Detection of Anti-Cardiolipin and Anti-β2glycoprotein I Antibodies Differs between Platforms without Influence on Association with Clinical Symptoms

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Thromb Haemost

Abstract

Background The anti-phospholipid syndrome (APS) is characterized by thrombosis and/or pregnancy morbidity with persistent presence of anti-phospholipid antibodies (aPL). Laboratory criteria include aPL detection by coagulation tests for lupus anticoagulant (LAC) or solid phase assays measuring anti-β2 glycoprotein I (aβ2GPI) or anti-cardiolipin (aCL) immunoglobulin (Ig) G/IgM antibodies. External quality control programs illustrate that commercially available aPL assays produce variable results.

Objective We aimed to investigate the agreement and diagnostic accuracy of solid phase assays.

Materials and Methods In this multi-centre study, 1,168 patient samples were tested on one site for aCL and aβ2GPI IgG/IgM antibodies by four solid phase test systems. Samples included APS patients, controls and monoclonal antibodies (MoAB) against different epitopes of β2GPI. LAC was determined by the local centre.

Keywords
- solid phase assays
- anti-cardiolipin
- β2 glycoprotein I
- epitope
- thrombosis

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Results aCL IgM assays resulted in the most discrepancies (60%), while aCL IgG and aβ2GPI IgM assays resulted in lower discrepancies (36%), suggesting better agreement. Discrepant samples displayed lower median aPL titers. Dependent on the solid phase test system, odds ratios (ORs) for thrombosis and pregnancy morbidity ranged from 1.98 to 2.56 and 3.42 to 4.78, respectively. Three platforms showed lower sensitivity for MoAB directed against the glycine (Gly) 40-arginine (Arg) 43 epitope of domain I of β2GPI.

Conclusion Poor agreement was observed between different commercially available aCL and aβ2GPI IgG/IgM assays, hampering uniformity in the identification of aPL-positive patients. Clinical association was globally concordant between solid phase test systems considering results of the four aPL together. An assay sensitive in detecting the MoAB against Gly40-Arg43 of domain I of β2GPI reached the highest OR for thrombosis.

Introduction

The anti-phospholipid syndrome (APS) is characterized by thrombosis and/or pregnancy morbidity with the persistent presence of anti-phospholipid antibodies (aPL).[1] Laboratory criteria include aPL detection by phospholipid-dependent prolongation of coagulation tests referred to as lupus anticoagulant (LAC) or by solid phase assays measuring anti-β2 glycoprotein I (aβ2GPI) and anti-cardiolipin (aCL) immunoglobulin (Ig) G/IgM antibodies.[1] For the classification of APS, at least one clinical and one persistent positive laboratory criterion is required.[1] Given the high frequency of clinical manifestations (thrombosis and pregnancy morbidity) associated with APS in the general population, laboratory tests are of utmost importance for the classification of patients with APS. Although LAC positivity is considered the strongest predictor of clinical manifestations of APS, aCL and aβ2GPI IgG/IgM antibodies have the same value in the current classification criteria.[1–5]

Anti-β2GPI antibodies are detected using β2GPI as antigen, while both cardiolipin and β2GPI are used as antigens in aCL immunoassays. The use of antigens from human sources is preferred above antigens from animal origin (e.g. bovine) to avoid false-positives.[6] In contrast to aβ2GPI immunoassays, antigens used in aCL immunoassays are not exclusively from human origin (►**Supplementary Table S1**, available in the online version). Traditionally, aCL and aβ2GPI antibodies are detected with an enzyme-linked immunosorbent assay (ELISA). Nowadays, more advanced and automated systems have become available for the detection of aCL and aβ2GPI aPL.[7–10] Automated systems make use of an alternative solid phase (e.g. magnetic beads) and use alternative detection methods, such as chemiluminescence or (enzyme-linked) fluorescence. A large variety of assays are used in clinical laboratories as there is no consensus on a 'gold standard' for the detection of aCL and aβ2GPI aPL.[6] Reports from external quality control programs illustrate that aPL assays produce variable results.[11,12] Detection of aβ2GPI antibodies is challenging as some antibodies may be directed against a cryptic epitope that is only exposed after conformational shape change.[13,14] Exposure of this cryptic epitope, spanning glycine (Gly) 40-arginine (Arg) 43 in the first domain of β2GPI, has been shown to vary across commercial aβ2GPI IgG assays.[7,15] In addition, variability between assays might be due to the heterogeneous origin of aPL, differences in local working conditions, differences in assay design and a lack of standardization.[6] As classification of APS heavily depends on the detection of aPL, variation within these tests will affect the treatment strategy.

Therefore, we aimed to investigate the agreement and diagnostic accuracy of commonly used commercially available solid phase assays measuring IgG and IgM aCL and aβ2GPI aPL. Samples and normal pooled plasma supplemented with monoclonal antibodies (MoAB) against different domains of β2GPI were tested with four assays at one location by a single technician to exclude inter-laboratory and inter-operator variation.

Materials and Methods

Patient Cohort

We obtained 1,168 samples from 8 European centres. Classification of APS was based on the Sydney criteria.[1] Patients were classified by the corresponding centre resulting in 259 thrombotic APS patients (APS thrombosis), 204 patients with a history of thrombosis and negative for laboratory criteria of APS (non-APS thrombosis), 122 obstetric APS patients (APS obstetric), 33 patients with pregnancy complications and negative for laboratory criteria of APS (non-APS obstetric), 196 patients with an autoimmune disease other than APS (autoimmune diseases), 100 individuals with a normal pregnancy (normal pregnancy), 194 controls that were referred for aPL testing for other reasons than the clinical criteria of APS, like subfertility and prolonged activated partial thromboplastin time (controls) and 60 women that were diagnosed with APS without information on the specification of the clinical manifestations (unspecified APS). Centres with the indicated number of samples included Ghent (469), London (196), Nîmes (164), Nancy (114), Kraków (101), Milan (52), Geneva (50) and Apeldoorn (22). The study was approved by the central and the local ethical committees.

Assays

aCL IgG, aCL IgM, aβ2GPI IgG and aβ2GPI IgM aPL were measured in the Ghent University Hospital (Ghent, Belgium) by four commercially available immunoassays: BioPlex2200 (Bio-Rad, Bio-Rad Laboratories, Hercules, California, United States), ImmunoCapEliA (Thermo Fisher Scientific/Phadia, Uppsala, Sweden), ACL AcuStar (Werfen/Instrumentation Laboratories, Bedford, Massachusetts, United States) and QUANTA Lite ELISA (Inova Diagnostics, San Diego, California, United States) (►**Supplementary Table S1**, available in the online version). Assays were selected based on frequently used assays in the External quality Control of diagnostic Assays and Tests program and the willingness of manufacturers for collaboration. Due to shortage of patient sample, three and two patients were excluded for aCL IgG and aB2GPI IgG detection by BioPlex2200, respectively. Manufacturers' recommended cut-off values were used upon confirmation in 20 healthy volunteers, in accordance with the Scientific and Standardization Committee (SSC) guidelines of the International Society on Thrombosis and Haemostasis.[16] Assays were performed according to the manufacturer's instructions. All four methods were performed in parallel in runs of 40 samples. According to the guidelines, single measurement was used on the automated systems (BioPlex2200, ImmunoCapEliA and ACL AcuStar) as the intra- and inter-run imprecision coefficient of variation was $< 10\%$ and duplicate measurement for the ELISA (QUANTA Lite ELISA).[16] aPL titers were expressed in arbitrary units (gram per litre, IgM antiphospholipid units [MPL], U/mL, Standard IgG unit and Standard IgM unit [SMU]). All samples were measured by the same technician and values below the calculated limit of detection (LOD) were replaced by the calculated LOD.

Monoclonal Antibodies

Two human-derived MoAB P2–6 and P1–117 were used to test the specificity and sensitivity of the four commercially available aCL and aβ2GPI IgG assays. P2–6 recognizes β2GPI independently of its conformation and P1–117 recognizes β2GPI in its open conformation binding to the Gly40-Arg43 epitope of the domain I.[15,17] Serial dilutions of antibodies (0–250 μg/mL) in normal pooled plasma were tested in duplicate for all platforms included in the study. Platforms were used according to the manufacturer's recommendations and these spiked samples were handled as patient samples and tested in the same conditions. The threshold for positivity corresponding to a positive titer of aCL or aβ2GPI was determined.

Statistical Analysis

Solid phase assays were compared pairwise as no 'gold standard' for aPL detection exists. Agreement between assays in positivity was assessed by 2×2 contingency tables in all measured samples. Comparison of discrepancies between aCL IgG, aCL IgM and aβ2GPI IgG and aβ2GPIgM positivity was calculated: positivity discrepancy (%) = (only positive for method A + only positive for method B) / (all positives) $\times$ 100. Median aPL titers within one platform were calculated with titers above the cut-off value. Correlation between solid phase assays was performed by a Spearman's rank correlation coefficient. Diagnostic accuracy was assessed by calculating odds ratios (ORs), sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV) and the receiver operator curve (ROC). The area under the ROC curve between solid phase assays was compared using DeLong et al's method.[18] Significance of differences was determined with the Mann–Whitney U test, as appropriate using the Statistical Package for Social Sciences (SPSS 23.0; SPSS, Chicago, Illinois, United States) and MedCalc Statistical Software version 17.7.2 (MedCalc Software bvba, Ostend, Belgium).

Results

We measured aCL IgG/IgM and aβ2GPI IgG/IgM aPL in 1,168 individuals with a mean age of 43 years ranging from 16 to 87 years old (►**Table 1**) with four commercially available assays (►**Supplementary Table S1**, available in the online version).

Laboratory criteria for the classification of APS require at least one positive aPL. We therefore compared positivity for at least one aPL between platforms using 2×2 contingency tables (►**Table 2**). Discrepancies varied between 79 (ACL AcuStar vs. BioPlex2200) and 164 (ACL AcuStar vs. ImmunoCapEliA) samples. aCL IgM and aβ2GPI IgG positivity resulted in the most discrepancies, varying from 69 to 162 and 44 to 153 samples, respectively (►**Table 3**). On the other hand, aCL IgG and aβ2GPI IgM positivity resulted in less discrepancies between assays, varying from 49 to 98 and 34 to 58 samples, respectively. Comparison of discrepancies between aCL IgG/IgM and aβ2GPI IgG/IgM positivity was calculated by the percentage of discrepancies from all positives, resulting in a maximum discrepancy of 36, 60, 53 and 36% for aCL IgG, aCL IgM, aβ2GPI IgG and aβ2GPI IgM positivity, respectively. In accordance, aCL IgM titers were less correlated between the solid phase assays compared with aCL IgG and aβ2GPI IgM with Spearman's rank correlation coefficients of $\geq$ 0.514 (95% confidence interval [CI], 0.471–0.555), $\geq$ 0.635 (95% CI, 0.599–0.668) and $\geq$ 0.738 (95% CI, 0.711–0.763), respectively (►**Table 4**).

aPL positive samples not in agreement across the platforms were characterized by lower median aPL titers than positives in agreement (►**Fig. 1**). However, for the majority of discrepancies observed, respective individuals suffered from clinical manifestations of APS (thrombosis and/or pregnancy morbidity) (►**Fig. 1**). Clinical implications of the observed (dis)agreements were assessed by calculating the sensitivity, specificity, NPV and PPV and OR for thrombosis, pregnancy morbidity or both clinical criteria (►**Table 5**). Positivity was defined when at least one aPL was positive. Clinically affected and non-clinically affected patients were set as outcome variable rather than APS/non-APS to be independent of aPL presence previously detected by the medical centres that collected the samples, minimizing selection bias. Independent from the assay used, a statistically significant association with thrombosis and/or pregnancy morbidity was found. ORs for thrombosis varied between 1.98 (95% CI, 1.46–2.69) and 2.56 (95% CI, 1.82–3.59) detected by ImmunoCapEliA and BioPlex2200, respectively. ORs for pregnancy morbidity ranged between 3.42 (95% CI, 2.32–5.05) and 4.78

Table 1 Demographic and clinical characteristics of the study population

	Patients (*n*)	Female	Age [year, mean (range)]	Pregnancy morbidity	Thrombosis				
					Venous	Arterial	Venous and arterial	Small vessel	Not specified
APS thrombosis	259	164 (63%)	50 (17–87)	22	160	55	26	4	14
Non-APS thrombosis	204	116 (57%)	46 (19–85)	0	149	47	5	0	3
APS obstetric	122	122 (100%)	35 (19–61)	122	4	2	0	0	4
Non-APS obstetric	33	33 (100%)	33 (20–52)	33	NA	NA	NA	NA	NA
Autoimmune diseases	196	158 (81%)	46 (16–83)	NA	NA	NA	NA	NA	NA
Normal pregnancy	100	100 (100%)	31 (27–36)	0	NA	NA	NA	NA	NA
Controls	194	170 (88%)	39 (18–82)	NA	NA	NA	NA	NA	NA
Unspecified APS	60	60 (100%)	48 (24–70)	NS	NS	NS	NS	NS	NS
Total population	1,168	923 (79%)	43 (16–87)	177	313	104	31	4	21

Abbreviations: APS, anti-phospholipid syndrome; NA, not applicable; NS, not specified.

Table 2 Number of samples positive for aCL IgG, aCL IgM, aβ2GPI IgG or aβ2GPI IgM by BioPlex2200, ImmunoCapEliA, ACL AcuStar and QUANTA Lite ELISA are compared pairwise

		BioPlex 2200		ImmunoCap EliA		ACL AcuStar	
		–	+	–	+	–	+
BioPlex 2200	–						
	+						
ImmunoCap EliA	–	755	37				
	+	108	268				
ACL AcuStar	–	795	11	717	89		
	+	68	294	75	287		
QUANTA Lite ELISA	–	792	64	751	105	753	103
	+	71	241	41	271	53	259

Abbreviations: aCL, anti-cardiolipin; aβ2GPI, anti-β2 glycoprotein I; Ig, immunoglobulin.

(95% CI, 3.14–7.27) detected by ImmunoCapEliA and QUANTA Lite ELISA, respectively. Although not the most sensitive aPL detection platform for thrombosis and/or pregnancy morbidity, the QUANTA Lite ELISA resulted in the highest specificity (87.76% [95% CI, 84.52–90.52%]) and OR (4.24 [95% CI, 3.10–5.79]). PPVs for thrombosis and/or pregnancy morbidity ranged from 74.73% (95% CI, 70.74–78.35%) to 80.77% (95% CI, 76.47–84.44%) and NPVs ranged from 49.71% (95% CI, 48.07–51.35%) to 50.23% (95% CI, 48.57–51.90%) as shown in ►**Table 5**. The area under the ROC curve of the solid phase assays for thrombosis and/or pregnancy morbidity was low as expected and was not significantly different among the tested solid phase assays.

BioPlex2200 aCL and aβ2GPI IgG showed the highest and equal sensitivity for both MoAB P2–6, that recognizes β2GPI independently of its conformation, and P1–117, which only recognizes β2GPI upon exposure of the Gly40-Arg43 epitope in domain I (►**Table 6**). ACL AcuStar aCL and aβ2GPI IgG sensitivity was slightly lower for P1–117, in the same extent for aCL and aβ2GPI. QUANTA Lite ELISA and ImmunoCapEliA showed much lower sensitivity for P2–6 and P1–117, with large difference in sensitivity for P2–6 and P1–117. Both aCL assays did not detect P1–117 at all. OR for thrombosis and/or pregnancy morbidity for aCL and aβ2GPI (►**Table 5**) ranged from 4.24 to 6.12 and 3.49 to 6.56, respectively. The platform (BioPlex2200) with the highest sensitivity in detecting the P1–117 MoAB also reached the highest OR for thrombosis, but not for pregnancy morbidity.

Discussion

APS classification strongly depends on the laboratory criteria. Besides the clinical criteria, thrombosis or pregnancy morbidity, often due to other causes than aPL, APS is defined by the persistent presence of aPL.[1] aPLs are detected by LAC assays or by semi-quantitative solid phase assays measuring aCL IgG,

Table 3 Discrepancies in aPL positivity detection between platforms

		BioPlex 2200		ImmunoCap EliA		ACL AcuStar	
		–	+	–	+	–	+
BioPlex 2200	–						
	+						
ImmunoCap EliA	–	892	59				
	+	25	190				
ACL AcuStar	–	905	37	913	30		
	+	12	212	40	185		
QUANTA Lite ELISA	–	897	78	913	46	919	58
	+	20	171	22	169	24	167
		BioPlex 2200		ImmunoCap EliA		ACL AcuStar	
		–	+	–	+	–	+
BioPlex 2200	–						
	+						
ImmunoCap EliA	–	895	69				
	+	18	184				
ACL AcuStar	–	874	5	867	14		
	+	39	248	99	188		
QUANTA Lite ELISA	–	909	115	964	62	877	149
	+	4	138	2	140	4	138
		BioPlex 2200		ImmunoCap EliA		ACL AcuStar	
		–	+	–	+	–	+
BioPlex 2200	–						
	+						
ImmunoCap EliA	–	930	28				
	+	119	91				
ACL AcuStar	–	989	9	897	101		
	+	60	110	61	109		
QUANTA Lite ELISA	–	971	27	906	92	946	52
	+	78	92	52	118	52	118
		BioPlex 2200		ImmunoCap EliA		ACL AcuStar	
		–	+	–	+	–	+
BioPlex 2200	–						
	+						
ImmunoCap EliA	–	1,007	23				
	+	29	109				
ACL AcuStar	–	1,028	26	1,024	30		
	+	8	106	6	108		
QUANTA Lite ELISA	–	997	19	996	20	1,006	10
	+	39	113	34	118	48	104

Abbreviations: aCL, anti-cardiolipin; aβ2GPI, anti-β2 glycoprotein I; aPL, anti-phospholipid antibodies; Ig, immunoglobulin.
Note: Number of samples positive for aCL IgG, aβ2GPI IgG, aCL IgM and aβ2GPI IgM by BioPlex2200, ImmunoCapEliA, ACL AcuStar and QUANTA Lite ELISA are compared pairwise.

aCL IgM, aβ2GPI IgG or aβ2GPI IgM antibodies.[1] Many studies on head-to-head comparisons of solid phase assays with different study designs, already have shown that solid phase assays differ in performance and agreement. In our study, we analysed a large cohort of APS and non-APS patients simultaneously with four different platforms, allowing comparison of different methods on the same patient population. LAC was determined by the local centre but excluded from the

Table 4 Correlation between aCL IgG, aCL IgM, aβ2GPI IgG and aβ2GPI IgM titers of solid phase assays

	aCL IgG (95% CI)	aCL IgM (95% CI)	aβ2GPI IgG (95% CI)	aβ2GPI IgM (95% CI)
BioPlex2200 vs. ImmunoCapEliA	0.712 (0.682–0.739)	0.514 (0.471–0.555)	0.784 (0.716–0.806)	0.739 (0.712–0.764)
BioPlex2200 vs. ACL AcuStar	0.767 (0.742–0.789)	0.803 (0.781–0.822)	0.900 (0.889–0.911)	0.813 (0.792–0.831)
BioPlex2200 vs. QUANTA Lite ELISA	0.744 (0.717–0.768)	0.586 (0.547–0.622)	0.676 (0.644–0.706)	0.701 (0.671–0.729)
ImmunoCapEliA vs. ACL AcuStar	0.635 (0.599–0.668)	0.521 (0.478–0.562)	0.758 (0.732–0.781)	0.775 (0.752–0.797)
ImmunoCapEliA vs. QUANTA Lite ELISA	0.716 (0.687–0.743)	0.562 (0.521–0.600)	0.686 (0.655–0.716)	0.764 (0.739–0.787)
ACL AcuStar vs. QUANTA Lite ELISA	0.673 (0.640–0.703)	0.580 (0.541–0.617)	0.632 (0.596–0.665)	0.738 (0.711–0.763)

Abbreviations: aCL, anti-cardiolipin; aβ2GPI, anti-β2 glycoprotein I; CI, Confidence interval; Ig, immunoglobulin.
Note: Spearman Rank Correlation rho coefficients with their respective 95% confidence intervals are shown

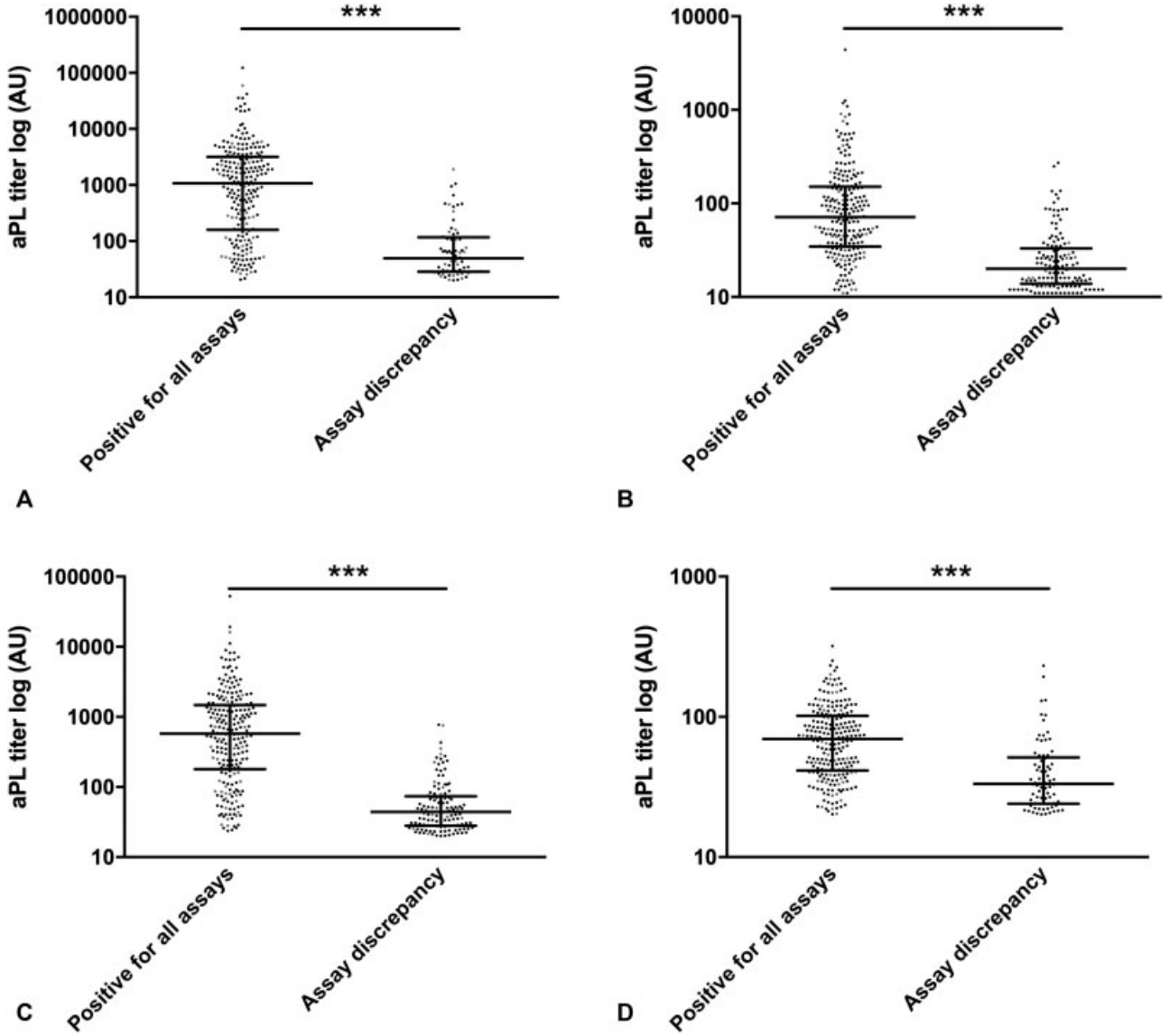

Fig. 1 Anti-phospholipid antibodies (aPL) titers of samples in (dis)agreement. Titers of samples positive for all assays and assay discrepancies are shown as detected with (**A**) BioPlex2200, (**B**) ImmunoCapEliA, (**C**) ACL AcuStar and (**D**) QUANTA Lite ELISA. Patients with thrombosis and/or pregnancy morbidity are indicated in black and without in red. Titers are expressed as the median value of positive aPL titers with interquartile ranges. $^{***}p < 0.001$. AU, arbitrary units.

Table 5 Diagnostic accuracy of aPL detection by BioPlex2200, ImmunoCapEliA, ACL AcuStar and QUANTA Lite ELISA

		Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	PPV (%, 95% CI)	NPV (%, 95% CI)	Odds ratio (95% CI)	AUC[a] (95% CI)
Thrombosis	BioPlex2200	31.32 (27.12–35.76)	84.87 (80.92–88.28)	71.08 (65.21–76.32)	51.00 (49.14–52.86)	2.56 (1.82–3.59)	0.58 (0.55–0.61)
$N = 853$[b]	ImmunoCapEliA	36.29 (31.90–40.85)	77.69 (73.23–81.73)	65.88 (60.75–70.66)	50.67 (48.5–52.84)	1.98 (1.46–2.69)	0.57 (0.54–0.60)
	ACL AcuStar	35.85 (31.48–40.41)	79.74 (75.41–83.62)	67.76 (62.50–72.59)	51.15 (49.04–53.26)	2.20 (1.61–3.00)	0.58 (0.54–0.61)
	QUANTA Lite ELISA	31.10 (26.91–35.54)	84.62 (80.65–88.05)	70.59 (64.71–75.86)	50.85 (48.99–52.71)	2.48 (1.77–3.48)	0.58 (0.55–0.61)
Pregnancy morbidity	BioPlex2200	38.06 (30.39–46.20)	87.55 (84.30–90.34)	49.17 (41.52–56.85)	81.71 (79.73–83.55)	4.32 (2.84–6.58)	0.63 (0.59–0.67)
$N = 645$[c]	ImmunoCapEliA	45.16 (37.17–53.35)	80.61 (76.83–84.02)	42.42 (36.45–48.63)	82.29 (80.01–84.36)	3.42 (2.32–5.05)	0.63 (0.59–0.67)
	ACL AcuStar	42.58 (34.68–50.77)	82.45 (78.79–85.71)	43.42 (37.06–50.01)	81.95 (79.76–83.95)	3.48 (2.35–5.17)	0.63 (0.59–0.66)
	QUANTA Lite ELISA	40.00 (32.22–48.17)	87.76 (84.52–90.52)	50.82 (43.22–58.38)	82.22 (80.19–84.08)	4.78 (3.14–7.27)	0.64 (0.60–0.68)
Clinical criteria	BioPlex2200	35.99 (32.37–39.73)	87.55 (84.30–90.34)	80.00 (75.60–83.78)	49.71 (48.07–51.35)	3.95 (2.90–5.39)	0.62 (0.59–0.65)
$N = 1{,}168$	ImmunoCapEliA	41.45 (37.71–45.26)	80.61 (76.83–84.02)	74.73 (70.74–78.35)	49.87 (47.96–51.79)	2.94 (2.24–3.86)	0.61 (0.59–0.65)
	ACL AcuStar	40.71 (36.98–44.51)	82.45 (78.79–85.71)	76.24 (72.19–79.87)	50.12 (48.26–51.99)	3.23 (2.44–4.26)	0.62 (0.59–0.64)
	QUANTA Lite ELISA	37.17 (33.52–40.93)	87.76 (84.52–90.52)	80.77 (76.47–84.44)	50.23 (48.57–51.90)	4.24 (3.10–5.79)	0.63 (0.60–0.65)

Abbreviations: aPL, anti-phospholipid antibodies; APS, anti-phospholipid syndrome; AUC, area under the curve; CI, confidence interval; LAC, lupus anticoagulant; NPV, negative predictive value; PPV, positive predictive value.
Note: Samples were defined as positive if at least one aPL was positive, excluding LAC.
[a]AUC from the receiver operator curve (ROC) were not significantly different between solid phase assays.
[b]APS thrombosis + non-APS thrombosis + AID + controls.
[c]APS obstetric + non-APS obstetric + AID + normal pregnancy + controls.

Table 6 Threshold for positivity: titer of MoAB corresponding to a positive titer of aCL or aβ2GPI IgG

	aCL IgG				aβ2GPI IgG			
	BioPlex 2200	ImmunoCap EliA	ACL AcuStar	QUANTA Lite ELISA	BioPlex 2200	ImmunoCap EliA	ACL AcuStar	QUANTA Lite ELISA
P2–6 (μg/mL)	1.95	125	15.63	62.5	1.95	31.25	1.95	62.5
P1–117 (μg/mL)	1.95	Neg[a]	31.25	Neg[a]	1.95	125	7.81	125

Abbreviations: aCL, anti-cardiolipin; aβ2GPI, anti-β2 glycoprotein I; Ig, immunoglobulin; MoAB, monoclonal antibodies.
[a]Negative results for all concentrations.

comparative analysis to investigate the variation solely introduced by solid phase assays. aPLs have a low prevalence (1–5%) in the general population, and APS is even more infrequent (40–50/100,000 persons).[19,20] As a consequence, studies comparing aPL detection methods are often based on a small patient group and/or lack diagnostic accuracy. We investigated the (dis)agreement and diagnostic accuracy of four commercially available solid phase assays for the detection of aCL IgG, aCL IgM, aβ2GPI IgG and aβ2GPI IgM antibodies in 1,168 samples from eight European centres.

As in the Sydney criteria, all individual aPL are part of the laboratory criteria for APS, diagnostic accuracy was assessed for positivity for at least one aPL detected by a solid phase assay (aCL IgG, aCL IgM, aβ2GPI IgG or aβ2GPI IgM).[1] Positivity for at least one aPL detected by a solid phase assay was significantly correlated with thrombosis and/or pregnancy morbidity, independent from the solid phase assay used. The highest OR for thrombosis was obtained by detection of aPL by BioPlex2200 (2.56 [95% CI, 1.82–3.59]). Detection of aPL by ImmunoCapEliA resulted in the lowest OR (1.98 [95% CI, 1.46–2.69]), mainly because of lower specificity. aPL detection by BioPlex2200 and QUANTA Lite ELISA resulted in similar OR for thrombosis and/or pregnancy morbidity, higher than OR obtained by detection with ImmunoCapEliA and ACL AcuStar. Calculated sensitivity,

NPV and AUC for thrombosis and/or pregnancy morbidity were low as expected, since we included patients with thrombosis and/or pregnancy morbidity without APS (negative for aPL). Inclusion of only APS patients would lead to a selection bias as diagnosis of APS is dependent on the aPL detection assays used by the local centre.

In our study, a maximum discrepancy of 36, 60, 53 and 36% for aCL IgG, aCL IgM, aβ2GPI IgG and aβ2GPI IgM positivity was found, respectively. Detection of aCL IgG and aβ2GPI IgM resulted in the best agreement. However, still a substantial number of samples were in disagreement. Conversely, a study comparing different kits detecting aCL and aβ2GPI IgG/IgM found better agreement between aβ2GPI ELISAs than aCL ELISAs.[21] This might be a consequence of the small number of aβ2GPI positive samples in this study.[21] Another study compared the performance of aCL IgG and IgM antibody detection of the ImmunoCapEliA with their in-house aCL ELISA in 1,143 routine samples.[22] The authors reached a good agreement ($>$ 90%) between the automated systems and their in-house ELISA.[22] As expected, positivity for aPL proved to be relatively rare, because of which the majority of routine samples were classified as negative, independent of the method used, resulting in a high agreement. Importantly, more samples were classified positive by only ImmunoCapEliA or only the in-house ELISA than by both methods (116/179 and 72/90 for aCL IgG and aCL IgM, respectively), suggesting a poor agreement comparable to our findings.[22] We observed large differences in positivity across platforms, even between automated systems sharing the same solid phase (BioPlex2200 vs. ACL AcuStar). In a collaborative study, the inter-laboratory variability of aβ2GPI IgG and aβ2GPI IgM antibodies was assessed in 30 serum samples from 22 centres.[23] Poor agreement was found between centres as positivity ranged from 50 to 93% and 13 to 70% for aβ2GPI IgG and aβ2GPI IgM detection, respectively.[23] We excluded inter-laboratory variability and found the best agreement between BioPlex2200 and ACL AcuStar. However, OR for clinical features of APS obtained by BioPlex2200 were more comparable with an ELISA (QUANTA Lite ELISA) than an assay sharing the same solid phase (ACL AcuStar). BioPlex2200 and QUANTA Lite ELISA were in poor agreement but characterized by a comparable diagnostic accuracy. Our results highlight the importance of measuring both the agreement between assays as well as their diagnostic accuracy. It seems also important that in daily practice the four aPLs are measured with a same platform. Although some small differences exist in the diagnostic performance of the tested platforms, the values of sensitivity and specificity for APS-related clinical symptoms and OR for clinical events are essentially comparable. This may be explained by the higher agreement that was observed in samples with higher levels of aPL (►**Fig. 1**). That newer generation ELISA and automated systems show low agreement but comparable diagnostic accuracy was also illustrated in other studies.[9,10,24–26]

In our study, guidelines from the SSC were followed by confirming manufacturer's cut-off values in at least 20 healthy volunteers.[16] In practice, most laboratories transfer their cut-off values similarly, predominately due to practical difficulties to calculate the 99th percentiles in a population of at least 120 healthy volunteers. Positivity for aPL not in agreement across the assays were characterized by lower median aPL titers. The majority of samples positive for aCL and/or aβ2GPI IgG/IgM detected by one assay, but not for all solid phase assays, fulfilled the clinical criteria of APS, suggesting that higher cut-off values result in reduced sensitivity for APS. The clinical relevance of aPL levels below the 99th percentile, needs to be further studied.[27] Lower levels of antibodies are observed especially in obstetric APS.[28,29]

Variability between aPL detecting assays is hypothesized to result from pre-analytical, analytical and post-analytical conditions, calibration and assay-specific issues.[30,31] In our study, detection of aPL and analysis was performed by a single operator, eliminating inter-laboratory and inter-operator variation. Traditionally, aCL and aβ2GPI antibodies are detected by ELISAs. Nowadays, automated systems have become available which are hypothesized to improve agreement.[8] Automation of assays indeed improved intra-laboratory and inter-laboratory reproducibility compared with non-automated ELISA. In general, ELISAs have shown large inter-laboratory variation and limited consensus in external quality control programs.[11,12] The lack of international calibration standards makes the comparison between assays challenging. Efforts have been made for standardization by international reference materials, such as the Harris standards (pool of patient material and thus limited in production) and Koike standards (directed against a single epitope, thus decreasing the sensitivity of the assay in which it is used as aPL), although not reflecting the real life since aPL of patients are a heterogeneous group of antibodies.[31] Variation in aPL detection might be introduced by the heterogeneous origin of aPL, differences in assay design and a lack of standardization. All four tested aβ2GPI antibody detection assays make use of an antigen of human origin. However, for detection of aCL antibodies only BioPlex2200 uses human β2GPI and non-animal derived cardiolipin. Human antigen source is considered more specific than animal CL and/or β2GPI.[6] Different preparations of human β2GPI have shown not to influence agreement in aβ2GPI IgG and IgM detection.[32] Indeed, BioPlex2200 and QUANTA Lite ELISA showed high specificity for thrombosis and/or pregnancy morbidity. However, our results show that the QUANTA Lite ELISA using purified cardiolipin and bovine β2GPI as antigen source resulted in the highest specificity for thrombosis and/or pregnancy morbidity.

Anti-β2GPI immunoassays detect antibodies against all five domains of β2GPI, including non-pathogenic antibodies, phospholipid-independent and low affinity aβ2GPI.[6,33] The use of negative surface charge of the solid phase in aβ2GPI ELISAs have shown to increase the antigen density and exposure of cryptic epitopes of β2GPI such as Gly40-Arg43 in domain I, mimicking the binding between negatively charged phospholipids and β2GPI.[6,13,14] Variation in solid phase may lead to variability in exposure of the epitope Gly40-Arg43.[15] Hence, antibodies against the Gly40-Arg43 epitope are considered pathogenic and have shown to highly correlate with thrombosis.[13,14,34] We used patient-derived MoAB P1–117 and P2–6 to verify and assess variability in exposure of epitopes of β2GPI.[7,15,17] We confirmed that the platform most sensitive in detecting P1–117

(the MoAB reactive against Gly40-Arg43 in domain I) by both the aCL and aβ2GPI IgG assays has the highest OR for thrombosis. Other platforms are less sensitive in detecting P1–117 compared with P2–6 (the MoAB recognizing β2GPI irrespective of its conformation), resulting in lower OR for thrombosis.

In conclusion, we found poor agreement between commercially available immunoassays detecting aCL and aβ2GPI IgG/IgM antibodies, which may hamper uniformity in the classification of aPL positive samples. However, computed OR for thrombosis and/or pregnancy morbidity in our study, considering results of the four aPL together, were globally concordant among solid phase test systems. Since our comparison between systems is based on considering measurement of the four aPL within one test system, classification and follow-up of patients for aPL is preferable when performed with the same system.

What is known about this topic?

- Detection of anti-phospholipid antibodies by solid phase assays is poorly standardized.
- Detection of patients positive for anti-cardiolipin and anti-β2GPI antibodies is assay dependent.

What does this paper add?

- Solid phase assays show comparable association for thrombosis and pregnancy morbidity.
- Exposure of Gly40-Arg43 of β2GPI in commercial assays is important to detect thrombotic APS.

Conflict of Interest
None declared.

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