Improving the clinical accuracy in patients with antiphospholipid antibodies using anti-phosphatidylserine/prothrombin and anti-beta2 glycoprotein I domain and particle-based multi-analyte technology

Improving the clinical accuracy in patients with antiphospholipid antibodies using anti-PhosphatidylSerine/Prothrombin and anti-Beta2Glycoprotein I Domain and Particle-based Multi-Analyte Technology

MassimoRadin[1], Silvia GraziettaFoddai[1,2], IreneCecchi[1], ElenaRubini[1], Alice Barinotti[1], Michael Mahler[3], Andrea Seaman[3], Carlos Ramirez[3], Sìlvia Casas[3], Dario Roccatello[1,4] and Savino Sciascia[1,4].

1. Center of Research of Immunopathology and Rare Diseases- Coordinating Center of Piemonte and Valle d'Aosta Network for Rare Diseases, S. Giovanni Bosco Hospital, Department of Clinical and Biological Sciences, University of Turin, Italy.
2. School of Specialization of Clinical Pathology, Department of Clinical and Biological Sciences, University of Turin, Italy.
3. Inova Diagnostics, San Diego, US.
4. Nephrology and Dialysis, Department of Clinical and Biological Sciences, S. Giovanni Bosco Hospital and University of Turin, Italy.

Running Title: Clinical accuracy of aPS/PT and β2GPI-D1 in APS

Corresponding Author: Massimo Radin

Center of Research of Immunopathology and Rare Diseases- Coordinating Center of Piemonte and Valle d'Aosta Network for Rare Diseases, S. Giovanni Bosco Hospital and Department of Clinical and Biological Sciences, University of Turin. Piazza donator di sangue 3, 10154, Turin, Italy.

Email: massimo.radin@unito.it;Tel +390112402056;Fax +390112402052

Key words: Autoantibodies; Autoimmunity;Clinical Laboratory Techniques; risk; Thrombosis

Highlights

1. It is unknown which aPL positive patients will develop APS clinical manifestations

2. aPS/PT and β2GPI- Domain 1 have been proposed to be relevant to APS and its clinical manifestations

3. In this study we tested 122 aPL positive patients do determine the best aPL clinical accuracy.

4. The combination of aPS/PT and β2GPI D1 might increase the diagnostic power of aPL testing

Antiphospholipid syndrome (APS) is the most common acquired thrombophilia, an autoimmune disorder characterized by arterial and/or venous thrombosis and/or pregnancy morbidity in the presence of persistent positivity for antiphospholipid antibodies (aPL) (1). The current classification criteria for APS includes three laboratory tests: lupus anticoagulant (LA), anticardiolipin (aCL), and anti-β2 glycoprotein-I (β2GPI).

Despite advances in the understanding ofthis condition, identifying which aPL positive patients are at higher risk for developing clinical manifestations of APS (thrombotic and/or pregnancy morbidity) is still an unmet clinical need and remains a major challenge in routine clinical practice. Along with cardiovascular risk factors, several studies have suggested that testing for aPL specificities beyond criteria aPL may help to identify patients who are at higher risk of developing APSclinical manifestations, as well as in situations where APS is strongly suspected, but criteria aPL are repeatedly negative or inconclusive (2). Many studies have in fact investigated patients with high clinical suspicion of having APS, but that were negative for criteria aPL, referred as the so-called "seronegative" APS. In recent years, new aPL specificities have been rising, in particular, phosphatidylserine/prothrombin antibodies (aPS/PT) and β2GPI- Domain 1 (β2GPI-D1) have been proposed to be particularly relevanttoAPS and its clinical manifestations, both when considering diagnosis and for risk stratification purposes (3).

The aim of this study was to evaluate the clinical accuracy of aPL specificities, both individually and/or in combination, in a large cohort of aPL positive patients to identify a panel of tests that may provide the best accuracy for clinical manifestations of APS.

We chart-reviewed patients who presented at San Giovanni Bosco Hospital over the past 5 years and tested persistently positive for at least one aPL (more than 2 occasions over a time of more than 12 weeks). The study was performed in compliance with the Declaration of Helsinki. Clinical and laboratory characteristics were retrospectively collected.

We enrolled 122 patients who met one of the following inclusion criteria:

1) Diagnosis of primary APS (pAPS) defined as per Sydney criteria (1): 38 Patients

2) Diagnosis of secondary APS (sAPS) defined as per Sydney criteria (1): 31 Patients

3) Tested persistently positive for aPL (1), with no clinical manifestations of APS (aPL positive asymptomatic): 23 Patients

4) Patients with thrombosis and/or pregnancy morbidity and high suspicion of having APS, but not completely fulfilling the laboratory criteria (1), (suspected APS) for the following reasons: a) inconsistent previous LA positivity; and/or b) fluctuating presence of aPL over the years after the first two positive determinations performed at least 12 weeks apart; and/or c) low-medium titers aPL [defined as levels of aCL IgG/IgM or β2GPI IgG/IgM 10-30 GPL/MPL]: 30 Patients

The aPL profile for inclusion in this study included aCL, LA, and β2GPI antibodies. aPL testing was performed as previously described (4). β2GPI-D1 IgG testing was performed with chemiluminescent immunoassay (QUANTA Flash®, Inova Diagnostics).

A particle-based multi-analyte technology (PMAT) system with a full-automated random accesssystem (Aptiva™, Inova Diagnostics), which allows the digital and simultaneous detection of aPL (extensively: IgG, IgA and IgM isotypes to CL, β2GPI and PS/PT), was usedas described elsewhere (4).

The cumulative GAPSS was calculated for each patient as previously reported by adding together all points corresponding to the score risk factors (5). Briefly, 5 points for aCL (IgG/IgM), 4 points for LA and β2GPI(IgG/IgM), 3 points for aPS/PT (IgG/IgM) and hyperlipidemia and 1 point for arterial hypertension.

Interrater agreement statistics methodology for our data is detailed in the supplementary materials. The significance of baseline differences was determined by the chi-squared test, Fisher's exact test or the unpaired t-test, as appropriate. Comparisons between groups were expressed asodds ratio (OR) with its 95% confidence interval (OR [95% CI]). Areas under the receiveroperating characteristic (ROC) curve (AUC) of different combinations of the five aPL tested and GAPSS score were computed. A two-sided p-value <0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 26.0 (IBM, Armonk, NY, USA).

A total of 122 patients were recruited and Table 1 summaries their clinical and other laboratory characteristics.

First, aPL positivity, without isotype distinction, were analyzed separately to determine the overall accuracy for clinical manifestations of APS (either thrombosis or pregnancy morbidity). The accuracy for clinical manifestations of APS for each aPL positivity when singularly analyzed were as follows: β2GPI-D1 (IgG) [AUC 0.64, 95% CI0.54–0.74]; aPS/PT (IgG/IgM) [AUC 0.62, 95%CI 0.52–0.73]; LA [AUC 0.61, 95%CI 0.50–0.72]; β2GPI (IgG/IgM) [AUC 0.58, 95%CI 0.47–0.69]; aCL (IgG/IgM) [AUC 0.56,95%CI 0.46–0.67].

Second, we analyzed the different aPL combinations based on the 5 tests as described in Table 1. The positivity for aPS/PT (IgG/IgM) and/or β2GPI-D1 (IgG) showed the best accuracy in predicting the clinical manifestations of APS [AUC 0.70, 95%CI0.60–0.79].

When comparing the different aPL combinations with the clinal risk assessment, as expressed by GAPSS, we found thatthe highest titers of aPS/PT (IgG and/or IgM) and/or β2GPI-D1 (IgG) were associated with higher levels of GAPSS (p>0.001). Patients with positivity for aPS/PT (IgG/IgM) and/or β2GPI-D1 (IgG) had significantly higher levels of GAPSS when compared to

the negative patients (mean GAPSS 14.4±5.2 vs. 8.3±5.4, respectively, p<0.001). The clinical accuracy for APS of the others combinations were as follows: triple criteria aPL (IgG) and β2GPI-D1 (IgG)[AUC 0.65, 95%CI 0.55–0.74], triple criteria aPL (IgG) [AUC 0.63, 95% CI 0.53–0.74], triple criteria aPL (IgG) and aPS/PT (IgG/M) [AUC 0.61, 95%CI 0.51–0.71].

Figure 1 illustrates the ROC curves of the clinical manifestations of APS and the various aPL combinations.

Third, analysis of different aPL profiles to identify the best combination for clinical recurrent events of APS, showed that triple IgG positivity for criteria aPL [AUC 0.72, 95% CI 0.57– 0.87] and positivity for aPS/PT (IgG/IgM) and/or β2GPI-D1 (IgG) [AUC 0.71, 95% CI 0.57 – 0.85] had the best performances.

When combining aPL positivity and cardiovascular risk factors, GAPSS score >14 had the best association with clinical recurrent events of APS (AUC 0.74, CI95% 0.61–0.88).

Examination of aPL asymptomatic patients and APS patients (both pAPS and sAPS), revealed that aPS/PT (IgG/IgM) and/or β2GPI-D1 (IgG) combined was the only combination statistically associated with the presence of clinical manifestations [OR 4.9, 95%CI 1.8-13.4, p <0.05] and GAPSS score>11 [OR 2.9, 95%CI 1.9-7.6, p<0.05].

Similarly, we found that a GAPSS score>11 was statistically observed more frequently in patients with clinical manifestations of APS [OR 2.9, 95%CI1.9-7.6, p<0.05]. Interestingly, when adding the patients with *suspected* APS to the analysis, aPS/PT (IgG/IgM) and/or β2GPI-D1 (IgG) combined was confirmed to be the only combinationstatistically associated with clinical manifestations [OR 2.5, 95%CI1.0-7.1 p<0.05].

When attending a subject found to be persistently positive for aPL, as often happens in patients with connective tissue diseases, the clinical question relies on the following "*Is that specific patient going to develop a thrombosis or not*?"

Therefore, improving the accuracy in identifying patients at increased risk of developing clinical manifestations in a cohort of aPL positive subjects still requires some investigation (6,7).

To date, the aPL profiling represents the most accurate risk stratification tool available for the treating clinician. Pioneering the field, Pengo *et al.* found that so-called triple aPL positivity (concomitant positivity for LA, aCL and β2GPI) was associated with a higher risk of thrombosis in APS (8). Recently, the GAPSS score, which combines aPL positivity and traditional cardiovascular risk factors, has been demonstrated to be a reliable tool for risk stratification (9).Recent studies have shown an emerging role for risk stratification in APS patients by inclusion of non-criteria aPL, with aPS/PT and β2GPI-D1 antibodies as the most promising non-criteria aPL (3,4).

Our results are in line with those reported by Nakamura et al (10). In fact, when cross-sectionally analyzing 157 patients (51 patients with APS and 106 with non-APS autoimmune diseases), they found that the combination of aPS/PT and β2GPI-D1 tests showed a high positive predictive value for the diagnosis of APS. Interestingly, the Japanese group concluded that the use of this combination as the first-line test for aPL has the potential to contribute to the simple and definite identification of APS with a high risk of thrombosis in clinical practice.

Building on these earlier examinations, the present study confirms the association of IgG/IgM aPS/PT and/or β2GPI-D1 positivity with the clinical manifestations of the syndrome and enrich the current debate suggesting that testing for both these tests might improve our ability of identifying patients at higher risk among all patients tested positive for any aPL.

Further, when compared to immune-enzymatic assays, PMAT technology offers a practical approachto implement a multi-level testing approach for aPL, and facilitating a personalized medicine approach to managing patients suspectedof APS. For instance, it is not uncommon in clinical practice to attend patients clinically suspected of APS whose aPL testing is

inconclusive (e.g.low titer aPL or fluctuating levels of aPL, LA equivocal or unreliable). Extending the aPL panel to aPS/PT and β2GPI-D1might help in providing further guidance for the management of these patients. This approach would be in line with our previous multicenter study, suggesting that aPS/PT might be a valid alternative when LA is not available or not reliable (2).

The limitations of the study include the retrospective nature of the study andthe heterogeneous previous medical history of APS patients. Further, in our analysis of binary outcomes for the different aPL combinations, we used the ROC curve to show the performance of our model. While this approach is heavily used and allowed to incorporate into the model also LA (whose outcome is intrinsically binary in nature -positive or negative) one could not exclude that use of aPL titers as continues variables would enrich future studies.

In conclusion, this study suggests a path for future new perspectives in aPL testing. The combination of aPS/PT and β2GPI D1 might increase the diagnostic power of aPL testing and allow improved risk stratification in patients with aPL positivity.

References

1. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006;4:295–306.

2. Sciascia S, Radin M, Cecchi I, Rubini E, Scotta A, Rolla R, et al. Reliability of Lupus Anticoagulant and Anti-phosphatidylserine/prothrombin Autoantibodies in Antiphospholipid Syndrome: A Multicenter Study. *Front Immunol* 2019;10:376.

3. Radin M, Cecchi I, Roccatello D, Meroni PL, Sciascia S. Prevalence and Thrombotic Risk Assessment of Anti-β<inf>2</inf> Glycoprotein I Domain I Antibodies: A Systematic Review. *Semin Thromb Hemost* 2017.

4. Sciascia S, Radin M, Ramirez C, at al, Evaluation of novel assays for the detection of autoantibodies in antiphospholipid syndrome, Autoimmunity Reviews, 2020, in press].

5. Sciascia S, Sanna G, Murru V, Roccatello D, Khamashta MA, Bertolaccini ML. The global anti-phospholipid syndrome score in primary APS. *Rheumatology (Oxford)* 2015;54:134–8.

6. Cervera R, Piette J-C, Font J, Khamashta MA, Shoenfeld Y, Camps MT, et al. Antiphospholipid syndrome: clinical and immunologic manifestations and patterns of disease expression in a cohort of 1,000 patients. *Arthritis Rheum* 2002;46:1019–27.

7. Otomo K, Atsumi T, Amengual O, Fujieda Y, Kato M, Oku K, et al. Efficacy of the antiphospholipid score for the diagnosis of antiphospholipid syndrome and its predictive value for thrombotic events. *Arthritis Rheum* 2012;64:504–12.

8. Pengo V, Ruffatti A, Legnani C, Gresele P, Barcellona D, Erba N, et al. Clinical course of high-risk patients diagnosed with antiphospholipid syndrome. *J Thromb Haemost* 2010;8:237–42.

9. Sciascia S, Radin M, Sanna G, Cecchi I, Roccatello D, Bertolaccini ML. Clinical utility of the global anti-phospholipid syndrome score for risk stratification: A pooled analysis. *Rheumatol (United Kingdom)* 2018;57.

10. Nakamura H, Oku K, Amengual O, Ohmura K, Fujieda Y, Kato M, et al. First-Line, Non-Criterial Antiphospholipid Antibody Testing for the Diagnosis of Antiphospholipid Syndrome in Clinical Practice: A Combination of Anti–β2-Glycoprotein I Domain I and Anti–Phosphatidylserine/Prothrombin Complex Antibodies Tests. *Arthritis Care Res* 2018;70:627–634. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28686816. Accessed April 1, 2020.

Authors' contribution

MR, SGF, IC, ER, AB, MM, AS, CR, SC: Significantly contributed to laboratory testing, patients recruitment, drafting of the manuscript and of figures and tables, critical analysis of the final draft of the manuscript.

DR and SS: Significantly contributed to the manuscript preparation, critical analysis of the figures and tables and final drafting of the manuscript.

Acknowledgments: MR, SGF, IC, ER, AB, MM, AS, CR, SC,DR and SS declare no acknowledgments

Disclosure of Conflicts of Interest:MR, SGF, IC, ER, AB, MM, AS, CR, SC,DR and SS declare no conflict of interest

Funding: SGF, IC, ER, AB, MM, AS, CR, SC,DR and SS declare no funding. MR is funded by a grant from the Italian Ministry of Health SG-2018-12368028.

Legend of Figures and Tables

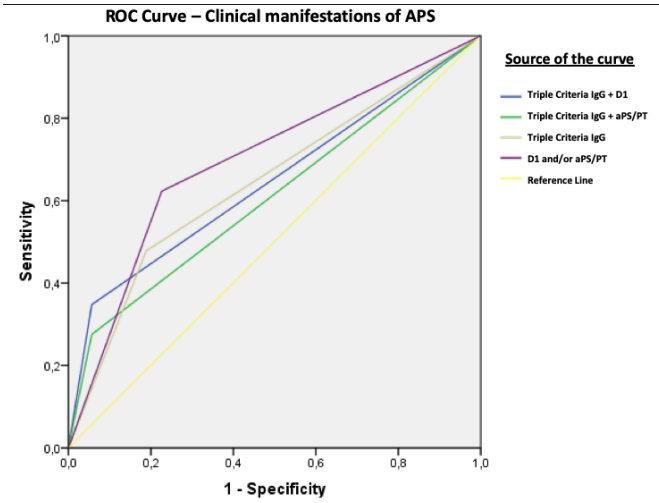
Table 1. Main clinical and laboratory characteristics of the patients included in the study.

Figure 1. ROC curves of the clinical manifestations of APS and the various aPL combinations

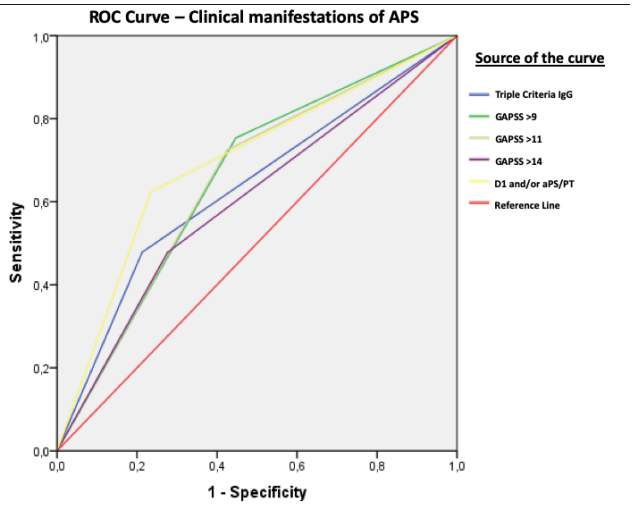
Table 1. Main clinical and laboratory characteristics of the patients included in the study.

	pAPS (n=38)	sAPS (n=31)	aPL+ (n=23)	Suspected APS (n=30)
Demographics				
Age at study inclusion; years (±SD)	50.2 (±13.7)	49.3 (±12.3)	48.8 (±12.8)	50.6 (±11.3)
Females; n, (%)	24 (63)	16 (52)	17 (74)	23 (77)
Clinical Characteristics				
Thrombosis; n, (%)	31 (82)	30 (97)	0	26 (87)
Arterial; n, (%)	21 (55)	16 (52)	0	14 (47)
Venous; n, (%)	15 (39)	16 (52)	0	13 (43)
Pregnancy Morbidity*; n, (%)	8 (21)	3 (10)	0	5 (17)
Recurrences of APS clinical manifestations; n, (%)	7 (18)	5 (16)	0	2 (7)
Laboratory Profile				
LA; n, (%)	32 (84)	26 (84)	21 (91)	16 (53)
aCL (IgG/M); n, (%)	25 (66)	22 (71)	15 (65)	14** (47)
β2GPI (IgG/M); n, (%)	26 (68)	23 (74)	15 (65)	14** (47)
Triple aPL (IgG/M); n, (%)	23 (61)	19 (61)	13 (57)	4** (13)
Triple aPL (IgG); n, (%)	17 (45)	16 (52)	10 (44)	0**
aPS/PT (IgG/M); n, (%)	24 (63)	20 (65)	15 (65)	6 (20)
aβ2GPI-D1; n, (%)	13 (34)	15 (48)	3 (13)	4 (13)
Triple aPL and aPS/PT (IgG/M); n, (%)	16 (42)	14 (45)	10 (44)	0**
Triple aPL and aβ2GPI-D1; n, (%)	12 (32)	12 (39)	3 (13)	0**
aPS/PT (IgG) and aβ2GPI-D1; n, (%)	6 (16)	10 (32)	1 (4)	1 (3)
aPS/PT (IgG) and/or aβ2GPI-D1; n, (%)	22 (58)	24 (77)	7 (30)	6 (20)
Cardiovascular Risk Factors				
Hypertension; n, (%)	15 (39)	14 (45)	5 (22)	9 (30)
Hyperlipidemia; n, (%)	14 (37)	11 (35)	2 (9)	7 (23)
Smoking; n, (%)	4 (11)	7 (23)	2 (9)	4 (13)
Diabetes; n, (%)	4 (11)	2 (6)	1 (4)	1 (3)
GAPSS; value (±SD)	13.8 (±6)	14 (±5.5)	12.6 (±5.3)	7.3 (±4.8)
GAPSS >9; n, (%)	30 (79)	22 (71)	14 (61)	7 (23)
GAPSS >11; n, (%)	28 (74)	22 (71)	14 (61)	6 (20)
GAPSS >14; n, (%)	17 (44)	16 (52)	11 (48)	2 (7)

APS – Antiphospholipid Syndrome; pAPS- Primary APS; sAPS – Secondary APS; aPL – antiphospholipid antibodies; LA – lupus anticoagulant; aCL – anticardiolpin antibodies; aβ2GPI – anti-βeta2Glycopritein 1; aβ2GPI-D1 – aβ2GPI Domain 1; GAPSS – Global APS Score;aPS/PT – anti-Phosphatidylserine/Prothrombin antibodies; Ig - Immunoglobulin.



Test Result Variable(s)	AUC
Triple Criteria IgG + D1	,646
Triple Criteria IgG + aPS/PT	,609
Triple Criteria IgG	,633
D1 and/or aPS/PT	,698



Test Result Variable(s)	AUC
Triple Criteria IgG	,633
GAPSS >9	,653
GAPSS >11	,650
GAPSS >14	,601
D1 and/or aPS/PT	,698

Figure 1. Receiver operating characteristic (ROC) curves of the clinical manifestations of APS and the various aPL combinations.

*aPS/PT refers to positivity for IgG and/or IgM

APS – Antiphospholipid Syndrome; aPL -antiphospholipid antibodies; D1 - Domain 1; GAPSS – Global APS Score; aPS/PT –anti-Phosphatidylserine/Prothrombin antibodies; Ig – Immunoglobulin; AUC – Area under the curve