correlated with LA and with the clinical manifestations of APS.[5] Furthermore, aPS/PT appeared as the strongest independent risk factor for obstetric complications.[27] These data suggest that aPS/PT testing might help in assessing the risk of thrombosis and pregnancy morbidity in patients suspected of suffering from APS.

An international task force of scientists analysed critical questions related to 'non-criteria' aPL tests in an evidence-based manner during the 13th International Congress on Antiphospholipid Antibodies. The task force members agreed that antiprothrombin antibody assays, in particular, aPS/PT, might potentially contribute to better recognition of APS patients.[28] However, the inclusion of antiprothrombin antibodies as one of the laboratory criteria of APS could not be warranted at that time, mainly due to poor standardisation of the aPS/PT assays. It was concluded that reproducibility of the strong correlations between aPS/PT and APS manifestations, which were presented by some of the investigators at the meeting, needed to be confirmed by larger collaborator studies. As a result, a retrospective and cross-sectional multicentre study was designed to evaluate the value of aPS/PT for the diagnosis of APS. After completing the initial study, a replication study was carried out in order to ensure the validity of the findings.[29]

Patients and methods

In the initial retrospective multicentre study, potential participating centres were asked to submit data and samples from patients with and without APS, as well as from control participants. At each participant institution, individuals were assigned to one of the following groups: (1) Patients with clinical APS in the presence or absence of concomitant systemic autoimmune diseases (APS group). Patients with clinical APS refers to patients with events consistent with APS-associated manifestations and positive for APS laboratory criteria,[1] and to patients with APS-associated manifestations for whom physicians have a strong suspicion of APS but without APS laboratory criteria, (2) patients without clinical APS, with and without systemic autoimmune diseases (non-APS group), and (3) apparently healthy individuals.

Demographics, medical history and laboratory data from all the participants were retrospectively collected in a questionnaire, and a serum/plasma sample from the individuals were prepared and stored until use.

All questionnaires were sent to the coordinating site at Hokkaido University for the assignment of a unique identification code for each participant. After receiving identification codes, each institution shipped the samples, with the identification code clearly typed on the tubes, to the analysis site at Inova Diagnostics Inc, San Diego CA, United States (US) (Inova).

At the analysis site, all samples were blindly evaluated for immunoglobulin (Ig)G aPS/PT

Table 1 Demographic characteristics of population

	APS group	*Non-APS group*	*Healthy participants*	*All population*
Initial study	*n = 126*	*n = 73*	*n = 48*	*n = 247*
Sex F:M (ratio)	106:20 (5.3)	54:19 (2.8)	26:22 (1.2)	186:61 (3.0)
Mean years (range)	40.8 (20–79)	46.9 (18–77)	32.9 (23–47)	41.0 (18–79)
Race (*n*)[a]				
- Caucasian	69 (55%)	37 (51%)	28 (58%)	134 (54%)
- Asian	39 (31%)	15 (21%)	20 (42%)	74 (30%)
- Hispanic	–	1 (1%)	–	1 (0.5%)
- Black	1 (1%)	1 (1%)	–	2 (1%)
Replication study	*n = 96*	*n = 67*	*n = 51*	*n = 214*
Sex F:M (ratio)	85:11 (7.7)	52:15 (3.5)	46:5 (9.2)	183:31 (5.9)
Mean years (range)	41.1 (14–74)	44.7 (25–88)	36.5 (21–60)	41.1 (14–88)
Race (*n*)[b]				
- Caucasian	70 (73%)	47 (70%)	41(80%)	158 (74%)
- Asian	23 (24%)	19 (28%)	8 (16%)	50 (23%)
- Hispanic	1 (1%)	1 (1%)	1 (2%)	3 (1%)
- Black	–	–	1 (2%)	1 (0.5%)

n: number; F: female, M: male; APS: antiphospholipid syndrome.
[a]In 35 individuals information was not provided. [b]In three individuals information was not provided.

by ELISA using kits provided by two manufacturers: QUANTA Lite™ aPS/PT IgG ELISA from Inova (US Food and Drug Administration (FDA) cleared) and PS/PT ELISA kit for IgG isotype from Medical and Biological Laboratories Co. Ltd, Nagano, Japan (MBL). Cut-offs levels were set up at ≥ 30 units for the QUANTA Lite™ aPS/PT IgG ELISA and >12 units for the MBL IgG PS/PT ELISA kit according to the manufacturers' instructions.

After completing the initial study in July 2013, a replication study was performed to ensure that results were reliable and valid.

The studies were approved by independent ethical committees or institutional review boards at all institutions involved. The studies were conducted in accordance with the Declaration of Helsinki and the Principles of Good Clinical Practice.

Statistical analysis

Statistical evaluation was carried out by Chi-squared test (χ^2) or Fisher's exact test, as appropriate. Pearson's correlation coefficient was used for analysing the correlations, and Cohen's kappa test was applied to compare the results obtained using different tests in the same sample. Titres of antibodies were compared using Mann–Whitney *U* test. Relative risk was approximated by odds ratio (OR) and 95% confidence interval (CI). Sensitivity, specificity and likelihood ratios (LRs) of aPS/PT for the diagnosis of APS were calculated. Receiver operating characteristic (ROC) curve analysis was used to assess the diagnostic accuracy of the assays. *P* values less than 0.05 were considered significant. All statistical analyses were performed using SPSS (Chicago, IL, USA).

Results

Initial study

In the initial study, 247 participants from eight centres in seven countries were included. Each site included at least 20 individuals (Appendix 1). The cohort comprised 199 patients and 48 apparently healthy people. Among the patients, 126 had clinical APS, of whom 77 (61%) had primary APS and in 49 (39%), APS was diagnosed in coexistence with other autoimmune diseases. In the group of patients without APS ($n = 73$), 42 had systemic autoimmune diseases and 31 had non-systemic autoimmune diseases. Demographics and clinical characteristics of the included population are presented in Table 1 and in Appendix 2. Seventy-five patients (60%) had thrombotic events (with/without obstetric complications), and 51 women (48%) had obstetric manifestations without thrombotic complications. All patients in the APS group were reported to have positive aPL laboratory criteria tests.

IgG aPS/PT were detected in 58% and 43% of patients with clinical APS using the Inova and MBL ELISA kits, respectively. Detailed data on the prevalence of IgG aPS/PT in each of the analysed groups is shown in Appendix 3.

There was a statistically significant correlation in the optical density (OD) values of IgG aPS/PT

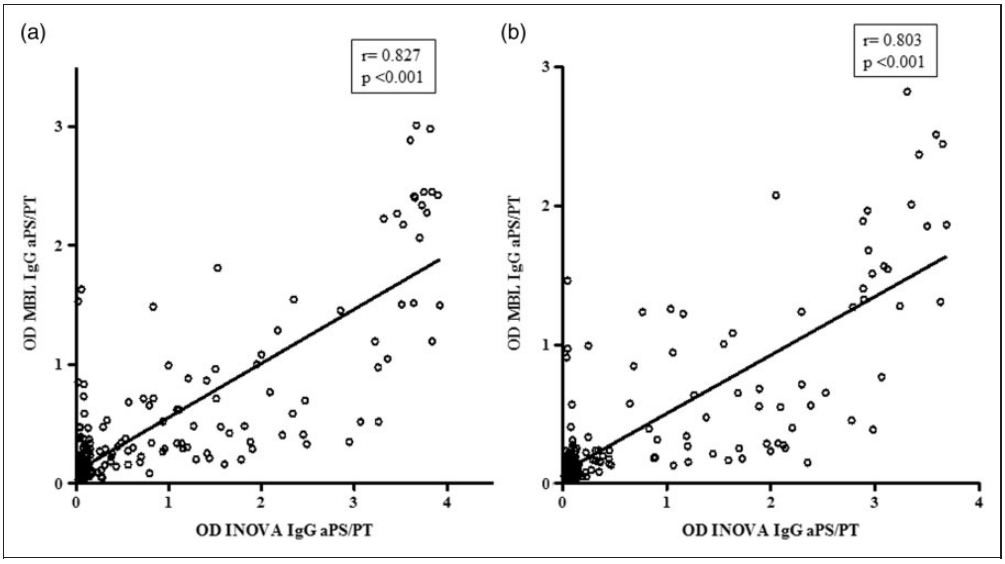

Figure 1 Correlation of IgG aPS/PT results obtained with commercial ELISAs, Inova and MBL, in samples from the initial and replication cohorts. (a) Correlation of the IgG aPS/PT optical density (OD) values between Inova and MBL ELISA kits in samples from the initial study. There was a statistically significant correlation in the IgG aPS/PT OD values obtained with both ELISAs (Pearson's correlation coefficient: $r = 0.827$, $p < 0.001$). (b) Correlation of the IgG aPS/PT OD values between Inova and MBL ELISA kits in samples from the replication study. A statistically significant correlation in the IgG aPS/PT OD values obtained with both ELISAs was observed (Pearson's correlation coefficient: $r = 0.803$, $p < 0.001$).
IgG aPS/PT: positive titres of phosphatidylserine-dependent antiprothrombin antibodies in Inova and MBL assays; ELISA: enzyme-linked immunosorbent assay; MBL: Medical and Biological Laboratories Co.

obtained with both ELISA kits (Pearson's correlation coefficient: $r = 0.827$, $p < 0.001$) (Figure 1(a)). A moderate agreement between the IgG aPS/PT Inova and MBL ELISA kits was observed ($k = 0.598$).

Concordant IgG aPS/PT results were considered when the sample gave a positive or negative result in both ELISA kits. A total of 204 samples displayed concordant IgG aPS/PT results and were subsequently analysed. Forty-three samples (17%) displayed discrepant IgG aPS/PT results between the Inova and MBL ELISA kits (Appendix 4).

Among the 204 samples with concordant results, 99 were obtained from patients with clinical APS, 58 from non-APS patients and 47 from healthy individuals. Positive titres of IgG aPS/PT were more prevalent in patients with clinical APS (51%) than in those without (9%), OR 10.8, 95% CI (4.0-29.3), $p < 0.0001$. Sensitivity, specificity, positive LR (LR+) and negative LR (LR–) of IgG aPS/PT for the diagnosis of APS were 51%, 91%, 5.9 and 0.5, respectively. The diagnostic accuracy of IgG aPS/PT for the diagnosis of APS, the thrombotic manifestations, and the pregnancy complications was assessed by ROC curves, and the area under the curve (AUC) values are presented in Table 2.

Table 2 Diagnostic value of IgG aPS/PT for antiphospholipid syndrome

	Inova AUC (95%CI)	*MBL AUC (95%CI)*
Initial study		
APS diagnosis	0.780 (0.716–0.844)[a]	0.769 (0.704–0.834)[a]
Thrombosis	0.719 (0.649–0.795)[a]	0.691 (0.612–0.770)[a]
Pregnancy complications	0.664 (0.563–0.766)[b]	0.721 (0.625–0.816)[a]
Replication study		
APS diagnosis	0.753 (0.679–0.827)[a]	0.770 (0.700–0.840)[a]
Thrombosis	0.808 (0.731–0.885)[a]	0.850 (0.789–0.910)[a]
Pregnancy complications	0.595 (0.483–0.706)[c]	0.549 (0.436–0.662)[c]

IgG: immunoglobulin G; aPS/PT: phosphatidylserine-dependent antiprothrombin antibodies; AUC: area under the curve calculated using receiver operating characteristic curve; CI: confidence intervals; MBL: Medical and Biological Laboratories Co.
[a] $p < 0.001$. [b] $p = 0.002$. [c] Not statistically significant.

Titres of IgG aPS/PT detected by either Inova or MBL ELISA assays were significantly higher in patients with clinical APS than in those without ($p < 0.001$), or in healthy controls ($p < 0.001$) (Figure 2(a)).

The presence of IgG aPS/PT significantly correlated with a history of thrombosis, OR 11.0,

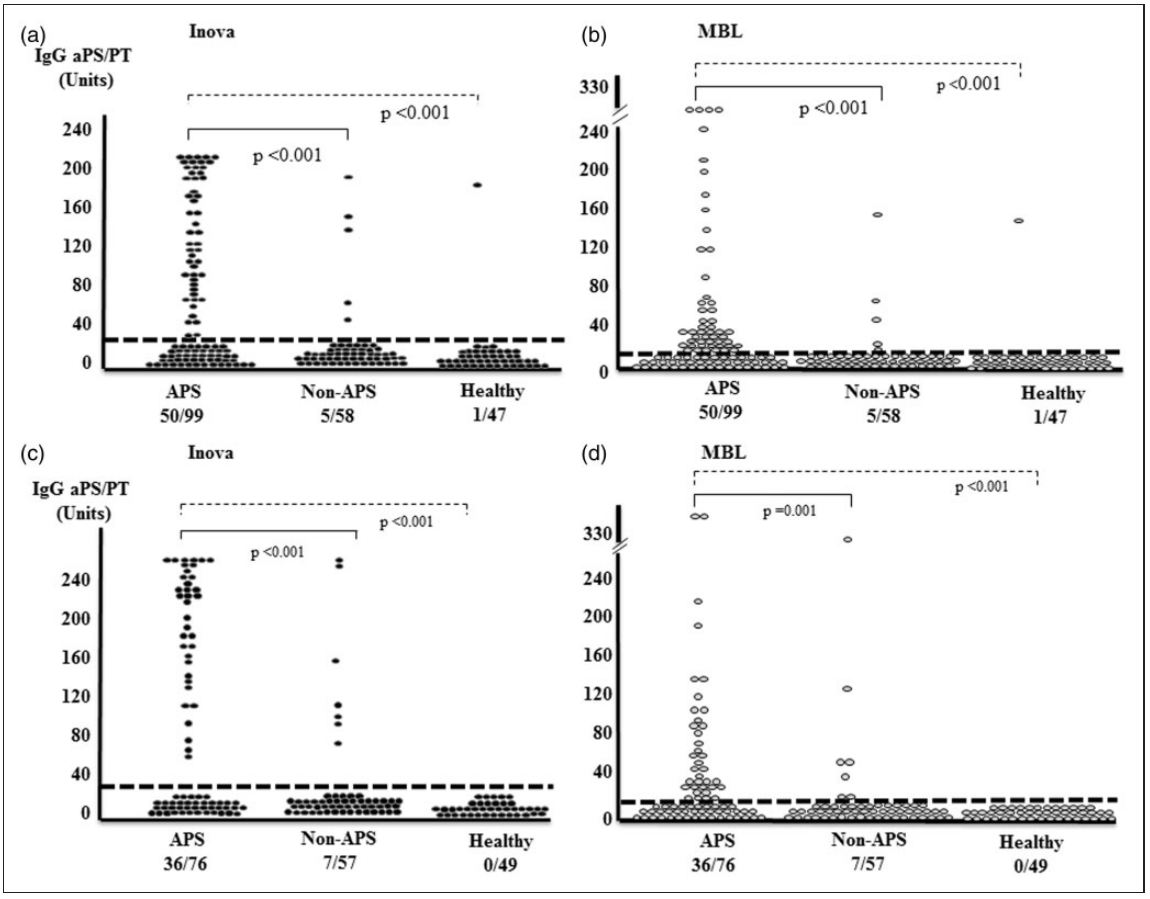

Figure 2 Distribution of IgG aPS/PT titres in samples from the initial and replication cohorts. Distribution of IgG aPS/PT titres in 204 samples with concordant results in the initial study ((a) and (b)) and 180 samples in the replication study ((c) and (d)). Titres of IgG aPS/PT detected by either Inova ((a), (c)) or MBL ((b), (d)) ELISA kits were significantly higher in patients with clinical antiphospholipid syndrome (APS) than in those without APS (non-APS), or in healthy controls. Data are shown as individual results. The dashed line indicates the cut-offs for positivity that were set up at ≥30 units (Inova) and >12 units (MBL).
IgG aPS/PT: positive titres of phosphatidylserine-dependent antiprothrombin antibodies in Inova and MBL assays; ELISA: enzyme-linked immunosorbent assay; MBL: Medical and Biological Laboratories Co.

95% CI (3.8–31.3), both arterial and venous thrombosis OR 8.1, 95% CI (2.5–26.0), and OR 12.7, 95% CI (4.1–40.0), respectively, all $p < 0.001$. In addition, IgG aPS/PT was more frequently found in patients with a history of obstetric APS, OR 10.6, 95% CI (3.5–32.1), $p < 0.001$ (Table 3).

Table 4 shows the aPL profiles of the analysed participants. In this cohort, IgG/M aCL, IgG/M anti-β2GPI and LA were frequently found in patients with clinical APS, with a prevalence of 68%, 55% and 90%, respectively. We evaluated the association between aPL criteria and APS manifestations. LA, but not IgG/M aCL nor IgG/IgM anti-β2GPI, was significantly correlated with a history of thrombosis. On the other hand, IgG/M aCL, IgG/IgM anti-β2GPI and LA significantly correlated with a history of pregnancy complications (data not shown).

Replication study

In order to validate the performance of IgG aPS/PT, we recruited a new sample set of 214 individuals from five new centres in five countries. Each individual site included at least 40 participants (Appendix 1). The new cohort comprised 163 patients and 51 apparently healthy people. Among the patients, the diagnoses were as follows: clinical APS ($n = 96$), systemic autoimmune disease without APS ($n = 45$), and non-systemic autoimmune disease ($n = 22$). Among patients with clinical APS, 55 (57%) had primary APS manifestations and in 41 patients (43%), APS was

Table 3 IgG aPS/PT and clinical manifestations of antiphospholipid syndrome

	Initial study				*Replication study*			
	Patients (n)	*IgG aPS P/T* n *(%)*	*OR 95% (CI)*[a]	p *value*[b]	*Patients* (n)	*IgG aPS P/T* n *(%)*	*OR 95% (CI)**	p *value*[b]
APS group	99	50 (51)			76	50 (47)		
Thrombosis	59	30 (51)	11.0 (3.8–31.3)	< 0.001	49	30 (61)	11.3 (4.2–30.0)	< 0.001
Arterial	30	13 (43)	8.1 (3.5–26.0)	< 0.001	22	14 (64)	12.5 (3.9–40.5)	< 0.001
Venous	33	18 (55)	12.7 (4.1–40.0)	< 0.001	25	16 (64)	12.7 (4.1–40.0)	< 0.001
Pregnancy complications	40	20 (50)	10.6 (3.5–32.1)	< 0.001	27	6 (22)	2.0 (0.6–6.8)	NS
Non-APS group	58	5 (9)			57	7 (12)		

IgG: immunoglobulin G; APS: antiphospholipid syndrome; IgG aPS/PT: positive titres of IgG phosphatidylserine-dependent antiprothrombin antibodies in Inova and MBL assays; OR: odds ratio; CI: confidence interval; MBL: Medical and Biological Laboratories Co.
[a]APS vs non APS. [b]p = Fisher exact test. NS: not statistically significant.

Table 4 Profile of antiphospholipid antibodies

	APS group	*Non-APS group*	*Healthy individuals*	χ^{2a}	p[a]
Sample positive/ Sample tested (%)					
Initial study	($n = 99$)	($n = 58$)	($n = 47$)		
aCL IgG	58/99 (59)	19/58 (33)	0/37 (0)	9.8	0.003
aCL IgM	23/81 (28)	11/58 (19)	0/37 (0)	1.6	NS
aCL IgG/M	65/95 (68)	27/58 (47)	0/37 (0)	7.2	0.01
Anti β2GPI IgG	31/67 (46)	5/37 (14)	0/32 (0)	11.3	0.001
Anti β2GPI IgM	14/66 (21)	6/36 (17)	0/29 (0)	0.3	NS
Anti β2GPI IgG/M	36/66 (55)	11/36 (31)	0/28 (0)	5.4	0.023
Lupus anticoagulant	80/89 (90)	11/41 (27)	0/38 (0)	53.1	<0.001
aPS/PT IgG	50/99 (51)	5/58 (9)	1/47 (2)	28.2	<0.001
Replication study	($n = 76$)	($n = 57$)	($n = 49$)		
aCL IgG	36/69 (60)	5/57 (9)	1/49 (2)	26.8	<0.001
aCL IgM	19/63 (30)	2/55 (4)	0/48 (0)	14.1	<0.001
aCL IgG/M	42/68 (62)	5/56 (9)	1/49 (2)	36.4	<0.001
Anti β2GPI IgG	34/65 (52)	4/56 (7)	0/47 (0)	28.5	<0.001
Anti β2GPI IgM	14/48 (29)	3/43 (7)	1/41 (2)	7.4	0.007
Anti β2GPI IgG/M	37/62 (60)	6/45 (13)	1/41 (2)	23.3	<0.001
Lupus anticoagulant	37/70 (53)	6/56 (11)	0/48 (0)	24.6	<0.001
aPS/PT IgG	36/76 (47)	7/57 (12)	0/49 (0)	18.3	<0.001

aCL: anticardiolipin antibodies; anti-β2GPI: anti-β2-glycoprotein I antibodies; IgG/M: immunoglobulin (Ig)G and/or IgM isotype; APS: antiphospholipid syndrome; aPS/PT: phosphatidylserine dependent antiprothrombin antibodies.
[a]APS patients vs. non-APS patients. p = Fisher exact test; NS: not statistically significant.

diagnosed concomitant with other systemic autoimmune diseases. Fifty-eight patients (60%) had thrombotic APS (with/without obstetric complications), and 37 women had obstetric APS in the absence of thrombotic complications. Seventeen patients had APS-associated manifestations without APS laboratory criteria. Nine patients had obstetric events, five patients had thrombotic events, and in three patients thrombotic and pregnancy complications were reported. The demographic characteristics of the population included in the replication study were similar to those of the initial study (Table 1, Appendix 2).

There was a statistically significant correlation among the OD values of IgG aPS/PT obtained with both ELISA kits (Pearson's correlation coefficient: $r = 0.803$, $p < 0.001$) (Figure 1(b)). A substantial agreement between the IgG aPS/PT Inova and MBL ELISA kits was observed ($k = 0.630$).

IgG aPS/PT were detected in 55% and 41% of patients with clinical APS using Inova and MBL ELISA kits, respectively. The detailed data of

prevalence of IgG aPS/PT in each of the analysed groups is shown in Appendix 3. IgG aPS/PT were detected as the only aPL in one out of 17 patients (6%) with clinical APS without APS laboratory criteria.

A total of 182 samples (85%) displayed concordant IgG aPS/PT results and were subsequently analysed. Discrepant results (15%) are shown in Appendix 4. Among 182 samples with concordant results, 76 were obtained from patients with clinical APS, 57 from non-APS patients and 49 from healthy individuals. IgG aPS/PT were more prevalently found to be positive in patients with clinical APS (47%) than in those without (12%), OR 6.4, 95% CI (2.6–16.0), $p < 0.0001$. Sensitivity, specificity, LR + and LR– of IgG aPS/PT for the diagnosis of APS were 47%, 88%, 3.9 and 0.6, respectively.

Titres of IgG aPS/PT detected by either Inova or MBL ELISA assays were significantly higher in patients with clinical APS than in those without APS ($p < 0.001$ and $p = 0.001$, respectively), or in healthy controls (both $p < 0.001$).

The accuracy of IgG aPS/PT for the diagnosis of APS, as well as for the history of thrombotic and the obstetric manifestations, were assessed by ROC curves and AUC values (Table 2).

The presence of IgG aPS/PT significantly correlated with thrombotic events, both arterial and venous (OR 11.3, 95% CI (4.2–30.0), $p < 0.001$) (Table 3). Conversely, IgG aPS/PT were not found more prevalent in women with pregnancy complications than in those without.

The aPL profile in the replication cohort is shown in Table 4. IgG/M aCL, IgG/IgM anti-β2GPI and LA significantly correlated with a history of thrombosis, but did not correlate with a history of pregnancy complications (data not shown).

Discussion

This multicentre study represents a collaborative effort to assess the value of IgG aPS/PT testing for the diagnosis of APS. The known existence of patients with clinical manifestations suggestive of APS in the absence of aCL, anti-β2GPI or LA has propelled the search for additional diagnostic tests that could contribute to a better identification of APS patients. Negative results using only the available criteria aPL tests for APS evaluation could be related to the inadequate performance of these assays, but also to the presence of autoantibodies with different antigenic specificities. Several studies have reported the presence of aPS/PT in patients with APS, usually in association with other aPL.[30,31] LA correlated with IgG aPS/PT and predicted thrombosis in APS.[5,27,31] However, the clinical relevance of aPS/PT determination in the routine evaluation of patients with suspicion of APS remains to be fully elucidated.

Results from this multicentre study confirmed the high prevalence of IgG aPS/PT in patients with clinical APS in two independent cohorts. Our findings are in concordance with previous reports showing that aPS/PT are autoantibodies frequently found in APS patients.[21,26] Our study evaluated the significance of IgG aPS/PT for the diagnosis of APS in a heterogeneous population of individuals with different clinical backgrounds. There are several assays currently used for aPS/PT testing. To avoid the variability in the aPS/PT results related either to the type of assay or to the assays' performance characteristics, all samples were blindly tested using two different IgG aPS/PT ELISA kits. All samples from the initial study were tested at the same time, and the same methodology was applied later in the replications study.

Previous works have reported the relationship between aPS/PT and arterial and venous thrombosis.[21,32] Some studies support the association of aPS/PT with arterial thrombosis, mainly in the setting of cerebrovascular events.[33] Others reports showed the correlation between aPS/PT and venous thrombosis.[27,31] In our study, in both cohorts arterial and venous thromboses were associated with IgG aPS/PT.

Antibodies to aPS/PT have also been related to pregnancy loss.[5,7,34] We observed an association between IgG aPS/PT and pregnancy complications in the initial study; however, the replication study failed to confirm such an association. The reason for this discrepancy might be related to the heterogeneity of patients collected at the several centres involved in this multicentre study, or might be due to the diversity in the definition of pregnancy complications. In the initial study, a history of obstetric complications was reported in 45% of women. Most of these women fulfilled APS obstetric criteria and were included in the APS group. In contrast, in the replication study, despite the fact that a history of obstetric complications was reported in 38% of females, some of these complications did not fulfil the APS obstetric criteria. Our study comprised a variety of patients with different obstetric complications reflecting the real population managed by clinicians at each site. Considering that we failed to find an association between aCL, anti-β2GPI,

LA and a history of obstetric complications in the replication cohort, aPS/PT would have behaved in a similar fashion.

Several clinical studies have demonstrated that the presence of LA activity is the most significant risk factor for thrombotic events.[35–37] In this study, LA was frequently found in patients with APS manifestations (90% and 53%, initial and replication study, respectively). The elevated percentage of LA in the initial study might be related to the heterogeneity in LA detection, reflecting the variation of the LA results among the laboratories. The presence of IgG aPS/PT was significantly associated with the positivity of LA, in both the initial and replication study, in agreement with previous publications (data not shown).[5,27,31,38] The association between aPS/PT and LA suggested that aPS/PT might be of help to confirm the presence of LA activity and useful in the evaluation of APS. aPS/PT have been reported in patients with thrombotic events in the absence of antibodies to cardiolipin or β2GPI.[39] Hoxha et al.[40] found aPS/PT in 9.4% of patients with clinical features suggestive of APS but negative for the three aPL criteria tests. In the present study, all APS patients from the initial cohort have APS laboratory criteria, precluding evaluation of the potential 'added value' of aPS/PT testing to the diagnosis of APS. In the replication cohort, in patients with clinical APS without positive aPL criteria tests ($n = 17$), IgG aPS/PT were detected in one patient (6%) with thrombosis and pregnancy complications. Our findings are in agreement with previous observations suggesting that, in case of APS suspicion and absence of criterial aPL, testing for aPS/PT might contribute to support the APS diagnosis.

An excellent agreement between the Inova and MBL IgG aPS/PT assays has been already reported.[41] We further showed moderate to substantial agreements between IgG aPS/PT results obtained with the two ELISAs, suggesting that comparable results can be obtained using these two commercial kits.

In our study, IgG aPS/PT showed a high specificity for APS (91% and 88% in the initial and replication cohorts, respectively). Moreover, higher titres of IgG aPS/PT were found in people with suspicion of or definite APS than in the other groups of individuals.

This study confirms, in two large cohorts of participants assembled from multiple institutions, previous observations indicating that IgG aPS/PT are associated with APS, despite the fact that they are not yet included in the APS laboratory criteria for APS classification. Testing for IgG aPS/PT could be useful for identifying patients at risk of developing APS.[26]

This study has some limitations. Samples were defined based on clinical and laboratory grounds. The definition of APS was based on clinicians' judgement. The different criteria used for the inclusion of patients in the APS group might influence the study findings. The number of patients included in the subgroup analysis is lower than what was expected. However, the sample sizes in both cohorts are large enough to indicate that IgG aPS/PT are frequently found in APS patients, and that their detection might contribute to a better identification of APS patients. Additional large prospective studies are needed to define the specific contribution of aPS/PT as a potential marker for APS diagnosis. Another limitation is the retrospective design of the study. Demographic, clinical and laboratory data were obtained from the medical records at each participant centre and some information was not available. Furthermore, results from laboratory investigation at each participant centre were referred using different units, range, and cut-offs precluding the specific analysis.

In conclusion, this international multicentre study using two different acceptable ELISA kits has shown that IgG aPS/PT are autoantibodies frequently found in patients with APS, playing a role as an additional marker of APS for clinical researchers, presumably as well as for clinical practice. Measurement of IgG aPS/PT, in conjunction with aCL, anti-β2GPI and LA, might contribute to a better and more complete identification of patients at risk of thrombotic complications. IgG aPS/PT should be considered as potential additional laboratory criterion for APS classification. Prospective clinical studies will clarify the risk of developing thrombosis and/or pregnancy morbidity related to classical aPL and/or IgG aPS/PT in the population at risk.

Acknowledgements

We thank Dr Yuichiro Fujieda and Dr Toshiyuki Watanabe for their help in the collection of samples and data.

This paper is dedicated to the late Professor Silvia Pierangeli, University of Texas Medical Branch, Galveston, TX, USA.

Declaration of conflicting interests

The authors declared the following potential conflicts of interest with respect to the research,

authorship, and/or publication of this article: Gary L Norman and Zakera Shums are employees of Inova Diagnostics Inc, San Diego, CA, USA. Jiro Arai is an employee of Medical and Biological Laboratories, Co. Ltd, Nagano, Japan. Atsuko Murashima has taken part in speakers' bureaus for Mitsubishi Tanabe Pharma Co., Takeda Pharmaceutical Co. Ltd., and Astellas Pharma Inc. Tatsuya Atsumi has received grant/research support from Chugai Pharmaceutical Co., Ltd., Eisai Co., Ltd., Bristol-Myers Squibb Co., Astellas Pharma Inc, Daiichi Sankyo Co., Ltd. and Mitsubishi Tanabe Pharma Co., and has taken part in speakers' bureaus for Astellas Pharma Inc. and Mitsubishi Tanabe Pharma Co. The other authors have no competing interests to disclose.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by grants from the Japanese Ministry of Health, Labor, and Welfare, and the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

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Appendix 1

	Number of participants
Centres participating in the initial study	
Hokkaido University Hospital, Sapporo, Japan	45
Favaloro University, Buenos Aires, Argentina	23
Nagoya City University Hospital, Nagoya, Japan	30
Fernando Fonseca Hospital, Lisbon, Portugal	38
University Medical Centre Ljubljana, Ljubljana, Slovenia	29
Hospital Universitario Cruces, Bizkaia, Spain	30
King's College, London, UK	20
University of Texas Medical Branch, Galveston, TX, USA	32
Total	247
Centres participating in the replication study	
University of Milan, Milan, Italy	41
National Center for Child Health and Development, Tokyo, Japan	48
Jagiellonian University, Cracow, Poland	41
Hospital Clínic, University of Barcelona, Barcelona, Catalonia, Spain	41
University of Utah and ARUP Laboratories, Salt Lake City, UT, USA	43
Total	214

Appendix 2. Population included in the initial and replication studies

	Initial study n	*Replication study* n
APS group	126 (51%)	96 (45%)
Primary APS	77 (61%)	55 (57%)
APS and systemic autoimmune diseases	49 (39%)	41 (43%)
SLE	49 (100%)	39 (95%)
Systemic sclerosis	–	1 (2%)
Raynaud	–	1 (2%)
Thrombotic APS	75 (60%)	59 (61%)
Obstetric APS	51 (40%)	37 (39%)
Non-APS group	73 (30%)	67 (31%)
Systemic autoimmune disease	42 (58%)	45 (67%)
SLE	18 (43%)	32 (71%)
RA	7 (17%)	2 (4%)
Vasculitis	5 (12%)	2 (4%)
Systemic sclerosis	5 (12%)	2 (4%)
Primary Sjögren's	3 (7%)	4 (9%)
Ankylosing spondylitis	2 (5%)	–
Psoriatic spondylitis	1 (2%)	–
Sharp syndrome	1 (2%)	–
Dermatomyositis	–	1 (2%)
Polymyalgia rheumatica	–	1 (2%)
Mixed connective tissue disease	–	1 (2%)
Other diseases	31 (42%)	22 (33%)
Renal disease	9 (29%)	–
Arterial hypertension	4 (13%)	6 (27%)
Osteoarthritis	4 (13%)	2 (9%)
Asthma	3 (10%)	–
Facial erythema	2 (6%)	–
Bipolar disorders	2 (6%)	–
Infection	1 (3%)	3 (14%)

(continued)

Continued

	Initial study n	*Replication study* n
Gout	1 (3%)	1 (5%)
Others	5 (16%)[a]	10 (45%)[b]
Healthy participants	48 (19%)	51 (24%)

APS: antiphospholipid syndrome; SLE: systemic lupus erythematosus; RA: rheumatoid arthritis; n = number of participants.
[a]Others include: diabetes mellitus ($n=1$), preeclampsia ($n=1$), breast cancer ($n=1$), speech problem ($n=1$), one episode of idiopathic thrombocytopenic purpura ($n=1$). [b]Others include: pain syndrome ($n=1$), schizophrenia ($n=1$), cellulitis ($n=1$), carpal tunnel syndrome ($n=1$), migraine ($n=1$), atrial fibrillation ($n=1$), celiac disease ($n=1$), adrenal insufficiency ($n=1$), thyroiditis ($n=1$), epidermolysis bullosa ($n=1$).

Appendix 3. Prevalence of IgG aPS/PT

	APS group Thrombotic		*APS group* Obstetric			*Non-APS group*			*Healthy*
participants	*AD* *27*	*P* *48*	*AD* *22*	*P* *29*	*All-APS* *126*	*AD* *42*	*Non-AD* *31*	A All non-APS 73	*48*
Study Initial									
Inova *n* (%)	20 (74)	23 (48)	18 (82)	12 (41)	73 (58)	6 (14)	5 (16)	*11 (15)*	2 (4)
3MBL *n* (%)	16 (59)	17 (35)	13 (59)	8 (28)	54 (43)	6 (14)	3 (10)	*9 (12)*	1 (2)
	AD 29	P 30	AD 12	P 25	All APS 96	AD 45	P 22	*All non-APS* *67*	51
Replication									
Inova *n* (%)	24 (83)	15 (50)	5 (42)	9 (36)	53 (55)	13 (29)	3 (14)	16 (24)	0 (0)
MBL *n* (%)	19 (66)	12 (40)	5 (42)	3 (12)	39 (41)	7 (16)	1 (5)	8 (12)	2 (4)

IgG aPS/PT: positive titres of IgG phosphatidylserine-dependent antiprothrombin antibodies in Inova and MBL assays; APS: antiphospholipid syndrome; n = number of participants; AD: autoimmune disease setting; P: antiphospholipid syndrome-associated manifestations in absence of concomitant autoimmune disease; MBL: Medical and Biological Laboratories Co.

Appendix 4. Concordant and discrepant IgG aPS/PT results between Inova and MBL ELISA kits

	Concordant results n *(%)*	*IgG aPS/PT (+) Inova/MBL* n *(%)*	*IgG aPS/PT (−) Inova /MBL* n *(%)*	*Discrepant results* n *(%)*	*IgG aPS/PT Inova (+) MBL (−)* n *(%)*	*IgG aPS/PT MBL (+) Inova (−)* n *(%)*
Initial study						
All population ($n=247$)	204 (83)	56 (23)	148 (60)	43 (17)	30 (12)	13 (5)
APS group ($n=126$)	99 (79)	50 (40)	49 (39)	27 (21)	23 (18)	4 (3)
Non-APS group ($n=73$)	58 (79)	5 (7)	53 (73)	15 (21)	6 (8)	9 (12)
Healthy subjects ($n=48$)	47 (98)	1 (2)	46 (96)	1 (2)	1 (2)	0 (0)
Replication study						
All population ($n=214$)	182 (85)	42 (20)	140 (65)	32 (15)	26 (12)	6 (3)
APS group ($n=96$)	76 (79)	35 (36)	41 (43)	20 (21)	17 (18)	3 (3)
Non-APS group ($n=67$)	57 (85)	7 (11)	50 (75)	10 (15)	9 (13)	1 (1)
Healthy participants ($n=51$)	49 (96)	0 (0)	49 (96)	2 (4)	0 (0)	2 (4)

IgG aPS/PT: positive titres of IgG phosphatidylserine-dependent antiprothrombin antibodies in Inova and MBL assays; APS: antiphospholipid syndrome; MBL: Medical and Biological Laboratories Co.; ELISA: enzyme-linked immunosorbent assay. Cut-offs: Inova IgG aPS/PT ≥ 30 units, MBL IgG aPS/PT > 12 units.