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New Tests to Detect Antiphospholipid Antibodies: Anti-Domain I Beta-2-Glycoprotein-I Antibodies

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Abstract Beta-2 glycoprotein I (β_2GPI) is the main antigenic target for antiphospholipid antibodies (aPL), the serological markers of antiphospholipid syndrome (APS). Domain I (DI) of β_2GPI has lately been identified as the main epitope targeted by antibodies reacting against β_2GPI. DI is a cryptic epitope, becoming available for autoantibody binding when β_2GPI opens from a circular to a fish-hook configuration. Antibodies targeting β_2GPI-DI are more frequently detected in patients with a full-blown syndrome than in asymptomatic aPL carriers or in patients with infectious diseases that have reactivity toward the whole molecule. Interestingly, anti-DI antibodies are strongly positively correlated with thrombotic and pregnancy manifestations, enabling identification of patients at higher risk of clinical events. However, available tests to detect anti-DI antibodies still lack standardization. Moreover, some APS patients develop antibodies reacting against β_2GPI epitopes other than DI, suggesting that other anti-β_2GPI antibody subsets may be clinically relevant. Available evidence on anti-DI antibodies in APS is herein critically reviewed.

Keywords Antiphospholipid antibodies · Detection · Antiphospholipid antibody syndrome · β_2 glycoprotein I · Domain I · New tests · Conformation · Anti-β_2 glycoprotein I antibodies · Anti-domain I antibodies · Pathogenicity · Thrombosis · Pregnancy complications

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Introduction

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by vascular thrombosis and/or pregnancy morbidity, associated with a persistent positivity for serum antiphospholipid antibodies (aPL). aPL are currently evaluated by use of two solid-phase assays, detecting antibodies against cardiolipin (aCL) and β_2–glycoprotein-I (anti-β_2GPI antibodies), and by use of a functional assay, lupus anticoagulant (LA) test [1].

aPL were initially believed to react against negatively charged phospholipids (PL); however, it soon became clear that aPL bind to proteins with affinity for PL. In particular, β_2–glycoprotein-I (β_2GPI), together with prothrombin, is the most important epitope targeted by aPL. Antibodies reacting against β_2GPI have also been identified as the main pathogenic subset of aPL in both in-vivo and in-vitro experiments.

Consistent with laboratory findings, anti-β_2GPI antibodies have been associated with increased risk of developing clinical manifestations of APS [2]. However, not all patients carrying anti-β_2GPI antibodies develop aPL-related clinical manifestations. This observation agrees with increasing evidence that anti-β_2GPI antibodies are rather heterogeneous. Within this autoantibody family are multiple antibody subsets, each with a different pathogenic potential. Such heterogeneity among anti-β_2GPI antibodies might be partially explained by

evidence that several epitopes of β_2GPI can be targeted by specific autoantibodies. However, a specific epitope in domain (D)I, a positively charged discontinuous structure located in the N-terminal of β_2GPI, has been identified as the most relevant antigenic target involved in β_2GPI/anti-β_2GPI antibody binding [3]. Over recent years, much research into APS has had the objective of better characterizing the pathogenic function and the clinical significance of antibodies specifically targeting DI. The implications of such a finding might have a strong effect on APS clinical diagnosis and management in the near future. It is therefore an appropriate time to critically review the available evidence regarding anti-DI antibodies and APS.

Beta-2 Glycoprotein-I: The Protein

β_2GPI, also known as apolipoprotein H, is a single-chain 43 kDa glycoprotein found in human plasma at concentrations in the range 50–400 μg mL^{-1}. This evolutionary conserved protein is synthesized by, among others, endothelial cells, hepatocytes, and trophoblast cells [4]. The physiological function of β_2GPI was determined only recently, when two independent groups revealed that the C-terminal of the protein interacts specifically with lipopolysaccharide (LPS). This observation led the investigators to hypothesize that β_2GPI may act as a carrier or as a scavenger for LPS [5••, 6]. Further evidence for an interaction between β_2GPI and LPS was provided by the in-vivo observation that LPS injection induces a 25 % reduction of baseline β_2GPI serum levels. Moreover, plasma levels of β_2GPI were found to inversely correlate with inflammatory markers, including tumor necrosis factor (TNF) α, interleukin (IL)-6, and IL-8 [5••]. A member of the complement control protein (CCP) family, β_2GPI consists of 326 aminoacidic residues arranged in five CCP repeat domains, termed "sushi" domains. DI–IV comprise 60 amino acids and each contain two disulfide bridges. DV is aberrant, including 82 amino acids resulting from a six-residue insertion and a 19-residue C-terminal extension crosslinked by an additional disulfide bond. DV is responsible for β_2GPI binding to PL via a cluster of positively charged amino acids (282–287); the same cluster also mediates the adhesion of β_2GPI to cells targeted by aPL, including the trophoblast and endothelial cells [2, 7].

Three configurations of β_2GPI have been described. Circulating plasma β_2GPI exists in a circular form, observed by use of electronic microscopy [8]. Upon binding to suitable anionic surfaces, for example to cardiolipin (CL) and other PL, or to LPS, the molecule opens up to a J-shaped fish-hook configuration, as revealed by its crystal structure [9, 10]. An intermediate S-shape of β_2GPI has been recently observed by use of small-angle X-ray scattering [11] (Fig. 1).

Beta-2 Glycoprotein-I and Reactivity Toward its Five Domains

In the late 90s, research focused on identifying the β_2GPI binding site for anti-β_2GPI antibodies. Some groups claimed that the epitope for binding of anti-β_2GPI antibodies was located on DIV, whereas other investigators suggested DV was involved [12]. It was observed that binding of anti-β_2GPI antibodies to β_2GPI was prevented when a clip was positioned at 316–317 in DV; Arvieux reported that anti-β_2GPI antibodies inhibited the binding of β_2GPI to CL [13–15]. Lastly, others reported anti-β_2GPI antibodies reacting with peptides that cover sequences in DI–IV [16]. These findings clearly revealed that anti-β_2GPI antibodies can bind to each of the five domains of β_2GPI. However, consistent evidence has led to the identification of DI as the immunodominant epitope.

Domain I as the Immunodominant Epitope

The reactivity of antibodies against DI was first described in 1998, when Iverson developed domain-deletion mutants of β_2GPI [17]. By use of surface plasmon resonance, it was consistently observed that the immunodominant binding epitope for anti-β_2GPI antibodies was localized in DI of β_2GPI [18]. Creation of human β_2GPI variants with point mutations in DI has enabled observation of the discontinuous nature of the main epitope: it involves arginine 39-arginine 43, aspartic acid 8-aspartic acid 9, and possibly the interlinking region between DI and DII, with R39 being the most important residue [19–21]. This epitope was later revealed to be a cryptic and conformation-dependent structure. In the circular conformation of β_2GPI, DI interacts with DV and the critical epitope is thus hidden. When β_2GPI adopts the S-shape, the epitope is covered by DIII–IV carbohydrate chains. These residues form a shield over DI, thus preventing antibodies from binding β_2GPI. It has been consistently observed that antibodies against DI are able to bind β_2GPI only when the carbohydrate chains have been removed; the antibodies have no reactivity toward the intact molecule [22]. Upon β_2GPI opening to a J-configuration, the critical epitope arginine 39-glycine 43 is exposed, thus becoming available for antibody binding. The three conformations of β_2GPI and the relative exposure of DI to the surface are represented in Fig. 1. The hypothesis that the immunogenicity of β_2GPI depends upon its conformation is supported by in-vivo evidence. Mice developed antibodies against DI only when injected with misfolded β_2GPI or with β_2GPI-CL, and production of anti-DI antibodies was observed when mice were injected with DI but not with DII–V [23••].

The conformation-dependent binding of anti-β_2GPI antibodies to their target antigen, together with the low avidity of these antibodies, might explain why β_2GPI/anti-β_2GPI

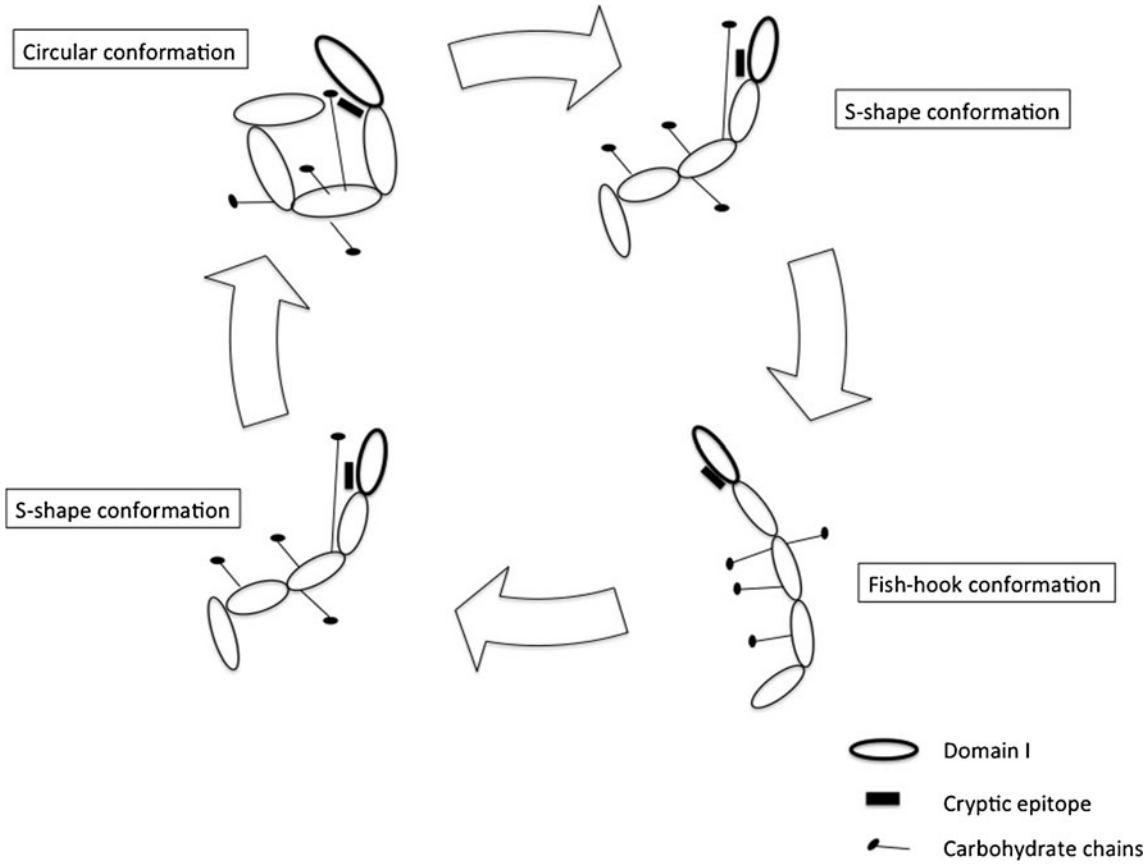

Fig. 1 Schematic representation of the three conformations of $\beta_2$glycoprotein I and the relative exposure of domain I

antibody immune complexes are not easily isolated from serum samples from APS patients. Overall, it is clear that β_2GPI conformation strongly affects anti-β_2GPI antibodies binding to the target epitope. Several factors might lead to the surface exposition of the critical epitope, including oxidative stress. In healthy persons, a free thiol form of β_2GPI, characterized by a broken disulfide bridge, predominates in the plasma. Under conditions of oxidative stress, disulfide bonds form at these sites, possibly exposing the critical B-cell epitope [24]. Compared with asymptomatic aPL carriers and healthy volunteers, APS patients were consistently found to have significantly higher oxidized plasma β_2GPI. Furthermore, anti-β_2GPI antibodies purified from β_2GPI-immunized animals and from APS patients had reduced binding to β_2GPI treated with oxidoreductase [25].

However, in rats primed with LPS, deposition of β_2GPI-dependent aPL human IgG on the endothelium was observed immediately after the infusion, implying that in-vivo anti-β_2GPI antibodies adhere to the vessel wall [26]. Therefore, β_2GPI might undergo a conformational change upon binding to the surface of target cells, exposing the critical epitope, which thus becomes available to specific autoantibodies. As a whole, the above evidence suggests that β_2GPI becomes immunogenic as a consequence of a conformational change: it could be hypothesized that, being cryptic, DI, in contrast with the other domains, does not induce immune tolerance. Consequently, DI could induce specific autoantibodies more easily than do other domains [27].

Anti-Domain I Antibodies in APS: Evidence of their Pathogenetic Function

Evidence of the pathogenicity of anti-DI antibodies comes from both in-vitro and in-vivo studies. First, anti-DI antibodies were repeatedly observed to induce in-vitro prolongation of clotting time [23••, 28]. Proof of their thrombogenic function was obtained in 2009, when passive infusion of a synthetic DI peptide in naïve mice was revealed to prevent, in a dose-dependent manner, the thrombus enhancement mediated by polyclonal aPL human IgG fractions. Furthermore, the infusion of the peptide inhibited, although not completely, the expression of adhesion molecules on aortic endothelial cells and the production of tissue factor (TF) by murine macrophages. Interestingly, mutations in DI associated with either an increase or a reduction in its affinity for IgG purified from APS patients correspondingly affected the ability of the mutant peptide to reverse the effects mediated by the same aPL fractions [29]. More recently, it was revealed that a greater increase in TF activity and significantly larger thrombi

were induced by eluted fractions rich in anti-DI antibodies, obtained from an APS patient, than by the anti-DI-antibody-poor serum recollected after affinity-purification [30]. Direct observation of the pathogenic effects of anti-DI antibodies has been recently obtained by use of a human monoclonal anti-DI IgG, infusion of which induced clotting and fetal loss in naïve mice. The anti-DI monoclonal was found to induce clotting only after the concomitant administration of LPS, in agreement with the "two-hit" hypothesis [31••].

Tests Detecting Anti-Domain I Antibodies

The conformational changes of β_2GPI might directly affect detection of anti-β_2GPI antibodies. It has been clearly revealed that β_2GPI might adopt peculiar conformations in different assays, caused by heterogeneity in purification methods or in coating procedures. Most recently, DI exposure has been revealed to be highly heterogeneous across commercially available ELISA assays, potentially affecting test results [32].

It has been consistently observed that anti-DI antibodies have reactivity toward their target epitope only when DI is coated onto hydrophobic, but not hydrophilic, plates, suggesting that β_2GPI undergoes conformational changes leading to DI being exposed to the surface only when coated on hydrophilic microtiter plates [28]. This conformation challenge contributes greatly toward the inter-assay variability regarding detection of anti-DI antibodies. To date, there is no commercial kit to detect anti-DI antibodies available on the market; however, several research assays have been developed.

Other than the two-step ELISA test using both hydrophilic and hydrophobic plates, a few additional ELISA assays have been developed to detect anti-DI antibodies, each using different molecular antigenic targets. Recently, a β_2GPI-DI chemiluminescence immunoassay (CIA, INOVA Diagnostics, San Diego, USA) has been developed, which uses a recombinant DI coupled to paramagnetic beads by use of the BIO-FLASH technology (Biokit, Barcelona, Spain) [33]. The ELISA and CIA research assays, both developed by INOVA Diagnostics (San Diego, CA, USA), have been directly compared, revealing that the two methods have the same specificity but a different sensitivity [34]. The CIA immunoassay has also been evaluated in comparison to a UK in-house ELISA test: a good agreement between the two tests was observed [35]. Although these preliminary data seem to suggest comparability between the solid phase assays and the CIA, multi-center prospective studies are warranted to more fully investigate the reproducibility of the different anti-DI antibody assays. The clinical significance of anti-DI antibodies and of anti-β_2GPI antibodies detected by CIA has been directly compared; the two assays had good qualitative and quantitative agreement and similar discrimination between APS patients and controls [36]. Lastly, two additional tests to detect anti-DI antibodies have been described: a capture ELISA method using N-terminally biotinylated DI on streptavidin plates, and a liquid phase inhibition assay using whole β_2GPI immobilized on the solid phase and synthetic β_2GPI-DI as inhibitor [37, 38•].

Anti-Domain I Antibodies and aPL-related Clinical Manifestations

To date, few studies have addressed the subject of anti-DI antibodies and aPL-related clinical manifestations. Details of these studies, i.e. study population, anti-DI antibody positivity, the assay used, and the observed association between anti-DI antibodies and aPL-related clinical manifestations, are shown in Table 1.

Positivity for DI-targeting antibodies among APS patients varies widely, depending on the selection of the study population and the test performed. In a cohort of 144 APS patients, anti-DI antibodies were detected in 85 % of cases by use of CIA, while Hollestelle has recently reported positivity as low as 33.3 % for anti-DI antibodies among APS patients [39, 40]. Positivity for anti-DI antibodies has been reported to be higher in primary APS than in APS associated with other systemic autoimmune diseases [36]. Despite these discrepancies, antibodies targeting DI—but notably not those reacting against the whole molecule—were found to be significantly associated with diagnosis of APS [40]. Furthermore, anti-DI antibodies as detected by use of CIA provide good specificity and high sensitivity for diagnosis of APS, both being approximately 85 % [41]. This observation fits well with the documented strong association between anti-DI antibodies and a positive LA test, which is the most powerful predictor of clinical events in APS [42].

Most of the available clinical studies on anti-DI antibodies focused on the association with thrombosis. Many authors confirmed the relationship between anti-DI antibodies and thrombotic events affecting the venous and the arterial vascular tree [28, 39, 43–46]. In particular, such an association emerged in the largest study published to date, a multi-center cohort comprising 442 APS patients. The investigators reported an odds ratio (OR) of 3.5 for anti-DI antibodies to predict thrombosis [43]. In contrast with the findings of the first study conducted by de Laat in 2005, anti-DI antibodies were also found to be related to pregnancy complications, although to a lesser extent than to thrombosis (OR 2.4) [28, 43]. Concordantly, anti-DI antibodies have been identified as the prevalent antibody specificities among APS patients who also have pure obstetric morbidity. Although the positivity was slightly lower among women with obstetric APS than among subjects with thrombosis (61.3 % versus 78.2 %), no significant differences in anti-DI antibody frequency were observed

Table 1 Studies assessing clinical function of anti-DI antibodies

Author, year, Ref.	Study population	anti-DI antibody positivity	Test	Main conclusions
De Laat, 2005, [28]	176 SLE patients 16 lupus-like disease 6 PAPS patients	NR	Home-made ELISA	anti-DI were strongly associated with thrombosis
De Laat, 2007, [48]	33 SLE/lupus-like/APS patients with: 10 β2GPI DI-dependent LA 12 β2GPI-independent LA 10 no LA	NR	Home-made ELISA	Patients with β2GPI DI-dependent LA had increased annexin A5 resistance
De Laat, 2009, [43]	442 APS patients	55 %	Home-made ELISA	anti-DI were correlated with thrombosis (OR 3.5) and obstetric morbidity (OR 2.4)
Andreoli, 2010, [47]	64 APS patients 57 1-year old children born to mother with SAD 33 children with atopic dermatitis	75 % 15.8 % 27.3 %	Research ELISA (INOVA Diagnostics)	anti-DI were the main antibody population in APS patients
Banzato, 2011, [38•]	22 triple positive APS patients 15 double positive APS patients 9 single positive APS patients 20 controls	NR	Competitive inhibition ELISA	anti-DI titers were significantly higher for patients with triple positivity
Albesa, 2012, [41][a]	72 APS patients with thrombosis 35 APS patients without thrombosis 272 controls	22.2 % 2.9 %	CIA	anti-DI were associated with a history of thrombosis
Albesa, 2012 (II), [39][a]	144 APS patients 200 healthy controls 72 patients with infectious diseases	85 % 0.5 % 14 %	CIA	anti-DI CIA had a sensitivity of 85 % and a specificity of 85 % for APS diagnosis
Levine, 2012, [50][a]	15 aPL + SLE patients 15 aPL-SLE patients	47 % 0 %	NR	anti-DI were significantly associated with aPL positivity 71 % of the patients with anti-DI were triple positive
Wahezi, 2013, [49]	183 children with SLE	25.1 %	Home-made ELISA	anti-DI were associated with reduced Annexin A5 anticoagulant activity
Hollestelle, 2013, [40][a]	24 APS patients 55 controls	33.3 % 7.2 %	CIA	anti-DI conferred an OR for diagnosis of APS of 1.9 anti-β2GPI were not associated with APS
Pelkmans, 2013, [38•]	176 SLE patients	13.6 %	Home-made ELISA	–
Andreoli, 2013, [46][a]	55 aβ2GPI + patients with thrombotic PAPS 31 aβ2GPI + women with obstetric PAPS 42 aβ2GPI + SLE/UCTD patients with aPL positivity 14 aβ2GPI + aPL carriers	78.2 % 61.3 % 59.5 % 50 %	Home-made ELISA	anti-DI were the prevalent specificity not only in thrombotic but also in obstetric APS
Agmon-Levin, 2013, [45][a]	178 APS patients 50 healthy subjects 47 sepsis patients	49 %	CIA	anti-DI were associated with arterial thrombosis High levels of anti-DI were associated with arterial thrombosis, multiple thrombotic events and CNS manifestations
Zohoury, 2013, [36][a]	273 APS patients	NR	CIA	

Table 1 (continued)

Author, year, Ref.	Study population	anti-DI antibody positivity	Test	Main conclusions
	1096 controls			anti-DI have sensitivity of 49.8 % and specificity of 99.6 % for APS diagnosis
Zuily, 2013, [44]ª	92 SLE/SLE aPL+/aPL+	NR	NR	anti-DI IgG were associated with a 3.6 fold increase in thrombotic risk

ª Abstract

CNS, central nervous system; SLE, systemic lupus erythematosus; SAD, systemic autoimmune diseases; UCTD, undifferentiated connective tissue disease; aPL, anti-phospholipid antibodies; PAPS, primary anti-phospholipid antibody syndrome; OR, odds ratio; NR, not reported

between the two subgroups of patients with different APS clinical manifestations. In addition, the same study reported no significant difference in anti-DI antibody titers between patients with thrombotic manifestations and women with pure obstetric complications [46]. In contrast, a few years ago other authors claimed that patients with thrombotic events had higher anti-DI antibody titers than those with non-vascular manifestations, although this finding was not replicated in further studies [41].

It is interesting to note that anti-DI antibodies provide the main epitopic specificities even for patients with autoimmune conditions other than APS. Indeed, aPL-positive subjects with systemic lupus erythematosus (SLE) or undifferentiated connective tissue diseases (UCTD), but no clinical features of APS, have positivity for anti-DI antibodies comparable to that of APS patients, whereas antibodies against DIV or DV are less frequent. On the other hand, in control populations the positivity for anti-DI antibodies has been revealed to be rather low. Anti-β_2GPI IgG isolated from sera obtained from aPL-positive asymptomatic carriers, individuals with leprosy, or children with atopic dermatitis have been revealed to preferentially recognize epitopes on DIV or V [46, 47]. Overall, these observations suggest that anti-DI antibodies may cluster in patients with systemic autoimmune diseases. As a consequence, it has been suggested that the ratio between antibodies targeting DI and those reacting against DIV and DV might be of use in discriminating between pathogenic anti-β_2GPI antibodies and those autoantibodies that, occurring in association with a wide variety of non immune-mediated clinical conditions, are a mere epiphenomenon with no pathogenic potential. This hypothesis needs to be tested by appropriately sampled studies; if confirmed, it would imply that screening for reactivity against specific domains of the protein would enable predictive and non-predictive anti-β_2GPI antibodies to be distinguished.

Further evidence for the pathogenic potential of anti-DI antibodies is provided by a good correlation with annexin A5 resistance assay, observed for cohorts of APS subjects and of adult and pediatric SLE patients [48–50]. Annexin A5 resistance is detected by use of a novel two-stage coagulation assay, and has been revealed to be reduced for aPL-positive patients with a history of thrombosis [51]. Annexin A5 is a potent anticoagulant protein mainly found in trophoblasts and vascular endothelial cells; β_2GPI-dependent aPL have been revealed to interfere with the protective shield that annexin A5 provides over the endothelium, thus favoring thrombosis [52].

Given that anti-DI IgG may have a more predictive aPL profile, anti-DI antibodies might be useful for risk-stratification of APS patients. APS patients at higher risk, i.e. those with triple aPL-positivity at medium-high titers, have been consistently observed to have a higher frequency and higher titers of anti-β_2GPI-DI antibodies [38•, 45, 46].

Anti-Domain I Antibodies: Potential use for Clinicians

Anti-β_2GPI antibodies specifically reacting with DI have a particular clinical importance, being more commonly detected among patients with APS and other autoimmune diseases than among those with transient aPL positivity caused by polyclonal B-cell activation, e.g. infections or atopic dermatitis. This observation implies that, compared with antibodies targeting the whole molecule, anti-DI antibodies have higher specificity for APS. As a result, routine testing for anti-DI antibodies in clinical practice would enable easy differentiation of subjects carrying clinically meaningful anti-β_2GPI antibodies from those individuals with a benign autoantibody profile.

Routine availability of an additional laboratory test would be valuable for risk-stratification of APS patients, because it is well established that APS subjects might be classified into different risk categories by their aPL profile [1, 53]. Indeed, antibodies targeting DI not only are more frequently detected in patients at highest risk but also, when at high titers, enable identification of patients with a more aggressive clinical presentation [38•, 45, 46].

No study has systematically addressed the persistency of anti-DI antibodies and the resultant need to confirm positivity 12 weeks apart, as currently recommended for the three criteria tests by international guidelines [1]. It has recently emerged that triple aPL-positivity at first detection is later confirmed for 98 % of cases, suggesting that robust and consistent autoantibody profiles do not require retesting [54]. Consequently, given the higher anti-DI antibody frequency among triple-positive patients, it can be inferred that positive anti-DI antibodies may have diagnostic value even when tested on a single occasion. This critical subject warrants further clarification in future prospective studies. Despite the high specificity for APS of anti-DI antibodies, it is far too soon to state whether isolated low-titer anti-DI antibodies have diagnostic significance, or whether they instead have limited clinical meaning as isolated low-titer aCL and anti-β_2GPI antibodies.

Conclusions

Increasing evidence suggests DI is the most relevant epitope targeted by anti-β_2GPI antibodies in patients with autoimmune conditions. Anti-DI antibodies have been consistently revealed to be clinically interesting, being significantly associated with both vascular and obstetric aPL-related events.

Despite the many corroborating findings, it is far too soon to recommend replacement of anti-β_2GPI antibody testing with anti-DI antibody assay. There are critical challenges caused by the current lack of standardization of the test, and, compared with the assay detecting antibodies against the whole molecule, tests for anti-DI antibodies have lower sensitivity for APS. Indeed, a low percentage of anti-β_2GPI-antibody-positive patients with a formal diagnosis of APS present with autoantibodies reacting with β_2GPI epitopes other than DI. These subjects could be misdiagnosed if testing for antibodies against the whole β_2GPI was not available.

Although anti-DI antibodies are significantly associated with APS clinical events and with a high-risk aPL profile, there is still no definite prospective evidence that this test may provide stronger risk factor than anti-b2GPI antibodies for aPL-related manifestations. However, anti-DI antibodies can enable more straightforward diagnosis and risk-stratification, possibly leading to a treatment strategy tailored to the individual clinical and laboratory characteristics of each patient.

As a whole, anti-DI antibodies are a very promising tool for managing APS; the coming years will be essential for clearly defining the diagnostic and prognostic value of anti-DI antibodies. We believe that, within a few years, testing for anti-DI antibodies will be part of routine clinical practice.

Compliance with Ethics Guidelines

Conflict of Interest Pier Luigi Meroni received an honorarium for attendance at an INOVA advisory board meeting. Cecilia Beatrice Chighizola and Maria Gerosa declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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•• Of major importance
• Of importance

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