PLOS ONE

RESEARCH ARTICLE

Measuring IgA Anti-β2-Glycoprotein I and IgG/IgA Anti-Domain I Antibodies Adds Value to Current Serological Assays for the Antiphospholipid Syndrome

Charis Pericleous[1]*, Isabel Ferreira[1], Orietta Borghi[2], Francesca Pregnolato[2], Thomas McDonnell[1], Acely Garza-Garcia[3], Paul Driscoll[3], Silvia Pierangeli[4], David Isenberg[1], Yiannis Ioannou[1,5], Ian Giles[1], Pier Luigi Meroni[2], Anisur Rahman[1]

1 Centre for Rheumatology Research, Division of Medicine, University College London, London, United Kingdom, **2** Laboratory of Immuno-rheumatology, IRCCS Istituto Auxologico Italiano, Milan, Italy, **3** Structural Biology, MRC National Institute for Medical Research, London, United Kingdom, **4** Division of Rheumatology, Department of Internal Medicine, University of Texas Medical Branch, Galveston, Texas, United States of America, **5** Arthritis Research UK Centre for Adolescent Rheumatology, UCL Hospital and Great Ormond Street Hospital, London, United Kingdom

* c.pericleous@ucl.ac.uk

OPEN ACCESS

Citation: Pericleous C, Ferreira I, Borghi O, Pregnolato F, McDonnell T, Garza-Garcia A, et al. (2016) Measuring IgA Anti-β2-Glycoprotein I and IgG/IgA Anti-Domain I Antibodies Adds Value to Current Serological Assays for the Antiphospholipid Syndrome. PLoS ONE 11(6): e0156407. doi:10.1371/journal.pone.0156407

Editor: Masataka Kuwana, Nippon Medical School Graduate School of Medicine, JAPAN

Received: February 2, 2016

Accepted: May 14, 2016

Published: June 2, 2016

Data Availability Statement: The corresponding and senior author may be contacted at c.pericleous@ucl.ac.uk or anisur.rahman@ucl.ac.uk. We have consulted our local Ethics Board on the issue of uploading data to a public repository. Their position is that data can only be stored in the way specified in our original ethics application in 2012. That application does not allow for placing any data in a public repository so we are not able to do that. We are happy to share all anonymised data from this paper on request. Each such request would be

Abstract

Introduction

Currently available clinical assays to detect antiphospholipid antibodies (aPL) test for IgG and IgM antibodies to cardiolipin (aCL) and β_2-glycoprotein I (aβ_2GPI). It has been suggested that testing for IgA aPL and for antibodies to Domain I (DI), which carries the key antigenic epitopes of β_2GPI, could add value to these current tests. We performed an observational, multicenter cohort study to evaluate the utility of IgG, IgM and IgA assays to each of CL, β_2GPI and DI in APS.

Methods

Serum from 230 patients with APS (n = 111), SLE but not APS (n = 119), and 200 healthy controls were tested for IgG, IgM and IgA aCL, aβ_2GPI and aDI activity. Patients with APS were further classified into thrombotic or obstetric APS. Logistic regression and receiver operator characteristic analyses were employed to compare results from the nine different assays.

Results

All assays displayed good specificity for APS; IgG aCL and IgG aβ_2GPI assays however, had the highest sensitivity. Testing positive for IgA aβ_2GPI resulted in a higher hazard ratio for APS compared to IgM aβ_2GPI. Positive IgG, IgM or IgA aDI were all associated with APS, and in subjects positive for aCL and/or aβ_2GPI, the presence of aDI raised the hazard

considered on an individual basis and some may require us to consult the ethics committee.

Funding: This work was supported by the following sources of funding: 1) Arthritis Research UK: Grant number 19423 - recipients AR, IG, YI, DI; 2) Arthritis Research UK: Grant number 20164 - recipient YI (www.arthritisresearchuk.org/); 3) University College London Hospitals National Institute for Health Research Biomedical Research Centre - support ethically approved work using human samples (www.uclhospitals.brc.nihr.ac.uk/); and 4) Medical Research Council: Grant number U117574559 - recipients AG, PD (www.mrc.ac.uk). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

ratio for APS by 3–5 fold. IgG aCL, aβ_2GPI, aDI and IgA aDI were associated with thrombotic but not obstetric complications in patients with APS.

Conclusion

Measuring IgG aDI and IgA aβ_2GPI and aDI may be useful in the management of patients with APS, particularly thrombotic APS.

Introduction

In clinical practice three tests are used to detect antiphospholipid antibodies (aPL), the serological hallmark of antiphospholipid syndrome (APS), a condition characterised particularly by vascular thrombosis (VT) and pregnancy morbidity (PM) [1]. Two of these tests are enzyme-linked immunosorbent assays (ELISAs) that measure anti-cardiolipin (CL, aCL) and anti-β_2-glycoprotein I (aβ_2GPI) aPL; the third is a functional clotting assay for lupus anticoagulant (LA). The ELISAs measure IgG and IgM aPL, while the LA test does not discriminate between antibody isotypes [1]. New, additional laboratory tests for APS may have benefits of easier standardisation and better prognostic value in asymptomatic aPL carriers, or for determining risk of recurrence of VT and/or PM in patients already diagnosed with APS. Proposed new tests include assays that measure IgA aPL and autoantibodies against domain I of β_2GPI (DI) [2, 3].

In comparison to IgG and IgM aPL, IgA aPL have been less-studied and are not included in standard serological tests for APS. Both IgA aCL and IgA aβ_2GPI have yet to be proven specific for APS, as they are also reported to be elevated in patients with systemic lupus erythematosus (SLE) (with or without APS). However, isolated positivity for IgA aβ_2GPI (in patients negative for IgG/IgM aCL/aβ_2GPI and LA) is associated with both VT and PM [4] and IgA aβ_2GPI have been shown to be prothrombotic *in vivo* [5].

The antibodies for which there is clearest evidence of a causal link to development of both thrombotic and obstetric complications in APS are IgG antibodies that can be detected either by binding to CL in the presence of β_2GPI (IgG aCL) or by binding to β_2GPI itself (IgG aβ_2GPI) [6–9]. β_2GPI, a 50kDa plasma glycoprotein of five domains (DI-DV), circulates primarily in a biochemically reduced state [10] in which DI interacts with DV to form a closed circular β_2GPI structure. Upon binding to anionic phospholipids on cell membranes via DV, β_2GPI changes conformation to an open fishhook structure, exposing DI [11, 12]. Antibodies directed against all individual domains of β_2GPI have been reported, of which IgG anti-DI antibodies (aDI) are most closely linked to the presence of APS [13–15]. IgG aDI titres are elevated in patients with APS compared to disease and healthy controls [16–22], and both affinity-purified IgG aDI from APS serum [23] and a human monoclonal IgG aPL that binds DI (IS4) [24] are prothrombotic *in vivo* [25, 26]. In the same mouse model, recombinant human DI abrogates aPL-induced thrombosis [27]. In two different *in vivo* models, a human monoclonal IgG aDI increases thrombosis and pregnancy loss [28]. Moreover, mice immunised with human or murine β_2GPI in the presence of CL vesicles, or with human DI, develop aβ_2GPI and aDI; whilst immunisation with human DII-V or β_2GPI alone does not induce production of these antibodies [29]. In light of these studies, there is increasing interest in validating assays to measure IgG aDI reliably and to assess their importance in APS.

The significance, if any, of IgM aPL against any of the five β_2GPI domains is unclear—de Laat and colleagues reported that IgM aDI were no better associated with VT than IgM aβ_2GPI [19]. For IgA aPL, two studies detected IgA aPL against DIV-V in over 50% of patients with

IgA aβ_2GPI [5, 30, 31], while the importance of IgA aDI to the pathogenesis of APS is unknown. One study used β_2GPI domain-deleted mutants to inhibit IgA aPL from binding to β_2GPI—only mutants containing DIV-V had inhibitory ability, while deletion of DI did not affect binding [32].

Given that both IgA aPL and IgG aDI are considered attractive candidates for new diagnostic tests in APS we designed this study to assess and compare the strength of association of circulating 'classical' IgG and IgM aCL and aβ_2GPI, IgA aCL and aβ_2GPI, and IgG, IgM and IgA aDI with APS and APS-related clinical manifestations.

Patients and Methods

Patients and controls

Sera from (n = 111) patients with APS—as defined by revised Sapporo criteria [1], (n = 119) patients with systemic lupus erythematosus (SLE) with or without aPL (fulfilling ACR SLE criteria [33, 34]) in the absence of APS—were collected by informed consent from institutions in UK, Italy and USA involved in this study. Ethical approval for this study was granted by the London-Hampstead Research Ethics Committee (reference 12/LO/0373). Sera from (n = 200) healthy controls (HC) were obtained as part of the Health Survey for England (HSE) 2006 [35] and provided to us by the Health and Social Care Information Centre together with anonymised data on age, gender, ethnicity and confirmation that they had no long-term illness or history of cardiovascular disease. Demographics for APS, SLE and HC subjects are listed in Table 1.

The clinical history of patients with APS (n = 111), summarised in Table 1, was recorded in accordance with APS classification criteria [1]. The majority (n = 70, 63%) had a history of VT. Of 93 women with APS, 61 had a history of PM and 20 of those had also suffered at least one thrombotic episode. Three patients had catastrophic APS (two female, one male). LA was measured at each patient's home institution clinical laboratory. Treatments at the time of sampling were recorded for patients with APS and SLE (Table 1).

Direct binding assays to detect aPL

For all assays, half of a 96-well plate was coated with antigen while the other half was treated with buffer alone. Net OD was obtained by subtracting the OD of the non-coated half from the OD of the antigen-coated half. The following anti-human horseradish peroxidase conjugates were used: IgG—A6029, Sigma UK; IgM—A6907, Sigma UK; IgA—ab97215, Abcam UK.

Sera were tested in duplicate at 1:50 dilution in the first instance; sera with activity above the highest calibrator were further diluted to determine exact activity. In all assays, activity of the highest calibrator corresponded to a net OD of 1.2–1.5. Inter- and intra-plate variations were $<$10% for all nine assays.

Detecting IgG, IgM and IgA aCL

We measured aCL as per consensus criteria protocols [36] and as previously described [37] using commercially sourced calibrators (Louisville APL Diagnostics, TX, USA). Activity was defined as IgG/IgM/IgA phospholipid units (GPLU/MPLU/APLU respectively). Serum activity was calculated as per manufacturer's instructions. The calibrators' activity ranges were 16-96GPLU; 16-96MPLU; and 2.7-120APLU.

Table 1. Demographic and clinical profile of subjects.

	APS	SLE	HC
No. of subjects (F:M)	111 (93:18)	119 (107:12)	200 (106:94)
Mean age (SD)	44.0 (10.9)	37.9 (13.0)*	44.1 (13.0)
Ethnicity[†]	3A; 4A/C; 101C; 2H; 1M	25A; 23A/C; 68C; 3M	23A; 13A/C; 164C
***Clinical manifestations*:**			
Thrombosis[‡]	70	-	-
Arterial[§]	46	-	-
Venous[§]	34	-	-
Pregnancy morbidity [‡]	61	-	-
≤10 weeks' gestation[∞]	27	-	-
>10 weeks' gestation[∞]	50	-	-
SLE-associated APS	26	-	-
Catastrophic APS	3	-	-
Lupus anticoagulant	87	28	-
***Treatments*:**			
Oral anticoagulants	55	1	-
Heparin	17	-	-
Antiplatelet agents	52	3	-
DMARDs	34	109	-
Oral steroids ≤5mg/day	15	40	-
Oral steroids >5mg/day	1	39	-
Rituximab	3	25	-

* SLE group was significantly younger compared to both APS and HC ($p<0.05$).

[†] A, Asian; A/C, African-Caribbean; C, Caucasian; H, Hispanic; M, Mixed.

[‡] 20 patients have a history of both thrombosis and pregnancy morbidity.

[§] 10 patients have a history of both arterial and venous thrombotic events.

[∞]16 patients have a history of both early and late stage pregnancy morbidity.

doi:10.1371/journal.pone.0156407.t001

Detecting IgG, IgM and IgA aβ_2GPI and aDI

We measured aβ_2GPI activity as previously described [37]. aDI were measured in the same manner; instead of human β_2GPI, plates were coated with human recombinant DI, expressed in-house in bacteria and refolded to adopt its physiological conformation [24, 38].

In-house calibrators were used for aβ_2GPI and aDI assays. For IgG assays, affinity purified IgG aDI isolated from the serum of a patient with APS was used. For IgM and IgA assays, serum from a different patient with high IgM and IgA aβ_2GPI & aDI activity was used. All calibrators were serially diluted to obtain a standard curve, and arbitrary activity units were assigned to each point. aβ_2GPI and aDI activity were defined as IgG/IgM/IgA β_2GPI units (GBU/MBU/ABU respectively) and DI units (GDIU/MDIU/ADIU respectively), and calculated as per aCL assays. For aβ_2GPI assays, calibrators' activity ranges were 3-100GBU; 13-100MBU; and 7-100ABU. For aDI, the ranges were 3-100GDIU; 9-100MDIU; and 2-100ADIU.

Statistical analysis

Logistic regression analysis was employed to determine possible associations between aPL titres and APS (within the entire cohort, n = 430). As we did not test for LA ourselves, we only had

robust LA data for APS but not SLE or HC subjects, and thus were unable to determine the strength of association between LA and APS in our cohort.

We additionally determined possible associations between aPL titres and: 'primary' versus 'secondary' APS; thrombotic versus obstetric APS; LA positivity (within the APS cohort, n = 111, excluding male patients where necessary). *P* values determined significant positive or negative associations. Hazard ratios (HR), or odds ratios, and 95% confidence intervals (95% CI) are reported. A significant association is determined when the 95%CI range excludes 1.0, where values >1.0 denote a positive association.

Receiver operating characteristic (ROC) analysis, performed to assess the discriminatory ability of each aPL test for APS, generated values for: accuracy (area under the curve, where a value of 1 represents a perfect test without false negatives or false positives); specificity (where 100% suggests no false positives); sensitivity (where 100% suggests no false negatives), and positive likelihood ratios (which reflects the proportion of patients who have APS and test positive to the proportion of patients who do not have APS but also test positive).

We performed logistic regression and ROC analyses using Stata10. Correlation tests (to compare different aPL titres of the same isotype), one-way ANOVA and Fisher's exact tests (to compare age and gender in APS, SLE and HC) were performed in GraphPad Prism 5.

Results

IgG aPL are present in a higher proportion of patients with APS than IgM or IgA antibodies and only IgG antibodies are associated with LA positivity

aPL positivity was defined as titres >99th percentile of the mean activity of our HC cohort. Cut-offs for positivity were determined to be: 17GPLU; 8GBU; 10GDIU for IgG aCL, aβ_2GPI and aDI respectively, 17MPLU; 16MBU; 21MDIU for IgM aCL, aβ_2GPI and aDI respectively, and 4APLU; 9ABU; 8ADIU for IgA aCL, aβ_2GPI and aDI respectively. Mean aPL activity for APS, SLE and HC, and the percentage of subjects from each of these groups that tested positive in each assay, are listed in Table 2. Results from individual subjects for all nine assays are graphically shown in Fig 1.

The ideal diagnostic test would be one in which a large proportion of APS and, crucially, only a minority of non-APS subjects would test positive. Comparing the three isotypes, our results show that the IgG aPL assays came closest to approaching this ideal. In patients with APS, positivity for IgG aPL was the most frequent (percentage positivity range 41–74%) and of higher mean titres than IgM or IgA aPL. Interestingly, in patients with APS, IgA aPL were more often positive (38–46%) than IgM aPL (27–35%) (Table 2, Fig 1).

IgG aCL were more prevalent in APS compared to aβ_2GPI and aDI. Importantly however, IgG aCL were also found in 30% of the SLE group (as previously reported [39]), as were IgA aCL. In fact, for all three isotypes, aβ_2GPI assays showed the best discrimination between APS and non-APS—no more than 8% of SLE or HC tested positive for aβ_2GPI of either of the three isotypes. Similarly, aDI were detected in very few SLE or HC (Table 2).

Looking at the group of 111 subjects with APS alone, being positive for IgG aCL was associated with increased likelihood of being LA positive (hazard ratio (HR) 1.9, 95%CI 1.1–3.3, p = 0.017) and this was also true for IgG aβ_2GPI (HR 1.8, 95%CI 1.1–3.1, p = 0.002) and IgG aDI (HR 2.2, 95%CI 1.1–4.5, p = 0.035). Conversely, positivity for IgM or IgA antibodies to any of these three antigens was not associated with LA (data not shown).

There were no significant differences in any of the nine assays tested between patients with APS but no other autoimmune disease ('primary' APS) and patients with SLE-associated ('secondary') APS. aPL titres were not associated with age or gender (data not shown).

Table 2. aPL activity and percentage of positivity in APS, SLE and HC groups.

	aCL		$a\beta_2$GPI		aDI	
	Mean titre (SD)	% positive	Mean titre (SD)	% positive	Mean titre (SD)	% positive
***IgG aPL*:**						
APS (n = 111)	55.8 (36.5)	74	35.4 (36.6)	65	22.8 (30.9)	41
SLE (n = 119)	17.2 (17.6)	30	6.2 (4.1)	8	6.9 (5.9)	11
HC (n = 200)	11.9 (1.7)	1	4.6 (1.1)	4	6.0 (1.4)	1
***IgM aPL*:**						
APS (n = 111)	18.9 (21.8)	27	28.3 (29.5)	33	25.5 (25.2)	35
SLE (n = 119)	11.6 (13.4)	7	14.9 (16.6)	6	11.0 (11.7)	8
HC (n = 200)	7.9 (2.8)	1	10.3 (1.8)	1	12.4 (4.5)	5
***IgA aPL*:**						
APS (n = 111)	17.1 (34.7)	38	16.2 (23.4)	46	12.5 (21.2)	41
SLE (n = 119)	5.9 (16.0)	30	7.9 (7.3)	8	6.4 (16.0)	7
HC (n = 200)	1.7 (0.7)	3	6.4 (0.9)	1	4.0 (1.3)	2

Mean aPL titres (standard deviation) for all APS, SLE and HC subjects.

doi:10.1371/journal.pone.0156407.t002

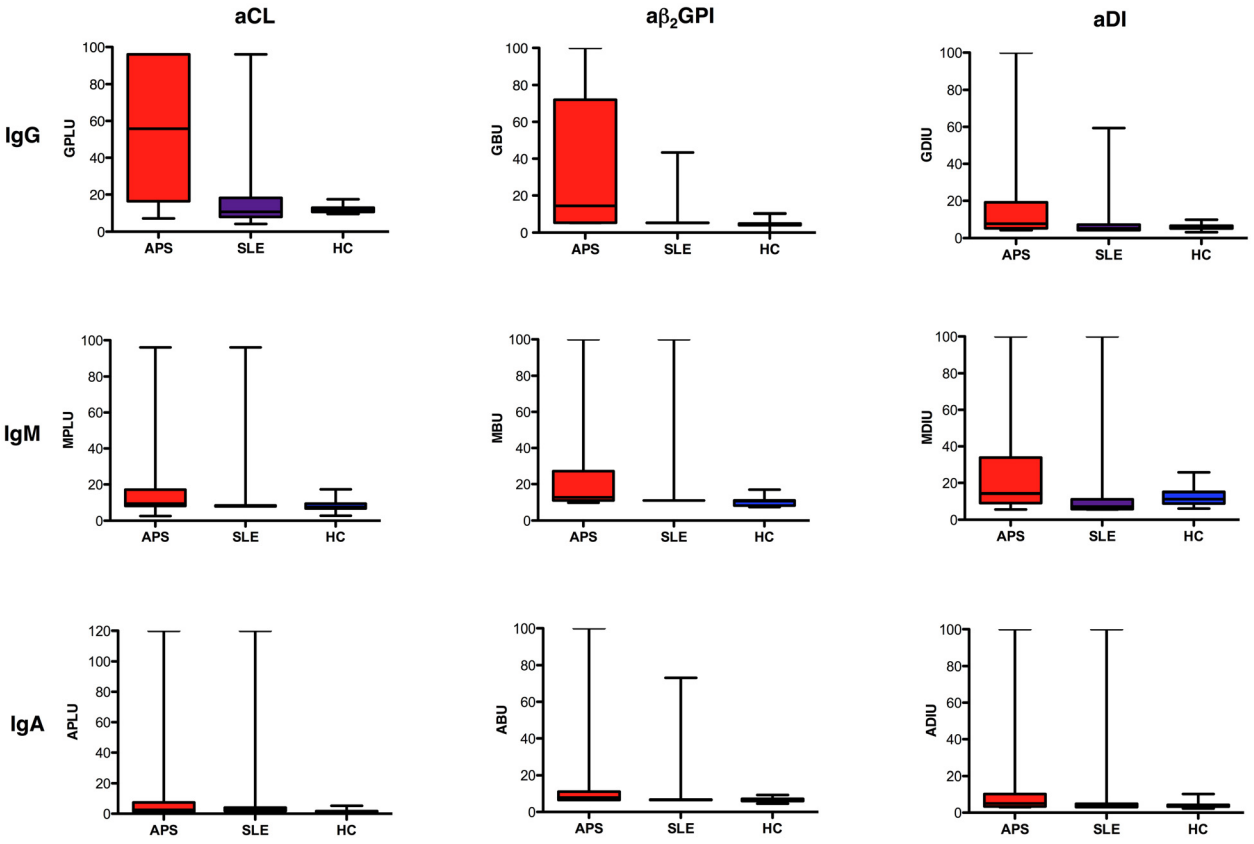

Fig 1. aPL titres in APS, SLE and healthy control (HC) subjects. Sera collected from a total of 111 patients with APS, 119 with SLE (but not APS) and 200 healthy controls were tested in nine aPL assays. Box and whisker plots running from top to bottom depict IgG, IgM and IgA titres of (running from left to right) aCL, $a\beta_2$GPI and aDI for each subject studied. The black line across data sets denotes mean activity (mean values are listed in Table 1). Abbreviations: GPLU, MPLU, APLU: IgG/IgM/IgA phospholipid units respectively; GBU, MBU, ABU: IgG/IgM/IgA β_2GPI units respectively; GDIU, MDIU, ADIU: IgG/IgM/IgA DI units respectively.

doi:10.1371/journal.pone.0156407.g001

ROC and logistic regression analysis indicate that IgG and IgA aβ$_2$GPI assays are the best discriminators of APS

ROC analysis (Table 3, Fig 2) confirmed that, for all three isotypes, aβ$_2$GPI were best associated with APS compared to aCL and aDI, with IgG aβ$_2$GPI positivity being the strongest discriminator for APS. For the purposes of this study, we report the specificity and sensitivity of each of the nine aPL for APS at the level of each assay's calculated cut-off for positivity. As seen in Table 3, all nine assays displayed excellent specificity for APS (~90% or above) but sensitivity was poorer in comparison.

Another way to evaluate each assay's performance is to compare the likelihood ratios (LR) generated from ROC analysis. We report the positive LR for each assay, which indicates how much the probability of having APS is increased if a subject tests positive. The LR is considered the most clinically relevant for diagnostic tests, and a positive ratio >10 is considered particularly significant [40]. Taking this into account, testing positive for aβ$_2$GPI of any of the three isotypes increased the probability of having APS by a factor of >13, followed by IgM aCL (LR 11.9), IgG aDI (LR 10.0) and IgA aDI (LR 9.5) (Table 3).

The results of logistic regression analysis for each assay are shown in Table 4 as the HR that a subject testing positive will have APS compared to a subject testing negative. This analysis was performed in two groups; (a) all subjects (n = 430) and (b) all SLE (n = 145, which included the 119 patients in our SLE/no APS group plus 26 patients from the APS group who also had SLE). The rationale for group (b) is to represent the common clinical scenario of testing patients with SLE to evaluate their risk of developing APS. For both (a) and (b), positivity in each of the nine assays showed a significantly positive HR for APS, with one exception—IgA aCL in the 145 patients with SLE, reflecting the similar prevalence of IgA aCL in both our APS and SLE/no APS patients (38% and 30% respectively, Table 2). Overall, IgG and IgA aβ$_2$GPI had the greatest HR for APS (33.4 and 33.9 respectively) (Table 4).

Table 3. ROC analysis: discriminatory ability of each aPL test for APS.

	aCL	aβ$_2$GPI	aDI
***IgG aPL*:**			
Area under curve (95% CI)	0.83 (0.78–0.89)	0.92 (0.89–0.94)	0.72 (0.66–0.78)
Sensitivity (95% CI)	72.9 (63.7–80.9)	64.8 (55.2–73.7)	40.5 (31.3–50.3)
Specificity (95% CI)	89.7 (85.8–92.8)	95.6 (92.8–97.6)	95.9 (93.1–97.8)
Likelihood ratio	7.1	14.8	10.0
***IgM aPL*:**			
Area under curve (95% CI)	0.74 (0.68–0.80)	0.80 (0.75–0.85)	0.67 (0.61–0.74)
Sensitivity (95% CI)	26.1 (18.3–35.3)	33.3 (24.7–42.9)	33.3 (24.7–42.9)
Specificity (95% CI)	97.8 (95.5–99.1)	97.5 (95.1–98.9)	93.7 (90.5–96.1)
Likelihood ratio	11.9	13.3	5.3
***IgA aPL*:**			
Area under curve (95% CI)	0.64 (0.57–0.71)	0.79 (0.74–0.84)	0.69 (0.62–0.75)
Sensitivity (95% CI)	36.9 (28.0–46.6)	40.5 (31.3–50.3)	38.7 (29.6–48.5)
Specificity (95% CI)	89.7 (85.8–92.8)	97.2 (94.7–98.7)	95.9 (93.1–97.8)
Likelihood ratio	3.6	14.4	9.5

Sensitivity, specificity and likelihood ratios shown are based on the cut off for positivity for each aPL. For all analyses, $p \leq 0.001$.

doi:10.1371/journal.pone.0156407.t003

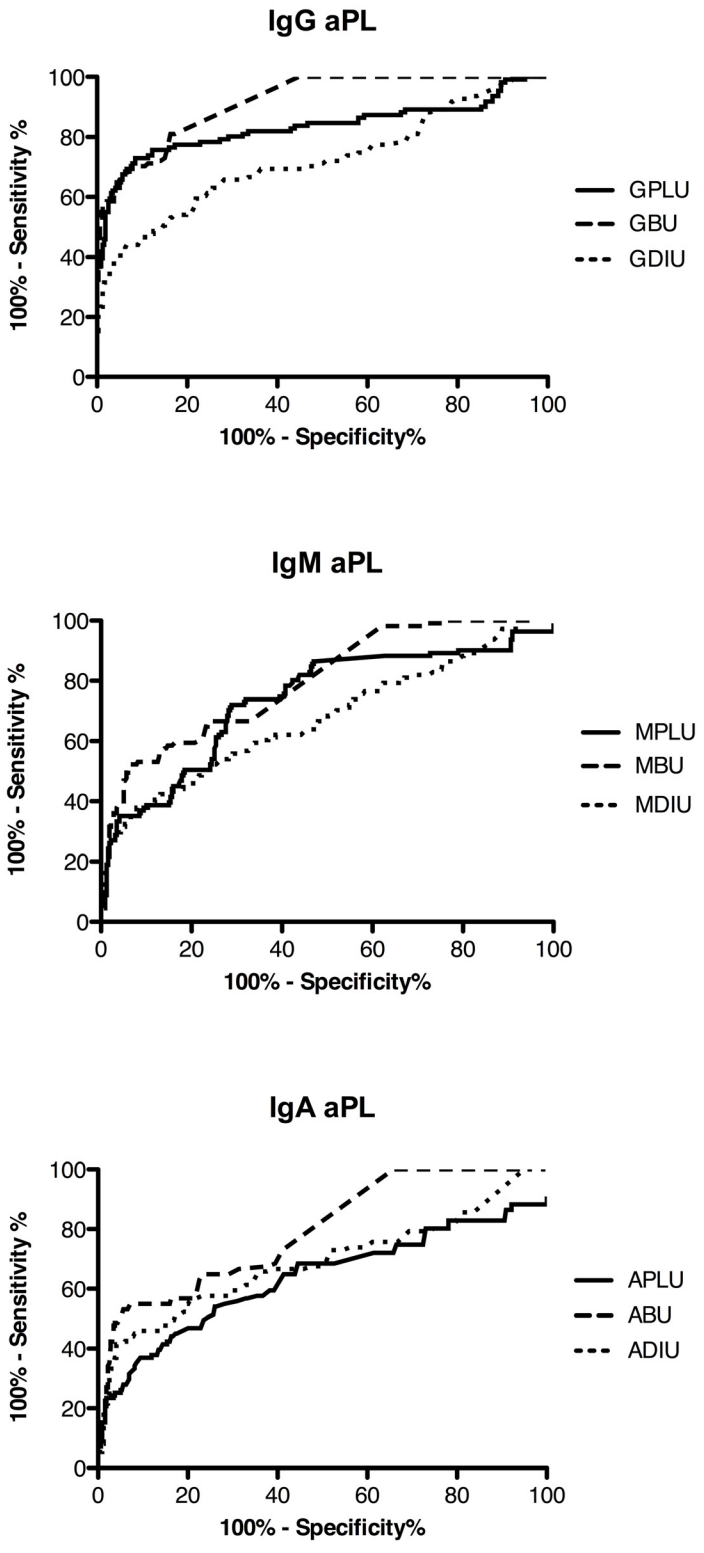

Fig 2. ROC analysis: aβ_2GPI tests best discriminate for APS. Receiver operating characteristic (ROC) analysis was performed to assess the ability of each of the nine aPL assays to discriminate between APS and non-APS subjects. For all three antibody isotypes, the resulting ROC curves illustrate the superiority of aβ_2GPI tests compared to aCL and aDI for APS diagnosis (numerical results are listed in Table 3). Abbreviations: GPLU, MPLU, APLU: IgG/IgM/IgA phospholipid units respectively; GBU, MBU, ABU: IgG/IgM/IgA β_2GPI units respectively; GDIU, MDIU, ADIU: IgG/IgM/IgA DI units respectively.

doi:10.1371/journal.pone.0156407.g002

Table 4. Regression analysis: hazard ratio for APS.

	aCL	aβ$_2$GPI	aDI
***IgG aPL*:**			
All subjects (n = 430)	8.6 (5.7–12.9)	33.4 (13.0–86.1)	6.6 (3.8–11.4)
All SLE (n = 145)*	4.2 (2.4–7.5)	9.8 (3.1–31.6)	3.5 (1.8–6.8)
***IgM aPL*:**			
All subjects (n = 430)	3.7 (2.4–5.7)	9.2 (4.6–18.4)	3.8 (2.6–5.5)
All SLE (n = 145)*	2.7 (1.3–5.4)	3.2 (1.7–6.2)	2.8 (1.5–4.9)
***IgA aPL*:**			
All subjects (n = 430)	2.1 (1.6–2.7)	33.9 (10.5–109.5)	4.5 (2.8–7.1)
All SLE (n = 145)*	1.3 (0.9–1.9)	5.3 (2.1–13.3)	2.2 (1.3–3.7)

Hazard ratio (95% CI) that subjects who test positive in an assay have APS compared to those who test negative in the assay. For all analyses, $p \leq 0.001$, with the following exceptions: in 'All SLE', IgM aCL p = 0.006; IgA aCL p = 0.15; IgA aDI p = 0.004.

* In the 'All SLE' group, 26 of 145 patients had APS.

doi:10.1371/journal.pone.0156407.t004

IgG, IgM and IgA aDI assays have high specificity but IgG aDI has lower sensitivity than IgG aβ$_2$GPI for APS

IgG, IgM and IgA aDI assays showed excellent specificity and similar sensitivity for APS compared to aβ$_2$GPI, with the exception of IgG aDI which was less sensitive than both IgG aβ$_2$GPI and IgG aCL (Table 3). Positivity for IgG, IgM and IgA aDI was strongly associated with APS to a similar or better level than the corresponding aCL assays, though not as strongly as with aβ$_2$GPI (Table 4).

For each immunoglobulin isotype, we noted a strong positive correlation between aDI and aβ$_2$GPI titres across the entire cohort of 430 subjects (r = 0.7–0.8 in all cases, $p<0.001$), however when only APS subjects were considered, the correlation between IgG aDI and aβ$_2$GPI dropped to a moderate level (r = 0.6, $p<0.001$). This discrepancy is because some APS patients had medium-high IgG aβ$_2$GPI but low aDI, or vice versa, thus some patients' reactivity against the whole molecule was different to reactivity against DI.

Does testing for aDI add value to current diagnostic tests?

The majority of patients testing positive for aDI were also positive for aCL, aβ$_2$GPI and LA (Table 5). We therefore next assessed whether the inclusion of a test for aDI positivity would add value in aCL and/or aβ$_2$GPI-positive cases. For this purpose, we compared the HR for APS in subjects positive for aCL and/or aβ$_2$GPI but not aDI [aCL/aβ$_2$GPI+(aDI-)] versus subjects with aDI [aCL/aβ$_2$GPI+(aDI+)] (Table 6).

Of 430 subjects, 136 were positive for IgG aCL/aβ$_2$GPI, 52 of which were also IgG aDI-positive; 52 subjects had IgM aCL/aβ$_2$GPI of which 33 were IgM aDI-positive; and 100 subjects had IgA aCL/aβ$_2$GPI of which 38 were IgA aDI-positive. For all isotypes, the presence of aDI increased the HR for APS by approximately 3-fold for IgG and IgM, and 5-fold for IgA (Table 6).

The same approach was applied to the group of 111 patients with APS in order to establish the HR for thrombosis or pregnancy morbidity associated with the aCL/aβ$_2$GPI+(aDI-) and aCL/aβ$_2$GPI+(aDI+) serological profiles (Table 6). Both IgG aCL/aβ$_2$GPI+(aDI-) (HR for thrombosis 3.2, 95%CI 1.1–9.1) and IgG aCL/aβ$_2$GPI+(aDI+) (HR for thrombosis 4.0, 95%CI 1.4–11.2) were associated with VT. No significant associations were seen for IgM or IgA

Table 5. Triple, double & single positivity per antibody isotype in APS.

	IgG aPL		IgM aPL		IgA aPL	
	All APS (n = 111)*	LA +ve APS (n = 87)†	All APS (n = 111)*	LA +ve APS (n = 87)†	All APS (n = 111)*	LA +ve APS (n = 87)†
Triple aCL/aβ$_2$GPI/aDI+	40	36	22	15	27	23
Double aCL/aβ$_2$GPI+	26	19	6	4	9	7
Double aCL/aDI+	4	2	1	0	1	1
Double aβ$_2$GPI/aDI+	1	0	6	6	5	1
aCL+ only	12	7	2	2	6	5
aβ$_2$GPI+ only	5	2	5	2	10	7
aDI+ only	0	0	7	7	13	10

The results for each different isotype in this table should be considered separately. Thus, a patient in the cell marked aCL+ only in the IgA aPL column does not have IgA aβ$_2$GPI or IgA aDI but may have IgG or IgM antibodies to those antigens. Overall, every patient in this Table tests positive for at least one of IgG aCL, IgG aβ2GPI, IgM aCL, IgM aβ2GPI or LA. Ten LA-positive patients tested negative in all nine aPL assays, and are not included in this table.

* Inclusive of all APS patients, with or without LA positivity.

† LA-positive APS patients only.

doi:10.1371/journal.pone.0156407.t005

serological profiles, except that the addition of IgA aDI positivity tripled the HR for thrombosis and converted it from non-significant (HR for thrombosis in aCL/aβ$_2$GPI+(aDI-) subjects 1.1, 95%CI 0.4–2.9) to significant (HR for thrombosis in aCL/aβ$_2$GPI+(aDI+) subjects 3.6, 95%CI 1.4–9.1). PM was not associated with either the aCL/aβ$_2$GPI+(aDI-) or aCL/aβ$_2$GPI+(aDI+) profile (Table 6). These findings are in agreement with results from individual regression analyses, where we determined the association of each of the nine assays with VT or PM and identified moderate positive associations between IgG aCL, aβ$_2$GPI, aDI, and IgA aDI with VT but not PM (data not shown). Of interest, LA positivity alone could not discriminate between thrombotic or obstetric complications in our APS cohort (for VT, HR 1.9, 95% CI 0.7–5.1; for PM, HR 0.8, 95% CI 0.3–2.1, $p>0.05$).

Discussion

In this study, we performed nine different assays using sera from 430 subjects. This large dataset allows the first rigorous comparison of IgG, IgM and IgA aCL, aβ$_2$GPI and aDI in patients with APS, SLE and healthy controls. We confirm the importance of IgG aCL and IgG aβ$_2$GPI tests, which had the highest sensitivity for APS and were strongly associated with LA positivity. We show that IgA aβ$_2$GPI are strongly associated with APS and are more common in our cohort than IgM aβ$_2$GPI, and demonstrate that aDI of all three isotypes are associated with APS with high specificity. Importantly, in subjects known to be positive for any isotype of aCL and/or aβ$_2$GPI, the additional finding of aDI positivity increases the likelihood of APS by between three and five times. Finally, we report that positivity for IgG or IgA aDI increases the strength of association between aCL/aβ$_2$GPI and thrombotic manifestations in APS.

While the pathogenicity of IgG aβ$_2$GPI is well characterised both *in vivo* and *in vitro* [6–9], IgA aβ$_2$GPI have been far less studied in comparison [4, 5]. In a recent comprehensive review, an international group of experts analysed published and unpublished data on IgA aCL and aβ$_2$GPI, highlighting the low quality of the data, variability of results and that many studies had been restricted to patients with SLE [41]. Due to lack of a substantial body of evidence, IgA aPL are not included in current APS diagnostic criteria [1], yet some published guidelines suggest

Table 6. Comparison of hazard ratios for APS, thrombosis and pregnancy morbidity in aCL and/or $a\beta_2$GPI positive subjects in the absence or presence of aDI.

	aCL/$a\beta_2$GPI+(aDI-)*	aCL/$a\beta_2$GPI+(aDI+)†
***IgG aPL*:**		
No. of subjects (no. of APS)	84 (43)	52 (45)
Association with:		
APS‡	**11.5 (6.3–21.0)**	**36.9 (17.7–76.9)**
Thrombosis§	**3.2 (1.1–9.1)**	**4.0 (1.4–11.2)**
Pregnancy morbidity∞	0.3 (0.1–1.0)	0.2 (0.1–0.9)
***IgM aPL*:**		
No. of subjects (no. of APS)	19 (12)	33 (29)
Association with:		
APS‡	**7.3 (3.0–17.5)**	**21.3 (9.1–50.4)**
Thrombosis§	1.4 (0.4–4.5)	2.3 (0.9–5.8)
Pregnancy morbidity∞	0.8 (0.2–3.2)	0.7 (0.3–2.0)
***IgA aPL*:**		
No. of subjects (no. of APS)	62 (15)	38 (32)
Association with:		
APS‡	**5.0 (2.7–9.2)**	**24.8 (12.3–49.9)**
Thrombosis§	1.1 (0.4–2.9)	**3.6 (1.4–9.1)**
Pregnancy morbidity∞	0.4 (0.1–1.4)	0.2 (0.1–0.7)

Hazard ratios (95% CI) shown for APS, thrombosis and pregnancy morbidity. Statistically significant associations are highlighted in bold.

*aCL/$a\beta_2$GPI+(aDI-) group includes double aCL/$a\beta_2$GPI and single aCL or $a\beta_2$GPI positives.

† aCL/$a\beta_2$GPI+(aDI+) group includes triple positives and double aCL/aDI or $a\beta_2$GPI/aDI positives.

‡ Analysis inclusive of all subjects (n = 430). For all significant (bold) associations, $p \leq 0.001$.

§Analysis inclusive of APS patients only (n = 111). Significant (bold) associations, IgG aCL/$a\beta_2$GPI+(aDI-) p = 0.027; IgG aCL/$a\beta_2$GPI+(aDI+) p = 0.008; IgA aCL/$a\beta_2$GPI+(aDI+) p = 0.007.

∞ Analysis inclusive of female APS patients only (n = 93).

doi:10.1371/journal.pone.0156407.t006

testing for IgA (particularly $a\beta_2$GPI) in patients who are IgG/IgM- and LA-negative but in whom APS is suspected [42]. Our data suggest that measurement of IgA $a\beta_2$GPI and aDI would be more valuable than measuring IgA aCL in patients with suspected APS, and we also note that IgA $a\beta_2$GPI are more commonly positive in our APS cohort compared to IgM $a\beta_2$GPI (Table 2). To confirm the validity of our IgA $a\beta_2$GPI results, we compared our IgA $a\beta_2$GPI test with the equivalent commercial test from Inova Diagnostics (QUANTA-Lite β_2GPI IgA assay). We tested a total of 32 serum samples– 13 APS; 8 SLE/no APS; and 11 healthy controls—in both our in-house and the Inova assays, and found that the two assays showed very good quantitative (Spearman's r = 0.9, $p<0.0001$) and qualitative agreements (93.75% of the observations agreed in terms of being positive or negative, *kappa* = 0.875).

The ability of human plasma purified β_2GPI to recognize aPL when immobilised on a plastic surface relies on its conformation and is a significant obstacle towards standardising $a\beta_2$GPI tests across laboratories. In contrast, we and others have successfully used recombinant human DI expressed in bacteria [38], baculovirus [16], and synthetically [43] to measure IgG aDI antibodies [15–22, 43–45], and thus DI could potentially be a more reliable source of antigen compared to whole β_2GPI. A number of different assays have been reported for measuring aDI (reviewed in [41]). Thus far, the most convincing published evidence arises from use of an assay dependent on comparing binding to DI on hydrophobic and hydrophilic plates [19, 21],

while we have employed our in-house single-plate solid-phase assay. Detecting aPL against a small part of a protein such as DI (~7kDa) is challenging however, and thus innovative approaches should be implemented to improve both the simplicity and sensitivity of any test aimed at measuring aDI in the clinical setting. New detection methods could help achieve this goal. Inova Diagnostics developed a chemiluminescent immunoassay (CIA) for IgG aDI that recently received clearance by the U.S. Food and Drug Administration for use in autoimmune disease testing, and report a sensitivity of 85% in a cohort of 144 patients with APS, compared to 0.5% and 14% for 200 healthy and 72 infectious disease controls respectively [14]. In a small study of 39 patients with APS and 77 disease and healthy controls, the IgG aDI CIA had a sensitivity of 36%, while an IgG aβ_2GPI CIA had a sensitivity of 46% [44]. Likewise, our IgG aβ_2GPI assay proved to have higher sensitivity for APS compared to aDI (Table 3). Moreover, positivity for IgG, IgM and IgA aDI was strongly associated with APS to a similar or better level than the corresponding aCL assays, though not as strongly as with aβ_2GPI (Table 4). The apparent superiority of aβ_2GPI assays is likely due to the presence of antibodies that target DII-V. Indeed, we recently utilised the Inova IgG aDI CIA and a further prototype CIA to measure IgG aβ_2GPI against domains IV-V (aDIV-V), demonstrating that both aDI and aDIV-V can be detected in the blood of aβ_2GPI-positive subjects. Importantly however, aDI were more frequently found in patients with systemic autoimmune disease compared to asymptomatic aPL carriers [15]. Of note, we reported good qualitative and quantitative agreement between our IgG aDI ELISA and the Inova CIA, as well as similar discrimination for APS compared to controls [41, 46].

In line with recently published studies [15, 45], we also found that aDI were more common in APS patients who were aCL, aβ_2GPI and LA-positive (Table 5). Interestingly, we identified two LA-positive APS patients with low IgG aCL and aβ_2GPI (patient 1, 20GPLU and 8GBU; patient 2, 27GPLU and 12GBU) but high IgG aDI (>50GDIU). One of these patients also had high IgM aDI (>100MDIU) despite low IgM aCL (20MPLU) and IgM aβ_2GPI (21MBU). Therefore, although rare, there are patients with APS with high aDI activity but low or negative classical IgG/IgM aPL activity, and in these cases aDI tests could certainly provide additional information to current tests available. Of relevance, we recently reported the presence of IgG aDI in 3 out of 40 'seronegative' APS patients, who fulfilled clinical but not serological APS criteria [47], further suggesting that detecting aDI could complement current criteria tests.

Our results concur with the largest published study for IgG aDI in 442 aβ_2GPI-positive subjects (of which 82% had APS), underlining the added value of measuring IgG aDI as well as aCL/aβ_2GPI in relation to increased risk of VT [21]. We additionally report that IgA aDI are of similar value for determining thrombotic risk (Table 6). Unlike the study of de Laat *et al* [21] however, we did not find any benefit of adding IgG aDI in terms of an association with PM. This dissimilarity may have arisen because the majority of our female APS patients (67%) who suffered pregnancy complications did not have a thrombotic history, compared to 42% in the original study. A very recent study also concluded that IgG aDI are associated with thrombosis but not pregnancy loss in a cohort of 65 IgG aβ_2GPI-positive subjects [45].

Conclusions

Based on our current findings and other groups' published reports, we consider aDI tests as a useful additional test rather than a replacement for tests using whole β_2GPI, since the latter would also pick up aβ_2GPI directed against other domains. Considering their pathogenic role, detecting IgG aDI may allow for risk stratification in established APS and help in the diagnosis of suspected APS, while the prevalence and clinical association of IgA aDI in APS requires further clarification. Moreover, prospective studies are imperative to determine if IgA aβ_2GPI and

IgG/IgA aDI have prognostic power. We are currently completing such a study in early samples from >500 patients with SLE, a proportion of whom later developed thrombosis or obstetric complications, and are performing longitudinal tests in order to establish if aPL levels remain constant or change before, near to and after an APS-related clinical event.

Acknowledgments

We would like to especially thank all patients (University College London Hospital, UK; Istituto Gaetano Pini, University of Milan, Italy; APL Standardisation Laboratory, TX, USA) for donating blood for our research. We would also like to thank Dr. Hannah Cohen (University College London Hospital, UK). The healthy control blood samples were used with the permission of the Health and Social Care Information Centre and NatCen Social Research. (Copyright 2008, All rights reserved).

Author Contributions

Conceived and designed the experiments: CP YI IG AR. Performed the experiments: CP. Analyzed the data: CP. Contributed reagents/materials/analysis tools: IF OB FP TM AG PD SP DI PLM. Wrote the paper: CP AR.

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. PMID: 16420554
2. Willis R, Harris EN, Pierangeli SS. Current international initiatives in antiphospholipid antibody testing. Semin Thromb Hemost. 2012; 38:360–74. doi: 10.1055/s-0032-1313564 PMID: 22576664
3. Swadzba J, Sydor WJ, Kolodziejczyk J, Musial J. Summary of the 9th meeting of the European Forum on Antiphospholipid Antibodies. Lupus. 2014; 23:395–9. doi: 10.1177/0961203314520841 PMID: 24474705
4. Andreoli L, Fredi M, Nalli C, Piantoni S, Reggia R, Dall'Ara F, et al. Clinical significance of IgA anti-cardiolipin and IgA anti-beta2glycoprotein I antibodies. Curr Rheumatol Rep. 2013; 15:343. doi: 10.1007/s11926-013-0343-1 PMID: 23754504
5. Murthy V, Willis R, Romay-Penabad Z, Ruiz-Limon P, Martinez-Martinez LA, Jatwani S, et al. Value of isolated IgA anti-beta2 -glycoprotein I positivity in the diagnosis of the antiphospholipid syndrome. Arthritis Rheum. 2013; 65:3186–93. doi: 10.1002/art.38131 PMID: 23983008
6. Galli M, Comfurius P, Maassen C, Hemker HC, de Baets MH, van Breda-Vriesman PJ, et al. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. Lancet. 1990; 335:1544–7. PMID: 1972485
7. McNeil HP, Simpson RJ, Chesterman CN, Krilis SA. Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2- glycoprotein I (apolipoprotein H). Proc Natl Acad Sci U S A. 1990; 87:4120–4. PMID: 2349221
8. Giannakopoulos B, Krilis SA. The pathogenesis of the antiphospholipid syndrome. New Engl J Med. 2013; 368:1033–44. doi: 10.1056/NEJMra1112830 PMID: 23484830
9. Meroni P, Chighizola C, Rovelli F, Gerosa M. Antiphospholipid syndrome in 2014: more clinical manifestations, novel pathogenic players and emerging biomarkers. Arthritis Res Ther. 2014; 16:209–22. PMID: 25166960
10. Ioannou Y, Zhang JY, Passam FH, Rahgozar S, Qi JC, Giannakopoulos B, et al. Naturally occurring free thiols within beta 2-glycoprotein I in vivo: nitrosylation, redox modification by endothelial cells, and regulation of oxidative stress-induced cell injury. Blood. 2010; 116:1961–70. doi: 10.1182/blood-2009-04-215335 PMID: 20551379
11. de Laat B, Derksen RH, van Lummel M, Pennings MT, de Groot PG. Pathogenic anti-beta2-glycoprotein I antibodies recognize domain I of beta2-glycoprotein I only after a conformational change. Blood. 2006; 107:1916–24. PMID: 16269621
12. Agar C, van Os GM, Morgelin M, Sprenger RR, Marquart JA, Urbanus RT, et al. Beta2-glycoprotein I can exist in 2 conformations: implications for our understanding of the antiphospholipid syndrome. Blood. 2010; 116:1336–43. doi: 10.1182/blood-2009-12-260976 PMID: 20462962

13. de Laat B, de Groot PG. Autoantibodies directed against domain I of beta2-glycoprotein I. Curr Rheumatol Rep. 2011; 13:70–6. doi: 10.1007/s11926-010-0144-8 PMID: 21046294

14. Mahler M, Norman GL, Meroni PL, Khamashta M. Autoantibodies to domain 1 of beta 2 glycoprotein 1: a promising candidate biomarker for risk management in antiphospholipid syndrome. Autoimmun Rev. 2012; 12:313–7. doi: 10.1016/j.autrev.2012.05.006 PMID: 22652408

15. Andreoli L, Chighizola CB, Nalli C, Gerosa M, Borghi MO, Pregnolato F, et al. Clinical Characterization of Antiphospholipid Syndrome by Detection of IgG Antibodies Against beta2 -Glycoprotein I Domain 1 and Domain 4/5: Ratio of Anti-Domain 1 to Anti-Domain 4/5 As a Useful New Biomarker for Antiphospholipid Syndrome. Arthritis Rheumatol. 2015; 67:2196–204. doi: 10.1002/art.39187 PMID: 25939498

16. Iverson GM, Victoria EJ, Marquis DM. Anti-beta2 glycoprotein I (beta2GPI) autoantibodies recognize an epitope on the first domain of beta2GPI. Proc Natl Acad Sci U S A. 1998; 95:15542–6. PMID: 9861005

17. Reddel SW, Wang YX, Sheng YH, Krilis SA. Epitope studies with anti-beta 2-glycoprotein I antibodies from autoantibody and immunized sources. J Autoimmun. 2000; 15:91–6. PMID: 10968891

18. Iverson GM, Reddel S, Victoria EJ, Cockerill KA, Wang YX, Marti-Renom MA, et al. Use of single point mutations in domain I of beta 2-glycoprotein I to determine fine antigenic specificity of antiphospholipid autoantibodies. J Immunol. 2002; 169:7097–103. PMID: 12471146

19. de Laat B, Derksen RH, Urbanus RT, de Groot PG. IgG antibodies that recognize epitope Gly40-Arg43 in domain I of beta 2-glycoprotein I cause LAC, and their presence correlates strongly with thrombosis. Blood. 2005; 105:1540–5. PMID: 15507529

20. Ioannou Y, Pericleous C, Giles I, Latchman DS, Isenberg DA, Rahman A. Binding of antiphospholipid antibodies to discontinuous epitopes on domain I of human beta(2)-glycoprotein I: mutation studies including residues R39 to R43. Arthritis Rheum. 2007; 56:280–90. PMID: 17195232

21. de Laat B, Pengo V, Pabinger I, Musial J, Voskuyl AE, Bultink IE, et al. The association between circulating antibodies against domain I of beta2-glycoprotein I and thrombosis: an international multicenter study. J Thromb Haemost. 2009; 7:1767–73. doi: 10.1111/j.1538-7836.2009.03588.x PMID: 19694946

22. Andreoli L, Nalli C, Motta M, Norman GL, Shums Z, Encabo S, et al. Anti-beta(2)-glycoprotein I IgG antibodies from 1-year-old healthy children born to mothers with systemic autoimmune diseases preferentially target domain 4/5: might it be the reason for their 'innocent' profile? Ann Rheum Dis. 2011; 70:380–3. doi: 10.1136/ard.2010.137281 PMID: 20971718

23. Pericleous C, Ruiz-Limon P, Romay-Penabad Z, Marin AC, Garza-Garcia A, Murfitt L, et al. Proof-of-concept study demonstrating the pathogenicity of affinity-purified IgG antibodies directed to domain I of beta2-glycoprotein I in a mouse model of anti-phospholipid antibody-induced thrombosis. Rheumatology (Oxford). 2015; 54:722–7.

24. Pericleous C, Miles J, Esposito D, Garza-Garcia A, Driscoll PC, Lambrianides A, et al. Evaluating the conformation of recombinant domain I of beta(2)-glycoprotein I and its interaction with human monoclonal antibodies. Mol Immunol. 2011; 49:56–63. doi: 10.1016/j.molimm.2011.07.024 PMID: 21899894

25. Pierangeli SS, Liu X, Espinola R, Olee T, Zhu M, Harris NE, et al. Functional analyses of patient-derived IgG monoclonal anticardiolipin antibodies using in vivo thrombosis and in vivo microcirculation models. Thromb Haemost. 2000; 84:388–95. PMID: 11019960

26. Giles I, Pericleous C, Liu X, Ehsanullah J, Clarke L, Brogan P, et al. Thrombin binding predicts the effects of sequence changes in a human monoclonal antiphospholipid antibody on its in vivo biologic actions. J Immunol. 2009; 182:4836–43. doi: 10.4049/jimmunol.0804241 PMID: 19342662

27. Ioannou Y, Romay-Penabad Z, Pericleous C, Giles I, Papalardo E, Vargas G, et al. In vivo inhibition of antiphospholipid antibody-induced pathogenicity utilizing the antigenic target peptide domain I of beta2-glycoprotein I: proof of concept. J Thromb Haemost. 2009; 7:833–42. doi: 10.1111/j.1538-7836.2009.03316.x PMID: 19220729

28. Agostinis C, Durigutto P, Sblattero D, Borghi MO, Grossi C, Guida F, et al. A non-complement-fixing antibody to beta2 glycoprotein I as a novel therapy for antiphospholipid syndrome. Blood. 2014; 123:3478–87. doi: 10.1182/blood-2013-11-537704 PMID: 24642748

29. de Laat B, van Berkel M, Urbanus RT, Siregar B, de Groot PG, Gebbink MF, et al. Immune responses against domain I of beta(2)-glycoprotein I are driven by conformational changes: domain I of beta(2)-glycoprotein I harbors a cryptic immunogenic epitope. Arthritis Rheum. 2011; 63:3960–8. doi: 10.1002/art.30633 PMID: 21898342

30. Akhter E, Shums Z, Norman GL, Binder W, Fang H, Petri M. Utility of antiphosphatidylserine/prothrombin and IgA antiphospholipid assays in systemic lupus erythematosus. J Rheumatol. 2013; 40:282–6. doi: 10.3899/jrheum.120084 PMID: 23378459

31. Sweiss NJ, Bo R, Kapadia R, Manst D, Mahmood F, Adhikari T, et al. IgA anti-beta2-glycoprotein I autoantibodies are associated with an increased risk of thromboembolic events in patients with systemic

lupus erythematosus. PLoS One. 2010; 5:e12280. doi: 10.1371/journal.pone.0012280 PMID: 20808864

32. Iverson GM, von Muhlen CA, Staub HL, Lassen AJ, Binder W, Norman GL. Patients with atherosclerotic syndrome, negative in anti-cardiolipin assays, make IgA autoantibodies that preferentially target domain 4 of beta2-GPI. J Autoimmun. 2006; 27:266–71. PMID: 17081732

33. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1982; 25:1271–7. PMID: 7138600

34. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1997; 40:1725.

35. Craig R, Mindell J. Health Survey for England—2006, CVD and risk factors for adults, obesity and risk factors for children. In: Centre HaSCI, editor. Leeds, UK2008.

36. Pierangeli SS, Harris EN. A protocol for determination of anticardiolipin antibodies by ELISA. Nat Protoc. 2008; 3:840–8. doi: 10.1038/nprot.2008.48 PMID: 18451792

37. Giles I, Haley J, Nagl S, Latchman D, Chen P, Chukwuocha R, et al. Relative importance of different human aPL derived heavy and light chains in the binding of aPL to cardiolipin. Mol Immunol. 2003; 40:49–60. PMID: 12909130

38. Ioannou Y, Giles I, Lambrianides A, Richardson C, Pearl LH, Latchman DS, et al. A novel expression system of domain I of human beta2 glycoprotein I in Escherichia coli. BMC Biotechnol. 2006; 6:8–18. PMID: 16472380

39. Petri M. Epidemiology of the antiphospholipid antibody syndrome. J Autoimmun. 2000; 15:145–51. PMID: 10968901

40. Florkowski CM. Sensitivity, specificity, receiver-operating characteristic (ROC) curves and likelihood ratios: communicating the performance of diagnostic tests. Clin Biochem Rev. 2008; 29 Suppl 1:S83–7. PMID: 18852864

41. Bertolaccini ML, Amengual O, Andreoli L, Atsumi T, Chighizola CB, Forastiero R, et al. 14th International Congress on Antiphospholipid Antibodies Task Force. Report on antiphospholipid syndrome laboratory diagnostics and trends. Autoimmun Rev. 2014; 13:917–30. doi: 10.1016/j.autrev.2014.05.001 PMID: 24824074

42. Lakos G, Favaloro EJ, Harris EN, Meroni PL, Tincani A, Wong RC, et al. International consensus guidelines on anticardiolipin and anti-beta2-glycoprotein I testing: report from the 13th International Congress on Antiphospholipid Antibodies. Arthritis Rheum. 2012; 64:1–10. doi: 10.1002/art.33349 PMID: 21953634

43. Banzato A, Pozzi N, Frasson R, De Filippis V, Ruffatti A, Bison E, et al. Antibodies to Domain I of beta (2)Glycoprotein I are in close relation to patients risk categories in Antiphospholipid Syndrome (APS). Thromb Res. 2011; 128:583–6. doi: 10.1016/j.thromres.2011.04.021 PMID: 21620443

44. Mondejar R, Gonzalez-Rodriguez C, Toyos-Saenz de Miera FJ, Melguizo-Madrid E, Zohoury N, Mahler M, et al. Role of antiphospholipid score and anti-beta2-glycoprotein I Domain I autoantibodies in the diagnosis of antiphospholipid syndrome. Clin Chim Acta. 2014; 431C:174–8.

45. Pengo V, Ruffatti A, Tonello M, Cuffaro S, Banzato A, Bison E, et al. Antibodies to Domain 1 (Dm1) of beta2-Glycoprotein 1 (beta2GP1) correctly classify patients at risk in Antiphospholipid Syndrome (APS). J Thromb Haemost. 2015; 13:1–6.

46. Willis R, Mahler M, Pregnolato F, Pericleous C, Rahman A, Ioannou Y, et al. Clinical evaluation of two anti-beta2glycoprotein I domain 1 autoantibody assays to aid in the diagnosis and risk assessment of the antiphospholipid syndrome. Arthritis Rheum. 2013; 65:S3.

47. Cousins L, Pericleous C, Khamashta M, Bertolaccini ML, Ioannou Y, Giles I, et al. Antibodies to domain I of beta-2-glycoprotein I and IgA antiphospholipid antibodies in patients with 'seronegative' antiphospholipid syndrome. Ann Rheum Dis. 2015; 74:317–9. doi: 10.1136/annrheumdis-2014-206483 PMID: 25359383