Autoimmunity Reviews 17 (2018) 1210–1218

Contents lists available at ScienceDirect

Autoimmunity Reviews

journal homepage: www.elsevier.com/locate/autrev

The clinical value of assays detecting antibodies against domain I of β2-glycoprotein I in the antiphospholipid syndrome

Dongmei Yin[a,b], Bas de Laat[a,b], Katrien M.J. Devreese[c], Hilde Kelchtermans[a,b,*]

[a] Cardiovascular Research Institute Maastricht, Maastricht University Medical Center, Maastricht, the Netherlands,
[b] Synapse Research Institute, Maastricht, the Netherlands
[c] Coagulation Laboratory, Department of Laboratory Medicine, Ghent University Hospital, Department of Diagnostic Sciences, Ghent University, Ghent, Belgium

ARTICLE INFO

Keywords:
Antiphospholipid syndrome
Antiphospholipid antibody
β2-glycoprotein I
Domain I
Assay
Thrombosis

ABSTRACT

As the clinical symptoms of the antiphospholipid syndrome (APS) frequently occur irrespective of the syndrome, diagnosis predominantly depends on the laboratory assays measuring the level or function of antiphospholipid antibodies (aPLs). β2-glycoprotein I (β2GPI) is increasingly accepted as the most important target of aPLs. Anti-β2GPI antibodies constitute a heterogeneous population, but current in vivo and in vitro evidence show that especially the first domain (DI) of β2GPI contains an important pathogenic epitope. This epitope containing Glycine40-Arginine43 (G40-R43) has proven to be cryptic and only exposed when β2GPI is in its open conformation. A previous study demonstrated a highly variable exposure of the cryptic epitope in commercial anti-β2GPI assays, with implications on correct patient classification. Unexpectedly, recent unpublished data revealed impaired exposure of the pathogenic epitope in the commercially available anti-DI chemiluminescence immunoassay (CIA) assay detecting specific antibodies directed to DI.

In this review we summarize the laboratory and clinical performance characteristics of the different anti-DI assays in published data and conclude with inconsistent results for both the correlation of anti-DI antibodies with clinical symptoms and the added value of anti-DI antibodies in the classification criteria of APS. Additionally, we hypothesize on possible explanations for the observed discrepancies. Finally, we highly advise manufacturers to use normal pooled plasma spiked with the monoclonal anti-DI antibodies to verify correct exposure of the cryptic epitope.

1. Introduction

APS is a systemic autoimmune disease characterized by thrombotic complications, either venous, arterial, or small-vessel thrombosis, or pregnancy-related morbidity. The latter includes fetal death, premature births attributed to placental insufficiency, eclampsia or severe pre-eclampsia and spontaneous abortions [1]. Given the high frequency of these clinical symptoms irrespective of the syndrome, apart from a clinical characteristic an APS patient needs to fulfil at least one of the laboratory criteria [2]. Current revised laboratory criteria for APS classification detect the presence of aPLs through a combination of laboratory assays, including one functional coagulation assay lupus anticoagulant (LAC) and two immunological assays measuring immunoglobulin (Ig) G and/or IgM anti-cardiolipin antibodies (anti-CL) and IgG and/or IgM anti-β2GPI antibodies (anti-β2GPI). To avoid false positive tests due to infections, positive tests should be repeated with an interval of at least 12 weeks [3]. Of note, the presence of these aPLs has also been demonstrated to associate with other clinical symptoms that are not included in the APS criteria, such as epilepsy and migraine [4,5].

Probably due to the fact that LAC measures a functional effect of the antibodies, LAC is a better predictor of thrombosis than the quantitative solid-phase immunoassays. However, currently it is not advised to carry out LAC tests during treatment with direct oral anticoagulants because of the risk of false-positive results [6]. The exact pathogenesis of APS is unknown, but aPLs have been described to activate monocytes, neutrophils, dendritic cells and the placental tissue (summarized in [7]). Despite the fact that many different proteins have been identified as being involved in the pathogenesis of APS, accumulating evidence from in vitro experiments as well as animal studies has revealed that β2GPI is the main target for aPLs [8–10]. Based on this evidence, one would expect that testing for antibodies with reactivity towards β2GPI has a

* Corresponding author at: Synapse BV, Oxfordlaan 70, Maastricht 6229 EV, the Netherlands.
E-mail addresses: d.yin@thrombin.com (D. Yin), b.delaat@thrombin.com (B. de Laat), Katrien.Devreese@uzgent.be (K.M.J. Devreese), h.kelchtermans@thrombin.com (H. Kelchtermans).

https://doi.org/10.1016/j.autrev.2018.06.011
Received 12 June 2018; Accepted 18 June 2018
Available online 11 October 2018

good correlation with clinical manifestations. Although numerous studies have demonstrated a significant correlation [11–13], a meta-analysis performed by Galli et al. failed to show a significant correlation between single anti-β2GPI positivity with a history of thrombosis or fetal loss [14]. The lack of correlation with thrombotic complications as well as pregnancy morbidity was also shown in more recent studies [15–17].

The reason for this variation may at least in part result from the availability of numerous commercial and home-made anti-β2GPI assays and the lack of standardization [18,19]. The available assays differ from each other in assay design [method/principle: enzyme-linked immunosorbent assay (ELISA) versus chemiluminescence immunoassays (CIA)], the source of β2GPI and coating principles, the calculation of cut-off values, the reference material and the units used to express positivity [20,21]. Additionally, various subsets of anti-β2GPI antibodies targeting different domains of the protein have been described with clear differences in clinical potential [22–25]. β2GPI consists of five homologous domains (DI-DV) arranged differently in its native versus open conformation [26]. Importantly, in the native circular or S-shaped β2GPI the critical DI epitope is not exposed. Upon binding to an anionic (phospholipid) surface through the positively charged patch on DV, β2GPI undergoes a conformational change. Consequently, the DI-IV spreads out resulting in a more open J shape, exposing a cryptic epitope G40-R43 in DI and allowing anti-DI β2GPI autoantibodies to bind [27–29]. This subpopulation of aPLs that recognize an epitope on DI of β2GPI comprising at least G40-R43 proved to be pathogenic [27,30–37] and displayed a significant correlation with clinical manifestations [16,38]. The detection of antibodies to DI versus DIV/V has therefore been suggested to help in the diagnosis of APS [39,40].

As to the in vivo evidence for the pathogenicity of antibodies directed to DI, administration of recombinant human DI was found to inhibit the induction of thrombosis by polyclonal human IgG from patients with APS in a mouse model [41,42]. In addition, a recombinant antibody recognizing DI of β2GPI induced thrombosis and fetal loss in animal models following priming with lipopolysaccharide (LPS), while a CH2-deleted version of this antibody prevented the procoagulant and abortion-inducing effect of aPLs from APS patients [43]. In a proof-of-concept study, using polyclonal IgG from patients with APS, anti-DI-rich IgG significantly induced larger thrombi and enhanced the procoagulant activity in vivo compared with anti-DI-poor IgG [44].

Interestingly, a study in our laboratory demonstrated that the exposure of this pathogenic epitope G40-R43 on domain I was highly variable in commercial full-length anti-β2GPI assays. This reduced exposure of G40-R43 may account for the variable results obtained concerning the clinical correlation of the assays as reduced exposure was found to result in false negative classification of APS patients [45]. More recently, various assays specifically measuring anti-DI antibodies have been developed. As these assays are measuring a specific pathogenic population, one would expect anti-DI assays to highly correlate with clinical symptoms. Nonetheless, as for the full length anti-β2GPI assays, no consensus has been reached for the anti-DI antibodies concerning the correlation with clinical symptoms. Whereas some studies have demonstrated a higher correlation with thrombosis compared to the full-length assays, other studies failed to show an added value of anti-DI assays. In this review, we summarize the laboratory and clinical performance characteristics of the various anti-DI assays and elaborate on the possible reasons for the observed discrepancies.

2. Available anti-DI assays

So far five major assays have been described in literature to specifically measure antibodies directed to DI of β2GPI (summarized in Table 1). These five assays differ in the source of DI, the coating and measuring principle and the interpretation of results. As these factors may influence the clinical performance of the test, we aim to summarize and compare the sensitivity and specificity, the odds ratio (OR) of anti-

Table 1
Overview of assays used for the detection of anti-domain I antibodies (anti-DI).

Assay	Principle of the method	Source of DI	Solid phase	Surface capacity	Exposure of the epitope G40-R43	Expression of result	Inter-assay CV	References
Two-step ELISA	Antibodies are detected against DI coated on a hydrophilic versus hydrophobic plate by an in-house ELISA	Baculovirus expression system	Hydrophobic and hydrophilic ELISA plate	Normal	Yes	Dichotomous values (the ratio of OD on hydrophobic plate/ hydrophilic plate > 2 is positive)	3.7–14.8%	[16,38,46]
Direct ELISA	Antibodies are detected against DI coated on a nickel plate by an in-house ELISA	Bacteria expression system	Nickel chelate ELISA plate	Normal	NS	Continuous values (measured in arbitrary units)	< 10.0%	[35,47–49]
Competitive inhibition ELISA	The percentage inhibition is calculated when IC50 of DI in solution is used to inhibit antibodies binding to β2GPI coated on a flexible plate by an in-house ELISA	Chemical synthesis	ELISA plate	Normal	NS	The median percentage inhibition of antibodies are calculated	10.8%	[50,51]
Commercial developed ELISA	Antibodies are detected against DI coated on ELISA plates by a commercially developed ELISA	Baculovirus expression system	ELISA plate	Normal	NS	Continuous values (measured in arbitrary units)	15.0%	[24,40,53]
CIA for anti-DI (HemosIL AcuStar®and QUANTA Flash®, INOVA)	Antibodies are detected against DI coated on paramagnetic beads by a CIA	Baculovirus expression system	Paramagnetic beads	Higher	NS	Continuous values (measured in arbitrary units)	< 10.0%	[15,17,54,56–69]

Abbreviations: β2GPI: β2-glycoprotein I; CIA: chemiluminescence immunoassay; CV: coefficient of variation; DI: domain I of β2GPI; ELISA: enzyme-linked immunosorbent assay; IC50: the concentration of DI able to inhibit 50% of patient IgG antibodies binding to β2GPI; NS: not specified; OD: optical density.

Table 2
Clinical performance characteristics of different IgG anti-DI assays: the sensitivity and specificity for APS.

Publication		Study population				IgG anti-DI				IgG anti-β2GPI			
Year	First author	Design	Patients	N	Control	N	Assay	Cut-off	Sensitivity	Specificity	Assay	Cut-off	Sensitivity	Specificity
2009	de Laat B [16]	R,multi-center	APS	364	SLE + LLD	78	Two-step ELISA	Mean ± 3SD	59.9%	67.9%	In-house ELISA	Mean ± 10SD	NA	NA
2014	Mondejar R [57]	R	APS	39	HC + RD	77	CIA	20.0 AU (99thp.)	35.9%	97.4%	CIA	20.0 AU (99thp.)	46.2%	90.9%
2015	Meneghel L [58]	R	PAPS	88	SN-APS + HC + RD	229	CIA	7.1 CU (99thp.)	54.5%	97.6%	CIA	34.9 U/ml (99^{th}p.)	56.8%	96.4%
2016	De Craemer AS [60]	R	APS	101	AID + DC + HC	325	CIA	20.0 CU (Manuf.)	53.5%	97.8%	CIA	60.0 IU/ml (99^{th}p.)	56.4%	99.1%
2016	Oku K [64]	R	APS	61	SLE + non-SLE CTD + DC + ID + HC	150	CIA	20.0 CU/ml (99^{th}p.)	52.5%	100.0%	CIA	20.0 CU/ml (99^{th}p.)	75.0%	90.2%
2016	Pericleous C [49]	R	APS	111	SLE + HC	319	Direct ELISA	10.0 GDIU (99^{th}p.)	40.5%	95.9%	In-house ELISA	8.0 GBU (99^{th}p.)	64.8%	95.6%
2016	Zhang S [17]	R	APS	86	DC + SLE + HC	143	CIA	20.0 CU (Manuf.)	46.5%	97.9%	CIA	NS	66.3%*	92.3%*
2017	Iwaniec T [65]	R,CS	APS	103	SLE	99	CIA	13.8 CU (99^{th}p.)	62.5%**	82.1%**	CIA	NS	82.3%**	71.7%**
2017	Nakamura H [66]	CS	APS	51	AID	106	CIA	20.0 CU (99^{th}p.)	60.8%	100.0%	In-house ELISA	2.2 U/ml (99^{th}p.)	62.8%	96.2%

Abbreviations: APS: antiphospholipid syndrome; AID: non- APS autoimmune diseases; AU: arbitrary unit; β2GPI: β2-glycoprotein I; CIA: chemiluminescence immunoassay; CS: cross-sectional study; CU: chemiluminescence units; CTD: connective tissue diseases; DC: disease control i.e. non-APS with clinical symptoms of APS (thrombosis, pregnancy complication); DI: domain I of β2GPI; ELISA: enzyme-linked immunosorbent assay; GDIU: IgG anti-DI units; GBU: IgG anti- β2GPI units; HC: healthy control; ID: infectious disease; IU: international units; LLD: lupus like disease; Mean ± 3 or 10SD: mean optical density (OD) plus 3 or 10 times standard deviation (SD); Manuf.: manufacturer; N: number of population; NA: not application; NS: not specified; SLE: systemic lupus erythematosus; non-SLE CTD: non-SLE connective tissue diseases; PAPS: primary antiphospholipid syndrome; R: retrospective study; RD: rheumatic disease; SN-APS: seronegative APS; U: units; 99^{th}p.: 99^{th} percentile.
*IgG and/or IgM positive, ** for APS thrombotic complications.

DI for clinical manifestations, as well as the added value of anti-DI to the existing laboratory criteria when measured with the different available assays in various patient populations (summarized in Tables 2-4).

2.1. In-house developed two-step anti-DI ELISA assay

In 2005, the first anti-DI assay was developed by de Laat et al. [38]. The DI used for coating was produced by a baculovirus expression system as described before [34]. In this assay, DI is coated on a hydrophobic as well as a hydrophilic plate. The arbitrary binding of DI on a hydrophobic plate will ensure satisfactory exposure of the G40-R43 epitope. On the contrary, on a hydrophilic plate the positive epitope G40-R43 will be strongly directed downwards making the epitope not available for binding. The result of the assay is expressed as a ratio between the optical densities (OD) measured with the hydrophobic plate versus the hydrophilic plate. A ratio higher than two indicates DI reactivity of the sample.

This two-step anti-DI ELISA has been utilized in three studies [16,38,46]. In a single-center study including 198 patients with various systemic autoimmune diseases both IgG anti-DI and IgG anti-β2GPI antibodies measured with an in-house ELISA correlated strongly with a history of thrombosis [OR 18.9, 95% confidence interval (95% CI) 6.8–52.3; OR 6.7, 95% CI 3.4–13.5, respectively] [38]. In contrast, those with affinity for other domains of β2GPI were not significantly correlated with thrombosis (OR 1.1, 95% CI 0.4–2.8). Interestingly, only 58% of IgG anti-β2GPI positive patients were IgG anti-DI positive, and 93% of the anti-DI positive samples proved to be LAC positive [38]. These findings were confirmed in the consecutive multicenter study conducted on 442 patients positive for IgG/IgM anti-β2GPI [16]. The prevalence of IgG anti-DI antibodies positivity was 57% and IgG anti-DI was significantly associated with thrombosis (OR 3.5, 95% CI 2.3–5.4) and with obstetric complications (OR 2.4, 95% CI 1.4–4.3). On the other hand, no significant positive correlation was found for IgG anti-β2GPI with obstetric complications (OR 1.5, 95% CI 0.6–3.7), nor for IgG reactive against other domains of β2GPI with thrombosis (OR 0.4, 95% CI 0.3–0.6) [16].

In a separate study, 25% of 183 children with systemic lupus erythematosus (SLE) were found to be anti-DI positive, whereas all pediatric controls were negative [46]. A multivariate analysis showed that both the presence of anti-DI antibodies and LAC are independently associated with reduced annexin A5 anticoagulant activity [46]. Due to the relatively low rate for histories of thrombosis (3.8%) in the population, the study was not sufficiently powered to determine the association of anti-DI antibodies with thrombosis.

2.2. In-house developed direct anti-DI ELISA

In addition to the baculovirus system for recombinant DI, a bacterial expression system to produce human DI [47] was used to develop a simple direct ELISA that does not require the usage of hydrophobic versus hydrophilic plates. In a population consisting of 22 APS patients, 20 SLE patients and 10 healthy controls, significantly higher binding was observed for polyclonal purified IgG from APS patients compared to that from the other groups [35]. Interestingly, the creation of multiple mutants of DI using the same bacterial system identified that aside from G40-R43 also R39, the domain I-II interlinker, and possibly D8 and D9 play a major role in the binding to antibodies [35].

This assay has been used for detection of IgG anti-DI in two studies [48,49]. In a population of 40 seropositive APS and 40 seronegative APS patients (i.e. individuals with typical clinical features highly suggestive for APS, but persistently negative for laboratory criteria), the prevalence of anti-DI positivity was 27.5% in seropositive APS and 7.5% in seronegative APS [48]. Additionally in a population of 111 APS patients, 119 SLE patients and 200 healthy controls, a lower sensitivity of the IgG anti-DI compared to IgG anti-β2GPI measured by an in-house

Table 3
Clinical performance characteristics of different IgG anti-DI assays: the correlation of IgG anti-DI with clinical manifestations of APS by odd radios (ORs).

Publication		Study			IgG anti-DI					IgG anti-β2GPI				
Year	First author	Design	Population	N	Assay	Cut-off	OR (95%CI) for T	OR (95%CI) for P	OR (95%CI) for T/P	Assay	Cut-off	OR (95%CI) for T	OR (95%CI) for P	OR (95%CI) for T/P
2005	de Laat B [38]	R	176 SLE + 16 LLD + 6 PAPS	198	Two-step ELISA	Mean ± 3SD	**18.9 (6.8–53.2)**	NS	NS	In-house ELISA	Mean ± 3SD	**6.7 (3.4–13.5)**	NS	NS
			IgG anti-β2GPI positive	52	Two-step ELISA	Mean ± 3SD	**10.7**	NA	NA	In-house ELISA	Mean ± 3SD	NA	NA	NA
2009	de laat B [16]	R,multicerter	IgG/ IgM anti-β2GPI positive (364 APS + 93 SLE+ 35 LLD)	442	Two-step ELISA	Mean ± 3SD	**3.5 (2.3–5.4)**	**2.4 (1.4–4.3)**[#]	NS	In-house ELISA	Mean ± 10SD	NA	1.5 (0.6–3.7)[#]	NA
			IgG anti-β2GPI positive	420	Two-step ELISA	Mean ± 3SD	**3.3 (2.1–5.2)**	NS	NS	In-house ELISA	Mean ± 10SD	NA	NA	NA
2013	Akhter E [53]	CS	SLE	326	Commercial ELISA	25.0 U (97thp.)	1.1 (0.4–2.9)	NS	NS	Commercial ELISA	NS	**3.3 (1.2–8.9)**	NS	NS
2014	Mondejar R [57]	R	39 APS + 47 RD + 30 HC	116	CIA	20.0 AU (99thp.)	NS	NS	**21.0 (4.5–98.9)**	CIA	20.0 AU (99thp.)	NS	NS	**8.6 (3.2–23.3)**
2015	Andreoli L [40]	R	IgG anti-β2GPI positive (87 PAPS+42 RD+ 30 aPLs-carriers)	159	Commercial ELISA	15.0 AU (95thp.)	1.7	2.4[#]	NS	Commercial ELISA	NS	NA	NA	NA
2015	Pengo V [59]	CS	IgG anti-β2GPI positive	65	CIA	14.2 CU (99thp.)	**7.3**	**3.7**[$]	NS	CIA	19.4 CU (99thp.)	NA	NA	NA
2016	De Craemer AS [60]	R	101 APS + 123 AID+ 82 DC + 120 HC	426	CIA	20.0 CU (Manuf.)	**14.4 (6.0–34.8)**	NS	17.0 (7.1–40.5)	CIA	60.0 IU/ml (99thp.)	**29.2 (8.8–95.9)**	NS	**36.2 (11.1–117.9)**
			IgG/ IgM anti-β2GPI positive	74	CIA	20.0 CU (Manuf.)	**31.5 (5.4–182.1)**	NS	**27.0 (5.0–145.9)**	CIA	60.0 IU/ml (99thp.)	NS	NS	NS
			IgG anti-β2GPI positive	60	CIA	20.0 CU (Manuf.)	10.3 (0.6–166.7)	NS	6.6 (0.5–89.8)	CIA	60.0 IU/ml (99thp.)	NA	NA	NA
2016	Mahler M [61]	CS	APS	106	CIA	20.0 CU (99.5thp.)	**4.0**	NS	NS	CIA	20.0 CU (99.5thp.)	**2.3**	NS	NS
					CIA	190.2 CU (optimized)	**8.7**	NS	NS	CIA	164.6 CU (optimize)	**4.1**	NS	NS
2016	Marchetti T [62]	CC	143 S-PEecl+199 NS-PEecl+195 HC	537	CIA	14.4 AU (99thp.)	NA	**14.6 (1.8–115.6)** *[#]	NS	CIA	17.0 IU/ml (99thp.)	NS	**16.9 (3.7–77.1)** **[#]	NA
2016	Zhang S [17]	R	86 APS + 62 DC + 42 SLE + 39 HC	229	CIA	20.0 CU (Manuf.)	**3.3 (1.6–6.7)**	1.6 (0.6–3.7) [§]	NS	CIA	NS	**2.8 (1.5–5.1)**	1.3 (0.6–2.7) [§]	NS
2017	Iwaniec T [53]	R,CS	103 APS + 99 SLE	202	CIA	13.8 CU (99thp.)	**7.6 (4.0–14.5)**	NS	NS	CIA	NS	**11.8 (6.0–23.0)**	NS	NS
2017	Lee JS [8]	R	hypercoagulability	180	CIA	40.0 CU (99th p.)	**15.4 (4.3–54.9)**	NS	NS	CIA	20.0 CU (99th p.)	2.5 (0.98–6.5)	NS	NS
2017	Nojima J [67]		SLE	138	CIA	NS	**9.2 (2.5–34.2)**®	NS	NS	ELISA	NS	NS	NS	NS
2018	Chighizola CB [54]	CC	women IgG anti-β2GPI positive	130	CIA	20,0 CU (NS)	**5.4 (2.4–12.0)**	**2.4 (1.2–5.0)**	NS	CIA	20.0 CU (NS)	NA	NA	NA

Abbreviations: APS: antiphospholipid syndrome; AID: non- APS autoimmune diseases; aPLs-carriers: asymptomatic antiphospholipid antibodies carriers; AU: arbitrary units; β2GPI: β2 glycoprotein I; CC: case-control; CIA: chemiluminescence immunoassay; CS: cross-sectional study; CU: chemiluminescence units; DC: disease control, i.e. non-APS with clinical symptoms of APS (thrombosis, pregnancy complication); DI: domain I of β2-glycoprotein I (β2GPI); ELISA: enzyme-linked immunosorbent assay; HC: healthy control; IU: international units; LLD: lupus like disease; Mean ± 3 or 10SD: mean optical density (OD) plus 3 or 10 times standard deviation (SD); Manuf.: manufacturer; N: number of population; NA: not application; NS: not specified; NS-PEecl: non-severe pre-eclampsia; OR (95% CI): odds ratio (95% confidence interval); P: pregnancy morbidity; PAPS: primary antiphospholipid syndrome; R: retrospective study; RD: rheumatic disease; SLE: systemic lupus erythematosus; S-PEecl: serve pre-eclampsia; T: thrombosis; U: units; 95th/97th/99th/99.5thp.: 95th/97th/99th/99.5th percentile.

*by univariate analysis; **by multivariate analysis.

®OR (95% CI) for venous thrombosis;[#]OR (95% CI) for P: only women with at least 1 previous pregnancy were included; [$]OR (95% CI) for P: only women were included; [§]OR (95% CI) for P: only married women of reproductive age were included.

Bold: significant association of assay with clinical symptoms.

ELISA for APS (40.5% versus 64.8%) was demonstrated [49]. Single positivity for IgG anti-DI (hazard ratio (HR) 6.6, 95% CI 3.8–11.4) was strongly associated with APS, but to a lesser extent than single IgG anti-β2GPI (HR 33.4, 95% CI 13.0–86.1). However, in the same study, of 136 patients positive for IgG anti-CL or anti-β2GPI, 52 were also IgG anti-DI positive, and the presence of IgG anti-DI positivity raised the HR for APS approximately 3-fold [(HR 36.9, 95% CI 17.7–76.9) versus (HR 11.5, 95% CI 6.3–21.0)]. In addition, positivity for IgG anti-DI increased the strength of association between anti-CL/anti-β2GPI positivity measured by an in-house ELISA and thrombotic manifestations in the group of 111 patients with APS, suggesting anti-DI positivity can be used for thrombotic risk stratification. None of the tested profiles was significantly associated with pregnancy morbidity [49].

2.3. In-house developed competitive inhibition ELISA

A competitive inhibition ELISA assay was developed in which a chemically synthesized DI [50] was used to inhibit binding of antibodies in APS patients plasma to whole β2GP1 immobilized on a 96 wells plate [51]. In this assay, at first the DI concentration able to inhibit 50% of patient IgG binding to β2GPI (IC50) was calculated. Consecutively, this concentration of DI was used to calculate the percentage inhibition obtained in different patient categories. The level of inhibition proved to be higher in samples from triple positive APS patients (positive for LAC, IgG anti-CL and IgG anti-β2GPI) compared to double (positive for IgG anti-CL and IgG anti-β2GPI) or single positive (positive only for IgG anti-β2GPI) APS patients and healthy controls [51]. Since triple positive APS patients are at high risk of developing future thromboembolic events [52], this result supports the idea that IgG anti-DI antibodies play an important pathogenic role [51]. Interestingly, in preliminary experiments, anti-DI antibodies were not detected by direct coating of DI onto ELISA plates. More specifically, when plasma from APS patients and healthy controls was tested for IgG binding to the same chemically synthesized DI coated on either a hydrophobic or a hydrophilic plate, the IgG anti-DI levels did not differ between APS patients and healthy controls [51].

2.4. Commercially developed anti-DI ELISA

The first commercially developed assay to measure anti-DI antibodies was from INOVA Diagnostics. Recombinant DI of β2GP1 was expressed and purified from the baculovirus expression system [34]. This assay has been used for detection of IgG anti-DI in three patient studies [24,40,53].

Using this ELISA, IgG anti-DI antibodies were found to be the most prevalent antibodies (75%) in 64 patients with APS [24]. A low prevalence of IgG anti-DI was reported in 57 healthy children born to mothers with various systemic autoimmune diseases (AID) and 33 children with atopic dermatitis (16% and 27%, respectively). On the other hand, IgG antibodies recognizing DIV/V of β2GPI were preferentially detected in children (37% and 33%, respectively), whereas isolated IgG anti-DIV/V was rare (5%) in APS and not associated with thrombosis. This study speculated that antibodies targeting DI are pathogenic, whereas those reactive with DIV/V are probably 'innocent' [24]. In another study including 159 anti-β2GPI positive patients (measured by an in-house ELISA) [40], 70% of the 87 patients with primary APS (PAPS) were positive for anti-DI reactivity. 30 asymptomatic aPLs-carriers displayed significantly lower levels of anti-DI IgG and higher levels of anti-DIV/V compared to the PAPS group and the rheumatic disease (RD) group. Interestingly, no association was found between IgG anti-DI and APS classification, thrombosis and obstetric complications in a multivariate logistic regression model, although compared to patients with a single event, those with recurrent thrombosis displayed significantly higher titers of IgG anti-DI [40]. Likewise, IgG anti-DIV/V positivity was not associated with APS clinical manifestations thrombosis and pregnancy morbidity [40,54]. However, positive anti-DI reactivity was associated with triple positivity, suggesting it may be used as a risk stratification tool in APS patients. Additionally, the ratio of anti-DI to anti-DIV/V antibodies in this study emerged as an informative tool to identify those subjects carrying "nonpathogenic" or "less-pathogenic" anti-β2GPI antibodies [40]. Another study including 326 SLE patients showed that IgG anti-β2GPI (measured by Quanta Lite ELISA,INOVA) but not IgG anti-DI reactivity was significantly associated with thrombosis (OR 3.3, 95% CI 1.2–8.9; OR 1.1, 95% CI 0.4–2.9, respectively) [53].

2.5. CIA for anti-DI

Using the same recombinant DI of β2GP1 from the baculovirus expression system [34] coupled to paramagnetic beads, more recently a CIA assay [55] has been developed for the measurement of anti-DI antibodies. After incubation of the paramagnetic beads with serum samples, isoluminol-labeled anti-human IgG antibodies are incubated with the aPLs previously captured by the paramagnetic particles. Finally, an agent is added to induce chemiluminescence. Currently, two systems are available that measure anti-DI antibodies using this technology, including the BIO-FLASH CIA from INOVA Diagnostics, Werfen, Austria and the HemosIL Acustar CIA from Instrumentation Laboratory, Bedford, MA, USA. The BIO-FLASH CIA and the HemosIL Acustar CIA are identical assays using the same analytic method and reagent kits. Since 2014, this commercially available CIA anti-DI assay has been evaluated in 17 published studies [15,17,54,56–69].

For the classification of APS, the CIA IgG anti-DI assay has a higher specificity (from 82.1% to 100.0%) and a lower sensitivity (from 35.9% to 62.5%) compared to IgG anti-β2GPI test (from 71.7% to 99.1% and from 46.2% to 82.3% for specificity and sensitivity, respectively) among seven studies (summarized in Table 2). For the association with clinical manifestations of APS, the ORs of IgG anti-DI assays varied in eleven studies, ranging from 3.3 to 31.5 for thrombosis and from 1.5 to 14.6 for obstetric symptoms, probably originating from different patient and control cohorts and different cut-off values (summarized in Table 3). Few studies failed to demonstrate a significant association of anti-DI reactivity with thrombosis [63,68]. In one study on thrombotic APS patients, 54% of the patients displayed anti-β2GPI antibodies, versus only 25% for anti-DI [63]. IgG anti-DI proved not to be associated with the site of the first event of thrombosis (OR 0.6, 95% CI 0.2–1.9), thrombosis recurrence (OR 1.0, 95% CI 0.4–2.7) nor pregnancy morbidity (OR 1.5, 95% CI 0.3–7.3) [63]. Another study in 178 SLE patients indicated that both the IgG anti-DI titer and IgG anti-B2GPI titer were not associated with venous events (n = 22), arterial events (n = 20), composite venous events or arterial events (n = 37), respectively (P = .90, 0.76 and 0.89 for IgG anti-DI titer and P = .86, 0.84 and 0.86 for IgG anti-B2GPI titer, respectively) [68]. Looking at a total population of 426 APS and control patients or at a subpopulation of 74 IgM/IgG anti-β2GPI positive patients, IgG anti-DI positivity was significantly associated with thrombosis (OR 14.4, 95% CI 6.0–34.8 and OR 31.5, 95% CI 5.4–182.1, respectively) [60]. However, in a subgroup of 60 anti-β2GPI IgG positive patients from the same study no significant association of IgG anti-DI positivity with thrombosis was observed (OR 10.3, 95% CI 0.6–166.7) [60]. On the other hand, three out of five studies indicated IgG anti-DI was associated with pregnancy morbidity [54,59,62]. In a cross-sectional study with 65 positive anti-β2GPI IgG patients, pregnancy loss was present in 16 out of 39 women (41%) positive for anti-DI reactivity and in three out of 19 women (16%) with negative values. The association of IgG anti-DI with obstetrical APS nearly reached statistical significance (P = .07) [59]. In a case-control study including 195 control women, 199 non-severe pre-eclampsia patients and 143 severe pre-eclampsia patients, anti-DI IgG reactivity was associated with severe pre-eclampsia patients in the univariate analysis. However, in the final multivariate analysis, positive anti-β2GP1 IgG but not positive IgG anti-DI was identified as a risk factor for severe pre-eclampsia [62]. A recently published study

Table 4
The prevalence of IgG anti-DI positive in triple, double and single positive patients.

Publication		Study			IgG anti-DI positive			
Year	First author	Design	Population	N	Assay	Cut-off	Prevalence	*P* value
2011	Banzato A [51]	R	Triple positive [A]	22	Competitive inhibition ELISA	NA	25.5%	S*
			Double positive	15			5.0%	
			Single positive	9			2.0%	
			Control	20			0.0%	
2015	Andreoli L [40]	R	Triple positive [B]	87	Direct ELISA	15.0 AU (95thp.)	80.4%	S*
			Double/single positive	72			48.6%	
2015	Meneghel L [58]	R	Triple positive [B]	NS	CIA	7.1 CU (99^{th}p.)	94.1%	S*
			Double positive	NS			45.5%	
			Single positive	NS			4.8%	
			SN-APS/HC	NS			1.6%/0.6%	
2015	Pengo V [59]	CS	Triple positive [A]	32	CIA	14.2 CU (99^{th}p.)	97.0%	S*
			Double positive	23			43.0%	
			Single positive	10			10.0%	
2016	De Craemer AS [60]	R	Triple positive [B]	62	CIA	20.0 CU (Manuf.)	83.9%	S*
			Double positive	4			50.0%	
			Single positive	4			0.0%	
			Triple negative	222			0.9%	
2016	Montalvão S [63]	R	Triple positive [B]	13	CIA	20.0 CU (Manuf.)	46.0%	S*
			Double/single positive	28			17.0%	
2016	Zhang S [17]	R	Triple/Double/single positive [B]	229	CIA	20.0 CU (Manuf.)	NS	S*
2017	Iwaniec T [65]	R,CS	Triple positive [B]	79	CIA	13.8 CU (99^{th}p.)	308.2 [&]	S*
			Double positive	10			6.2 [&]	
			Single positive	14			2.0[&]	
2017	Lee JS [15]	R	Triple positive [A]	17	CIA	40.0 CU (99^{th}p.)	58.8%	S*
			Double positive	27			7.4%	
			Single positive	93			0.0%	
			Triple negative	43			0.0%	
2018	Chighizola CB [54]	CC	Triple positive [B]	82	CIA	20.0 CU (NS)	195.0 ± 628.3[§]	S*
			Double/single positive	53			4.0 ± 11.0[§]	
2018	Marchetti T [68]	Longitudinal study	Triple positive [B]	22	CIA	14.4 AU (99 thp.)	365.4 ± 596.1[§]	S*
			Double positive	13			137.9 ± 434.1[§]	
			Single positive	40			3.8 ± 8.7[§]	

Abbreviations: AU: arbitrary units; CIA: chemiluminescence immunoassay; CS: cross-sectional study; CU: chemiluminescence unit; DI: domain I β2-glycoprotein I (β2GPI); ELISA: enzyme-linked immunosorbent assay; HC: healthy control; Manuf.: manufacturer; N: number of population; NA: not applicable; NS: not specified; R: retrospective study; SN-APS: seronegative APS; 95th/99thp.: 95^{th}/99^{th} percentile; S*: significantly higher prevalence or levels of IgG anti-DI positive were found in patients with triple positivity, compared with patients with double and single aPL positivity; &: median value of IgG anti-DI; §: mean ± standard deviation (SD) value of IgG anti-DI; A: IgG triple positive = positive lupus anticoagulant (LAC), IgG anti-CL, IgG anti-β2GPI; B: Triple positive = positive LAC, IgG or IgM anti-CL, and IgG or IgM anti-β2GPI.

including 135 well-characterized female patients with persistent medium-high titer of anti-β2GPI antibodies and at least one pregnancy showed that reactivity against DI is a predictor for pregnancy morbidity (OR 2.4, 95% CI 1.2–5.0) [54]. More specifically, IgG anti-DI significantly predicted late pregnancy morbidity (OR 7.3, 95% CI 2.1–25.5) [54]. In addition to the clinical symptoms of APS included in the criteria, one study in 32 APS patients indicated that the non-criteria manifestations livedo reticularis ($n = 8$) and heart value disease ($n = 9$) were associated with higher levels of IgG anti-DI ($p = .005$ and $P = .01$, respectively) [56].

Furthermore, several studies assessed whether testing for anti-DI adds value to current criteria laboratory tests (summarized in Table 3). Upon comparison of IgG anti-DI assays and IgG anti-β2GPI assays, in four of seven studies the ORs of IgG anti-DI exceeded those of IgG anti-β2GPI for clinical manifestations (three for thrombosis, one for thrombosis and pregnancy morbidity) [15,17,57,61]. In the remaining three studies, despite significant correlation of IgG anti-DI with the clinical manifestations of APS, ORs of IgG anti-DI positivity proved to be lower than IgG anti-β2GPI positivity [60,62,65]. In severe pre-eclampsia patients, a significant association was shown for IgG anti-β2GPI but not IgG anti-DI in the final multivariate analysis [62]. Of note, one study also investigated the clinical significance of testing for IgG targeting other domains of β2GPI: neither thrombosis nor pregnancy morbidity was significantly correlated with IgG targeting other domains of β2GPI [17].

Different score systems have been formulated to quantify the risk of thrombosis/obstetric events in APS, including triple positivity (i.e. positive for LAC, IgG or IgM aCL, IgG or IgM anti-β2GPI) [70] and antiphospholipid score (aPL-S) [71]. Eleven studies showed that triple positive patients tend to have a significantly higher prevalence and higher levels of IgG anti-DI than those with double-positive or single-positive profile (summarized in Table 4). In 180 patients with hypercoagulability, the thrombotic risk of the newly defined triple positive group (positive for LAC, IgG anti-CL, IgG anti-DI) was more than twice than that of the triple positive group (positive for LAC, IgG anti-CL, IgG anti-β2GPI) [15]. Similarly, in 138 SLE patients, the test results of IgG anti-DI raised the accuracy of predicting thrombosis compared to the test results of anti-CL/ anti-β2GPI-ELISA, resulting in an increased area under the receiver operating characteristic (ROC) curve (AUC) (0.84 versus 0.80, respectively) [67]. However, in a retrospective study including 202 AID patients adding positivity for anti-DI to the triple positivity profile did not increase the predicting capacity for APS thrombotic complications [65]. Similar results were obtained in a cohort study in which no added value was demonstrated for anti-DI to the criteria panel [60]. Also in this study, patients with a high aPL-S were shown to display higher titers of anti-DI IgG [60]. In another study, an adjusted aPL-S was determined, measuring reactivity against DI instead of the whole molecule β2GPI (aPL-S-DI) [57]. When comparing the aPL-S-DI with the traditional aPL-S reaching the same specificity of 95%, the aPL-S-DI resulted in slightly lower OR for clinical symptoms of APS. However, when the optimal cut-off for each aPL-S was calculated, the aPL-S -DI resulted in the highest relative risk of having clinical

manifestations of APS [57]. In a separate study, testing IgG anti-DI and IgG/M anti-phosphatidylserine/prothrombin (PS/PT) showed a high positive predictive value for the diagnosis of APS and a strong correlation with the aPL-S was obtained [66]. Similarly, the thrombotic risk associated with the combination of IgG anti-PS/PT and IgG anti-DI was elevated 4.5 times compared to double positivity for IgG anti-PS/PT and IgG anti-β2GPI [15].

3. Combined results on the clinical value of anti-DI antibodies

3.1. Inconsistency on clinical value of anti-DI antibodies

Most studies have shown that anti-DI positivity significantly correlates with clinical manifestations of APS (**Tables 2, 3 and 4**). In terms of clinical performance, compared with the anti-β2GPI assays the anti-DI assays in general seem to be less sensitive, but (slightly) more specific for the diagnosis of APS (Table 2). As the anti-DI assays detect a pathogenic subpopulation of antibodies, the ORs for manifestations of APS were expected to be higher than the ORs of full length anti-β2GPI assays. However, results were inconsistent and dependent on the assays used to detect both anti-β2GPI and anti-DI reactivity (Table 3). Nonetheless, significantly higher titers and prevalence of anti-DI antibodies were found in high risk patients with triple positivity compared with double and single positive patients (Table 4). Moreover, adding of IgG anti-DI or IgG anti-DI instead of IgG anti-β2GPI in combined positive profile markedly raised the correlation with the risk of thrombosis in APS in several studies [15,49,66,67]. Furthermore, as with triple positivity, positive and negative values of IgG anti-DI in initial test were consistently confirmed after 12 weeks, illustrating that IgG anti-DI positivity is a robust and reproducible marker [59,72].

For different assays detecting IgG anti-DI, the two-step anti-DI ELISA in particular showed that the ORs of IgG anti-DI are markedly higher than the OR of IgG antibodies targeting the whole length β2GPI or other domains of the protein [16,38]. Moreover, this assay emphasized the exposure of epitope G40-R43 domain I is important for antibody binding. CIA is currently the most widely used method to detect IgG anti-DI antibodies. IgG anti-DI measured by CIA seem to represent a strong indicator for clinical manifestations of APS. However, the results of the added clinical value of IgG anti-DI are not consistent. Some studies showed that anti-DI display no added clinical value to the classical aPLs panel [60,62,65]. None of the remaining three assays, the direct anti-DI ELISA, the competitive inhibition ELISA and the commercial developed INOVA anti-DI ELISA showed an added value of IgG anti-DI compared to IgG anti-β2GPI. Taken together, the observed inconsistency probably explains why anti-DI antibodies have not yet been included in the laboratory criteria [19].

3.2. Towards an explanation for the observed discrepancies

The studies included in this review differ in study design, study population and the methodology to measure the anti-DI antibodies. These factors, together with the absence of standardization or calibration, make it very difficult to compare results of clinical studies and accurately assess the clinical value of measuring anti-DI. Even using the same assay for detection of anti-DI has led to discrepant results in determination of the added value of anti-DI antibodies. Differences in methods of calculating cut-off values, statistical analysis method, laboratory-specific handlings or protocols and different study populations may affect the interpretation of the results and lead to the observed discrepancies. Two studies independently showed the effect of different cut-off values: increasing cut-off values resulted in significantly higher ORs [61,73]. One study including patients with APS, AID, disease controls and healthy controls showed different results for correlation of IgG anti-DI positivity with thrombosis in the total population of 426 patients versus in 60 IgG anti-β2GPI positive patients [60].

A similar situation is observed for the anti-β2GPI assays, in which external quality assessment program reports show a wide variability in results over different centers [74–77]. Previous research has shown the importance of a certain antigen density to enable divalent binding of antibodies [78,79], as well as a hydrophilic solid phase surface to coat β2GPI thereby inducing a conformational change resulting in the exposure of a cryptic pathogenic epitope in DI. Antibodies directed to this cryptic epitope G40-R43 are a major pathological subset of antibodies [27–29,35–38,41]. Hence, results obtained by anti-β2GPI assays depend on the density and the conformation of the coated β2GPI (i.e. the exposure of the epitope G40-R43), which are affected by the type of solid phase surface used to immobilize β2GPI and source of protein. As in anti-DI assays DI is coated instead of the full protein, similar problems were not expected. However, taken into account the positive charge of epitope G40-R43, the charge of the coating surface possibly influences the availability of the G40-R43 epitope. A neutral coating surface is hypothesized to result in an arbitrary orientation of the DI, resulting in exposure of epitope G40-R43. On the other hand, a negative surface binds the positive epitope preventing its availability for antibodies. Based on this hypothesis and the evidence provided with the two-step ELISA, we assume that the differences in the type of solid phase surface used to immobilize DI and hence the exposure of the pathogenic DI epitope in the available anti-DI assays that add up to the variation in results. Looking at the other anti-DI assays, a possible charge of the beads cannot be excluded, rendering the exposure of epitope G40-R43 on DI uncertain. Exactly the same question can be made for the other available assays, as except for the two- step anti-DI ELISA, the charge of the solid phase surfaces used to immobilize DI are unknown. This may provide an explanation why both studies using the commercially developed anti-DI ELISA from INOVA Diagnostics to measure anti-DI showed that there is no significant association with thrombosis [40,53]. A clinical study using an in-house developed direct anti-DI ELISA showed significantly but less clinical value of anti-DI compared to anti-β2GPI [49]. Similarly, although with a competitive inhibition anti-DI ELISA a significant difference was found in patients with triple positivity compared with patients with double or single positivity and healthy controls, a direct anti-DI ELISA with the same DI did not find differences in IgG anti-DI between APS patients and controls, independent of using hydrophobic or hydrophilic plates, different coating/washing/blocking buffers and concentrations of DI [51]. In addition, although CIA can provide a greater surface for antibodies binding to DI, three studies showed significantly but less clinical value of IgG anti-DI compared to IgG anti-β2GPI [60,62,65]. This problem seems to be avoided in the in-house developed two-step ELISA, where the ratio between the OD measured with a hydrophobic plate versus hydrophilic plate is used to determine positivity.

We aimed to verify our hypothesis by determining the exposure of the pathogenic G40-R43 epitope in the commercially available aDI CIA method. Therefore, normal pooled plasma was spiked with two human-derived monoclonal antibodies P1-117 and P2-6. Antibody P1-117, recognizing epitope G40-R43 only available when β2GPI is in its open conformation, and P2-6, recognizing domain I independently of its conformation [80]. Our unpublished results show that P2-6 can be detected while no signal is obtained for P1-117 (data not shown). Contrary to the expectation, this anti-DI assay thereby does not expose the epitope G40-R43 on the surface of the beads, therefore patient samples with antibodies recognizing the epitope G40-R43 will be missed. Our results are consistent with previous results obtained with the CIA anti-β2GPI assay (HemosIL Acustar) in which P1-117 showed lower reactivity compared to P2–6, demonstrating a reduced G40-R43 availability [81]. In addition, a high agreement (69% ~ 92%) was observed between anti-β2GPI and anti-DI in the same CIA device [17,59,60,65], suggesting that both assays measure almost the same antibody population directed against domain I but not to the G40-R43 epitope. On the other hand, two studies detecting IgG anti-DI using the two-step ELISA found that approximately half (58% and 57%, respectively) of IgG anti-β2GPI bound DI [16,38]. Taken together, these

results suggest that the two-step ELISA measures a more specific anti-DI antibody population directed against G40-R43, compared to the commercially available anti-DI CIA assay detecting all antibodies to domain I, as well as to other domains. As a result, the two-step ELISA did show an added clinical value of the anti-DI positivity compared to the full-length anti-β2GPI, demonstrated by the higher OR for thrombosis and pregnancy morbidity [16,38]. Our data highlight the importance of not only measuring G40-R43 exposure in the full length anti-β2GPI assays, but also in the anti-DI assays to be certain that at least this pathogenic antibody population is not missed.

Apart from the coating principle, also the density of the coated DI and the source of DI may influence the results. Regarding the density, especially the CIA with paramagnetic beads provide a three-dimensional platform and larger surface for antibodies binding to DI [82]. Three different sources of DI are used in the available assays: DI obtained through a baculovirus expression system, an *Escherichia coli* expression system or by a chemical synthesis. So far, the effect of production on the conformation of DI and hence the availability of the pathogenic G40-R43 epitope is not known.

4. Conclusion

In summary, given all the in vitro and in vivo evidence, anti-DI assays were expected to be very powerful in the classification of APS patients. The majority of clinical studies did find a significant association of anti-DI antibodies with clinical symptoms of APS and a higher specificity was demonstrated compared with anti-β2GPI antibodies. As specifically antibodies to DI are detected, a more uniform result was expected, resulting in an improved risk stratification and tailored treatment for APS patients. However, multiple anti-DI assays are available and suffer from the same problems as the full-length anti-β2GPI assays. To assess the added value of anti-DI assays, several important issues urgently need to be addressed. As for anti-β2GPI assays, standardization of anti-DI assays is of utmost importance. This standardization in our view also includes the confirmation of a satisfactory exposure of the epitope G40-R43 to ascertain that at least one specific pathogenic antibody population is measured. Additionally, so far only retrospective studies were performed to determine the added value of DI antibodies. Prospective, well designed multicenter studies are urgently required to clarify the clinical utility of the anti-DI assays.

Declaration of interest

All authors declare that they have no conflicts of interests.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

[1] Ruiz-Irastorza G, Crowther M, Branch W, Khamashta MA. Antiphospholipid syndrome. Lancet 2010;376:1498–509.
[2] Ortel TL. Antiphospholipid syndrome: laboratory testing and diagnostic strategies. Am J Hematol 2012;87(Suppl. 1):S75–81.
[3] Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 2006;4:295–306.
[4] Asiful Islam M, Alam F, Cavestro C, Calcii C, Sasongko TH, et al. Antiphospholipid antibodies in epilepsy: a systematic review and meta-analysis. Autoimmun Rev 2018. https://doi.org/10.1016/j.autrev.2018.1001.1025. [Epub ahead of print].
[5] Islam MA, Alam F, Wong KK. Comorbid association of antiphospholipid antibodies and migraine: a systematic review and meta-analysis. Autoimmun Rev 2017;16:512–22.
[6] Hoxha A, Banzato A, Ruffatti A, Pengo V. Detection of lupus anticoagulant in the era of direct oral anticoagulants. Autoimmun Rev 2017;16:173–8.
[7] van den Hoogen LL, van Roon JA, Radstake TR, Fritsch-Stork RD, Derksen RH. Delineating the deranged immune system in the antiphospholipid syndrome. Autoimmun Rev 2016;15:50–60.
[8] Fischetti F, Durigutto P, Pellis V, Debeus A, Macor P, et al. Thrombus formation induced by antibodies to beta2-glycoprotein I is complement dependent and requires a priming factor. Blood 2005;106:2340–6.
[9] Arad A, Proulle V, Furie RA, Furie BC, Furie B. Beta(2)-Glycoprotein-1 autoantibodies from patients with antiphospholipid syndrome are sufficient to potentiate arterial thrombus formation in a mouse model. Blood 2011;117:3453–9.
[10] Jankowski M, Vreys I, Wittevrongel C, Boon D, Vermylen J, et al. Thrombogenicity of β2-glycoprotein I–dependent antiphospholipid antibodies in a photochemically induced thrombosis model in the hamster. Blood 2003;101:157–62.
[11] Kelchtermans H, Pelkmans L, de Laat B, Devreese KM. IgG/IgM antiphospholipid antibodies present in the classification criteria for the antiphospholipid syndrome: a critical review of their association with thrombosis. J Thromb Haemost 2016;14:1530–48.
[12] Abou-Nassar K, Carrier M, Ramsay T, Rodger MA. The association between antiphospholipid antibodies and placenta mediated complications: a systematic review and meta-analysis. Thromb Res 2011;128:77–85.
[13] Gris JC, Bouvier S, Nouvellon E, Lissalde-Lavigne G, Mercier E, et al. Antiphospholid antibodies and the risk of pregnancy complications. Thromb Res 2017;151(Suppl. 1):S34–7.
[14] Galli M, Luciani D, Bertolini G, Barbui T. Anti-beta 2-glycoprotein I, anti-prothrombin antibodies, and the risk of thrombosis in the antiphospholipid syndrome. Blood 2003;102:2717–23.
[15] Lee JS, Gu J, Park HS, Yoo HJ, Kim HK. Coexistence of anti-beta2-glycoprotein I domain I and anti-phosphatidylserine/prothrombin antibodies suggests strong thrombotic risk. Clin Chem Lab Med 2017;55:882–9.
[16] de Laat B, Pengo V, Pabinger I, Musial J, Voskuyl AE, 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.
[17] Zhang S, Wu Z, Chen S, Li J, Wen X, et al. Evaluation of the diagnostic potential of antibodies to beta2-glycoprotein 1 domain 1 in Chinese patients with antiphospholipid syndrome. Sci Rep 2016;6:23839.
[18] Devreese K. Standardization of antiphospholipid antibody assays.Where do we stand? Lupus 2012;21:718–21.
[19] Devreese KM, Ortel TL, Pengo V, de Laat B. Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibodies. Laboratory criteria for antiphospholipid syndrome: communication from the SSC of the ISTH. J Thromb Haemost 2018;16:809–13.
[20] Bertolaccini ML, Amengual O, Andreoli L, Atsumi T, Chighizola CB, et al. 14th International Congress on Antiphospholipid Antibodies Task Force. Report on antiphospholipid syndrome laboratory diagnostics and trends. Autoimmun Rev 2014;13:917–30.
[21] Devreese KM, Pierangeli SS, de Laat B, Tripodi A, Atsumi T, et al. Testing for antiphospholipid antibodies with solid phase assays: guidance from the SSC of the ISTH. J Thromb Haemost 2014;12:792–5.
[22] Pelkmans L, de Laat B. Antibodies against domain I of beta2-glycoprotein I: the one and only? Lupus 2012;21:769–72.
[23] Pengo V, Ruffatti A, Tonello M, Hoxha A, Bison E, et al. Antibodies to Domain 4/5 (Dm4/5) of beta2-Glycoprotein 1 (beta2GP1) in different antiphospholipid (aPL) antibody profiles. Thromb Res 2015;136:161–3.
[24] Andreoli L, Nalli C, Motta M, Norman GL, Shums Z, 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.
[25] Artenjak A, Locatelli I, Brelih H, Simonic DM, Ulcova-Gallova Z, et al. Immunoreactivity and avidity of IgG anti-beta2-glycoprotein I antibodies from patients with autoimmune diseases to different peptide clusters of beta2-glycoprotein I. Immunol Res 2015;61:35–44.
[26] de Groot PG, Meijers JC. Beta(2) -Glycoprotein I: evolution, structure and function. J Thromb Haemost 2011;9:1275–84.
[27] 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.
[28] Agar C, van Os GM, Morgelin M, Sprenger RR, Marquart JA, et al. Beta2-glycoprotein I can exist in 2 conformations: implications for our understanding of the antiphospholipid syndrome. Blood 2010;116:1336–43.
[29] de Laat B, van Berkel M, Urbanus RT, Siregar B, de Groot PG, 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.
[30] By Makoto Igarashi EM, Igarashi Yoshiko, Nagae Hisato, Ichikawa Kenji, Triplett Douglas A, Koike Takao. Human β2-Glycoprotein I as an Anticardiolipin Cofactor Determined using Deleted Mutants Expressed by a Baculovirus System. Blood 1996;87:3262–327.
[31] Iverson GM, Victoria EJ, Marquis DM. Anti-β2 glycoprotein I (β2GPI) autoantibodies recognize an epitope on the first domain of β2GPI. Proc Natl Acad Sci U S A 1998;95:15542–6.
[32] 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.
[33] Patricia A, JSD McNeeley, Furie Richard A, Jack Richard M, Ortel Thomas L, Triplett Douglas A, et al. beta2-glycoprotein I-dependent anticardiolipin antibodies preferentially bind the amino terminal domain of beta2-glycoprotein I. Thromb Haemost 2001;86:590–5.
[34] Iverson GM, Reddel S, Victoria EJ, Cockerill KA, Wang YX, et al. Use of single point mutations in domain I of 2-glycoprotein i to determine fine antigenic specificity of antiphospholipid autoantibodies. J Immunol 2002;169:7097–103.
[35] Ioannou Y, Pericleous C, Giles I, Latchman DS, Isenberg DA, et al. 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.
[36] Giannakopoulos B, Krilis SA. The pathogenesis of the antiphospholipid syndrome. N Engl J Med 2013;368:1033–44.
[37] de Groot PG, de Laat B. Mechanisms of thrombosis in systemic lupus erythematosus and antiphospholipid syndrome. Best Pract Res Clin Rheumatol 2017;31:334–41.
[38] 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.
[39] Pengo V, Bison E, Zoppellaro G, Padayattil Jose S, Denas G, et al. APS - Diagnostics and challenges for the future. Autoimmun Rev 2016;15:1031–3.
[40] Andreoli L, Chighizola CB, Nalli C, Gerosa M, Borghi MO, 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.
[41] Ioannou Y, Romay-Penabad Z, Pericleous C, Giles I, Papalardo E, 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.
[42] McDonnell T, Pericleous C, Laurine E, Tommasi R, Garza-Garcia A, et al. Development of a high yield expression and purification system for domain I of beta-2-glycoprotein I for the treatment of APS. BMC Biotechnol 2015;15:104.
[43] Agostinis C, Durigutto P, Sblattero D, Borghi MO, Grossi C, et al. A non-complement-fixing antibody to beta2 glycoprotein I as a novel therapy for antiphospholipid syndrome. Blood 2014;123:3478–87.
[44] Pericleous C, Ruiz-Limon P, Romay-Penabad Z, Marin AC, Garza-Garcia A, 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 antiphospholipid antibody-induced thrombosis. Rheumatology (Oxford) 2015;54:722–7.
[45] Pelkmans L, Kelchtermans H, de Groot PG, Zuily S, Regnault V, et al. Variability in exposure of epitope G40-R43 of domain i in commercial anti-beta2-glycoprotein I IgG ELISAs. PLoS One 2013;8:e71402.
[46] Wahezi DM, Ilowite NT, Wu XX, Pelkmans L, Laat B, et al. Annexin A5 anticoagulant activity in children with systemic lupus erythematosus and the association with antibodies to domain I of beta2-glycoprotein *I*. Lupus 2013;22:702–11.
[47] Ioannou Y, Giles I, Lambrianides A, Richardson C, Pearl LH, et al. A novel expression system of domain I of human beta2 glycoprotein I in Escherichia coli. BMC Biotechnol 2006;6:8.
[48] Cousins L, Pericleous C, Khamashta M, Bertolaccini ML, Ioannou Y, et al. Antibodies to domain I of β-2-glycoprotein I and IgA antiphospholipid antibodies in patients with 'seronegative' antiphospholipid syndrome. Ann Rheum Dis 2015;74:317–9.
[49] Pericleous C, Ferreira I, Borghi O, Pregnolato F, McDonnell T, et al. Measuring IgA anti-beta2-glycoprotein I and IgG/IgA anti-domain i antibodies adds value to current serological assays for the antiphospholipid syndrome. PLoS One 2016;11:e0156407.
[50] Pozzi N, Banzato A, Bettin S, Bison E, Pengo V, et al. Chemical synthesis and characterization of wild-type and biotinylated N-terminal domain 1-64 of beta2-glycoprotein I. Protein Sci 2010;19:1065–78.
[51] Banzato A, Pozzi N, Frasson R, De Filippis V, Ruffatti A, 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.
[52] Pengo V, Ruffatti A, Legnani C, Gresele P, Barcellona D, et al. Clinical course of high-risk patients diagnosed with antiphospholipid syndrome. J Thromb Haemost 2010;8:237–42.
[53] Akhter E, Shums Z, Norman GL, Binder W, Fang H, et al. Utility of antiphosphatidylserine/prothrombin and IgA antiphospholipid assays in systemic lupus erythematosus. J Rheumatol 2013;40:282–6.
[54] Chighizola CB, Pregnolato F, Andreoli L, Bodio C, Cesana L, et al. Beyond thrombosis: Anti-β2GPI domain 1 antibodies identify late pregnancy morbidity in antiphospholipid syndrome. J Autoimmun 2018;90:76–83.
[55] Mahler M, Radice A, Yang W, Bentow C, Seaman A, et al. Development and performance evaluation of novel chemiluminescence assays for detection of anti-PR3 and anti-MPO antibodies. Clin Chim Acta 2012;413:719–26.
[56] Cieśla M, Wypasek E, Undas A. IgA antiphospholipid antibodies and anti-domain 1 of beta 2 glycoprotein 1 antibodies are associated with livedo reticularis and heart valve disease in antiphospholipid syndrome. Adv Clin Exp Med 2014;23:729–33.
[57] Mondejar R, Gonzalez-Rodriguez C, Toyos-Saenz de Miera FJ, Melguizo-Madrid E, Zohoury N, et al. Role of antiphospholipid score and anti-beta2-glycoprotein I Domain I autoantibodies in the diagnosis of antiphospholipid syndrome. Clin Chim Acta 2014;431:174–8.
[58] Meneghel L, Ruffatti A, Gavasso S, Tonello M, Mattia E, et al. Detection of IgG anti-Domain I beta2 Glycoprotein I antibodies by chemiluminescence immunoassay in primary antiphospholipid syndrome. Clin Chim Acta 2015;446:201–5.
[59] Pengo V, Ruffatti A, Tonello M, Cuffaro S, Banzato A, et al. Antiphospholipid syndrome: Antibodies to Domain 1 of beta2-glycoprotein 1 correctly classify patients at risk. J Thromb Haemost 2015;13:782–7.
[60] De Craemer AS, Musial J, Devreese KM. Role of anti-domain 1-beta2 glycoprotein I antibodies in the diagnosis and risk stratification of antiphospholipid syndrome. J Thromb Haemost 2016;14:1779–87.
[61] Mahler M, Albesa R, Zohoury N, Bertolaccini ML, Ateka-Barrutia O, et al. Autoantibodies to domain 1 of beta 2 glycoprotein I determined using a novel chemiluminescence immunoassay demonstrate association with thrombosis in patients with antiphospholipid syndrome. Lupus 2016;25:911–6.
[62] Marchetti T, de Moerloose P, Gris JC. Antiphospholipid antibodies and the risk of severe and non-severe pre-eclampsia: The NOHA case-control study. J Thromb Haemost 2016;14:675–84.
[63] Montalvão S, Elídio PS, da Silva Saraiva S, de Moraes Mazetto B, Colella MP, et al. Clinical implications of the detection of antibodies directed against domain 1 of beta2-glycoprotein 1 in thrombotic antiphospholipid syndrome. Thromb Res 2016;148:32–7.
[64] Oku K, Amengual O, Kato M, Bohgaki T, Horita T, et al. Significance of fully automated tests for the diagnosis of antiphospholipid syndrome. Thromb Res 2016;146(1–6).
[65] Iwaniec T, Kaczor MP, Celinska-Lowenhoff M, Polanski S, Musial J. Clinical significance of anti-domain 1 beta2-glycoprotein I antibodies in antiphospholipid syndrome. Thromb Res 2017;153:90–4.
[66] Nakamura H, Oku K, Amengual Olga, Ohmura Kazumasa, Fujieda Yuichiro, et al. First-line, non-criterial antiphospholipid antibody testing for the diagnosis of antiphospholipid syndrome in clinical practice: a combination of anti-beta2-glycoprotein I domain I and phosphatidylserine-dependent antiprothrombin antibodies. Arthritis Care Res 2017:1–46.
[67] Nojima J, Motoki Y, Hara K, Sakata T, Tsuneoka H, et al. Detection of antibodies against domain 1 of β2-glycoprotein I is key in predicting thromboembolic complications in patients with systemic lupus erythematosus. Thromb Res 2017;153:83–4.
[68] Marchetti T, Ribi C, Perneger T, Trendelenburg M, Huynh-Do U, et al. Prevalence, persistence and clinical correlations of classic and novel antiphospholipid antibodies in systemic lupus erythematosus. Rheumatology 2018;57(8):1350–7. (Oxford). [Epub ahead of print].
[69] Zohoury N, Bertolaccini ML, Rodriguez-Garcia JL, Shums Z, Ateka-Barrutia O, et al. Closing the serological gap in the antiphospholipid syndrome: The value of "non-criteria" antiphospholipid antibodies. J Rheumatol 2017;44:1597–602.
[70] Sciascia S, Cosseddu D, Montaruli B, Kuzenko A, Bertero MT. Risk scale for the diagnosis of antiphospholipid syndrome. Ann Rheum Dis 2011;70:1517–8.
[71] Otomo K, Atsumi T, Amengual O, Fujieda Y, Kato M, 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.
[72] Pengo V, Ruffatti A, Del Ross T, Tonello M, Cuffaro S, et al. Confirmation of initial antiphospholipid antibody positivity depends on the antiphospholipid antibody profile. J Thromb Haemost 2013;11:1527–31.
[73] De Craemer AS, Devreese KM. Role of anti-domain 1 ß2glycoprotein I antibodies in the diagnosis and risk stratification of antiphospholipid syndrome: reply. J Thromb Haemost 2016;14:2078–80.
[74] Audrain MA, Colonna F, Morio F, Hamidou MA, Muller JY. Comparison of different kits in the detection of autoantibodies to cardiolipin and beta2glycoprotein 1. Rheumatology (Oxford) 2004;43:181–5.
[75] Reber GTA, Sanmarco M, de Moerloose P, Boffa MC. Proposals for the measurement of anti-b2-glycoprotein I antibodies. Standardization Group of the European Forum on Antiphospholipid Antibodies. J Thromb Haemost 2004;2:1860–2.
[76] Tincani A, Allegri F, Balestrieri G, Reber G, Sanmarco M, et al. Minimal requirements for antiphospholipid antibodies ELISAs proposed by the European Forum on antiphospholipid antibodies. Thromb Res 2004;114:553–8.
[77] Pengo V, Biasiolo A, Bison E, Chantarangkul V, Tripodi A, et al. Antiphospholipid antibody ELISAs: Survey on the performance of clinical laboratories assessed by using lyophilized affinity-purified IgG with anticardiolipin and anti-beta2-Glycoprotein I activity. Thromb Res 2007;120:127–33.
[78] Urbanus RT, de Groot PG. Antiphospholipid antibodies–we are not quite there yet. Blood Rev 2011;25:97–106.
[79] de Laat B, de Groot PG. Autoantibodies directed against domain I of beta2-glycoprotein I. Curr Rheumatol Rep 2011;13:70–6.
[80] Dienava-Verdoold I, Boon-Spijker MG, de Groot PG, Brinkman HJ, Voorberg J, et al. Patient-derived monoclonal antibodies directed towards beta2 glycoprotein-1 display lupus anticoagulant activity. J Thromb Haemost 2011;9:738–47.
[81] Devreese K, Kelchtermans H, de Laat B. Differences in sensitivity of two automated panels for anticardiolipin and anti-beta2glycoprotein I antibodies in the laboratory diagnosis of antiphospholipid syndrome due to the exposure of the domain I epitope of beta2glycoprotein I on the solid phase [abstract]. Thromb Haemost 2014;12:55.
[82] Mahler M, Bentow C, Serra J, Fritzler MJ. Detection of autoantibodies using chemiluminescence technologies. Immunopharmacol Immunotoxicol 2016;38:14–20.