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Reactivity against the Five Domains of β_2 Glycoprotein I: A Focus on Systemic Lupus Erythematosus

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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), a systemic autoimmune disease characterized by vascular thrombosis and/or pregnancy morbidity. aPL are detected in 20 to 50% of patients with systemic lupus erythematosus (SLE), representing a poor prognostic factor. Indeed, thrombotic events heralds high morbidity and mortality, being regarded as the strongest predictors of death in the first five years after SLE onset. It would be thus very important to identify a reliable laboratory tool such as anti-domain antibodies to better risk-stratify lupus patients according to the aPL profile, in order to tailor treatment strategies. Domain I (DI) of β_2GPI has lately been identified as the main epitope targeted by antibodies reacting against β_2GPI isolated from APS patients, well correlating with thrombotic as well as obstetric manifestations. Interestingly, anti-DI antibodies have been shown to well correlate with lupus anticoagulant and to predict the clinical manifestations of the syndrome, thrombotic events as well as pregnancy complications. Further, anti-DI antibodies allow to identify patients at highest clinical risk, presenting a good specificity for APS. On the other hand, anti- β_2GPI antibodies from aPL asymptomatic carriers or subjects with infectious diseases preferentially display reactivity towards DIV or DV of the molecule.

Few studies have to date evaluated the domain profile of anti-β_2GPI antibodies in subjects with SLE, even though it would be very relevant from a clinical point of view to understand whether among patients with SLE the domain specificities of anti-β_2GPI antibodies display a clinical meaning comparable to the one they exert in APS. It is therefore rather appropriate to review the available evidence about anti-domain specificities with a particular focus on SLE.

Keywords: Antiphospholipid antibodies; Systemic lupus erythematosus; Detection; Antiphospholipid antibody syndrome; β_2 glycoprotein I; Domains; Domain I; Domain IV; Domain V; New tests; Conformation; Anti-β_2 glycoprotein I antibodies; Anti-domain I antibodies; Anti-domain IV antibodies; Anti-domain V antibodies; Pathogenicity; Thrombosis; Pregnancy complications

Introduction

The anti-phospholipid antibodies (aPL) were first described in the early 80s in patients with systemic lupus erythematosus (SLE), being only later recognized as serological markers of a distinct clinical entity, the anti-phospholipid syndrome (APS). APS is a systemic autoimmune disease characterized by recurrent vascular thrombosis and/or pregnancy morbidity. The Sapporo revised classification criteria define patients with primary disease (PAPS) those with an APS diagnosis in the absence of any underlying autoimmune condition, and patients with secondary APS (SAPS) those who develop APS clinical manifestations in the context of autoimmune disorders. aPL are currently evaluated by three tests acknowledged in the international criteria for APS classification: two solid-phase assays detecting antibodies against beta-2 glycoprotein I (anti-β_2GPI antibodies) and against cardiolipin (aCL) plus a functional assay, the lupus anticoagulant (LA) [1]. Although aPL were initially thought to react against negatively charged phospholipids (PL), it soon became clear that aPL bind to proteins with affinity for anionic PL. In particular, beta-2 glycoprotein I (β_2GPI) provides, together with prothrombin, 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 [2]. Multiple epitopes in the β_2GPI can be bound by anti-β_2GPI antibodies, even autoantibodies purified from a given patient may react against several epitopes in the same molecule. Such phenomenon might be secondary to an intramolecular epitope spreading, that may occur following initiation of the immune response by a single epitope in the β_2GPI molecule. The heterogeneity across the several epitopes targeted by different anti-β_2GPI antibody subsets might explain why anti-β_2GPI antibodies exert a variable pathogenic potential. Indeed, not all patients carrying anti-β_2GPI antibodies develop aPL-related clinical events. A particular epitope in a positively charged discontinuous structure located in the N-terminal β_2GPI named domain (D) I has been identified as the most relevant antigenic target involved in β_2GPI/anti- β_2GPI antibody binding among APS patients [3]. Conversely, anti-β_2GPI antibodies from non-APS subjects with aPL positivity due to polyclonal B activation have been shown to preferentially react against DIV and/or DV [3].

Few studies have to date evaluated the domain profile of anti-β_2GPI antibodies in subjects with SLE. It is indeed very relevant from a clinical point of view to understand whether the domain specificities of anti-β_2GPI antibodies are similarly distributed in patients with SLE

and those with APS. We thus believe it is rather appropriate to review the available evidence about anti-domain specificities, with a particular focus on SLE.

Anti-phospholipid Antibodies in Systemic Lupus Erythematosus

aPL are among the most common autoantibodies in lupus patients, being detected in approximately 20 to 50% of SLE subjects. In particular, the aPL positivity rate among subjects with SLE ranges from 12% to 44% for aCL, from 15% to 34% for LA, and from 10% to 19% for anti- β_2GPI antibodies [4]. In the Europhospholipid series, patients with full-blown SLE represented the 37% of the total cohort, while 4% of the included subjects presented a lupus-like disease [5]. Longitudinal studies show that APS may develop in 50 to 70% of aPL positive SLE patients after 20 years of follow-up. Conversely, up to 30% of lupus patients carrying aCL does not develop any clinical manifestations peculiar of APS over an average follow-up of seven years. A greater frequency of both thrombosis and pregnancy loss has been observed in SAPS compared to primary APS [4]. In particular, a positivity for LA at diagnosis is associated with a 50% chance of developing deep venous thrombosis during a 20 year follow-up. To precisely weight the burden aPL carry among lupus patients, it should be considered that vascular events are regarded as the strongest predictors of death in the first five years after SLE onset [6]. Such increase in cardiovascular morbidity occurs early in disease history, with a 50 times higher risk of myocardial infarction among young women with a SLE diagnosis compared to age-matched controls. In this context, aPL positivity has been identified as the strongest determinants of thrombotic events among lupus patients. This large amount of vascular morbidity and mortality is largely attributable to the accelerated atherosclerosis observed in lupus patients, even though traditional cardiovascular risk factors have been shown not to correlate with the atheromatous burden in SLE. aPL positivity, together with the chronic systemic inflammation peculiar of SLE, directly contributes to atherogenesis, in line with the well-established hypothesis that atherosclerosis itself is an inflammatory condition.

A diagnosis of SAPS has been shown to carry a 3.1-fold increase in the risk of pregnancy loss, in particular when considering late events (after 20 weeks of gestation). Moreover, it was an independent risk factor for further pregnancy losses in a cohort study of 166 pregnancies at the Hopkins Lupus Centre. Conversely, a positivity for aCL and/or LA without the clinical criteria for APS did not increase the risk for pregnancy loss in the same series [4].

Since the first description of the disease, the spectrum of clinical manifestations ascribable to aPL has notably extended and APS is now regarded as a systemic disease. Thrombocytopenia, heart valve disease (valve thickening, vegetations and regurgitation), nephropathy, livedo reticularis and skin ulcers are relatively common features of APS, although not included in the classification criteria [5]. In lupus cohorts, positivity for LA has been identified as a predictor of organ damage and as a risk-factor for both renal and central nervous system involvement, such as seizure and depression. Furthermore, persistent aPL positivity has been related to cognitive impairment in SLE patients, even if recent studies conducted in very large SLE cohorts did not confirm such association. When evaluating aPL positive lupus patients with renal disease, it should be considered that a renal small artery vasculopathy involving both arterioles and glomerular capillaries, defined as aPL associated nephropathy (APLN), have been described both in primary and SLE-APS. APLN can be clinically silent or manifest in a minor proportion of patients (<3%) with systemic hypertension, proteinuria and inconstant haematuria. At renal biopsy, changes consistent with APLN were described independently of lupus nephritis in two thirds of SLE-APS patients, one third of SLE/non-APS patients with aPL and in only 4% of SLE patients without aPL [5].

As a whole, it emerges clearly that aPL represent a poor prognostic factor among SLE patients, with thrombotic events heralding high morbidity and mortality. It would be thus very important to identify a reliable laboratory tool, such as anti-domain antibodies, to better risk-stratify lupus patients according to the aPL profile, in order to tailor treatment strategies.

The Molecular Structure of β_2 Glycoprotein I

β_2GPI is a single-chain 43-kDa glycoprotein whose serum concentration ranges between 50 and 400 μg/mL. Since it appears in the lipoprotein fractions, it is also known as apolipoprotein H. This evolutionary conserved protein is synthesized by many cell types, as endothelial cells, hepatocytes and trophoblast cells [7]. It was first described in 1961 by Schultz as a perchloric acid soluble human plasma protein with an unknown function. It took quite a while to unravel the physiologic role of β_2GPI: only recently, two independent groups demonstrated that the C-terminal of the protein interacts specifically with lipopolysaccharide (LPS). Such observation suggests β_2GPI may act as a carrier or as a scavenger for LPS [8,9]. The interaction between β_2GPI and LPS is further supported by the inverse correlation between plasma levels of β_2GPI and inflammatory markers such as tumour necrosis factor (TNF) α, interleukin (IL)-6 and IL-8. Moreover, *in vivo* LPS injection induced a 25% reduction of baseline β_2GPI serum levels [8].

Member of the complement control protein (CCP) family, β_2GPI consists in a single polypeptide chain composed of 326 aminoacidic residues arranged in 5 CCP repeat motis, termed "sushi" domains. DI-IV comprise each 60 amino acids with a framework of four conserved half-cysteine residues involved in the formation of two internal disulfide bridges. Conversely, DV is aberrant, as it includes 82 amino acids due to a 6-residue insertion and a 19-residue C-terminal extension crosslinked by an additional disulfide bond. A lysine-rich locus in DV is critical for binding to anionic PL such as cardiolipin (CL); the same cluster of positively charged amino acids (282-287) mediates the adhesion of β_2GPI to cells targeted by aPL, such as the trophoblast and endothelial cells. β_2GPI presents five oligosaccharide attachment sites, with an overall carbohydrate content of 17% [10].

Reactivity towards the Five Domains of β_2 Glycoprotein I

From the early 90s on, research in APS field focused on the identification of the β_2GPI binding site for β_2GPI-dependent aCL and anti-β_2GPI antibodies. Antibodies targeting β_2GPI have been shown to bind to each of the five domains of β_2GPI. Indeed, Shoenfeld et al. evaluated the distribution of reactivity against six linear peptides spanning the different domains of β_2GPI: most commonly antibodies from primary APS patients reacted against peptides corresponding to DV (52.9-64.6%), followed by those binding to peptides corresponding to DIV (45.8%), DI-II (33.1%) and DIII (20.5%). This picture clearly shows that anti-β_2GPI antibodies are a rather heterogenous family, being often polyreactive [11] (Figure 1).

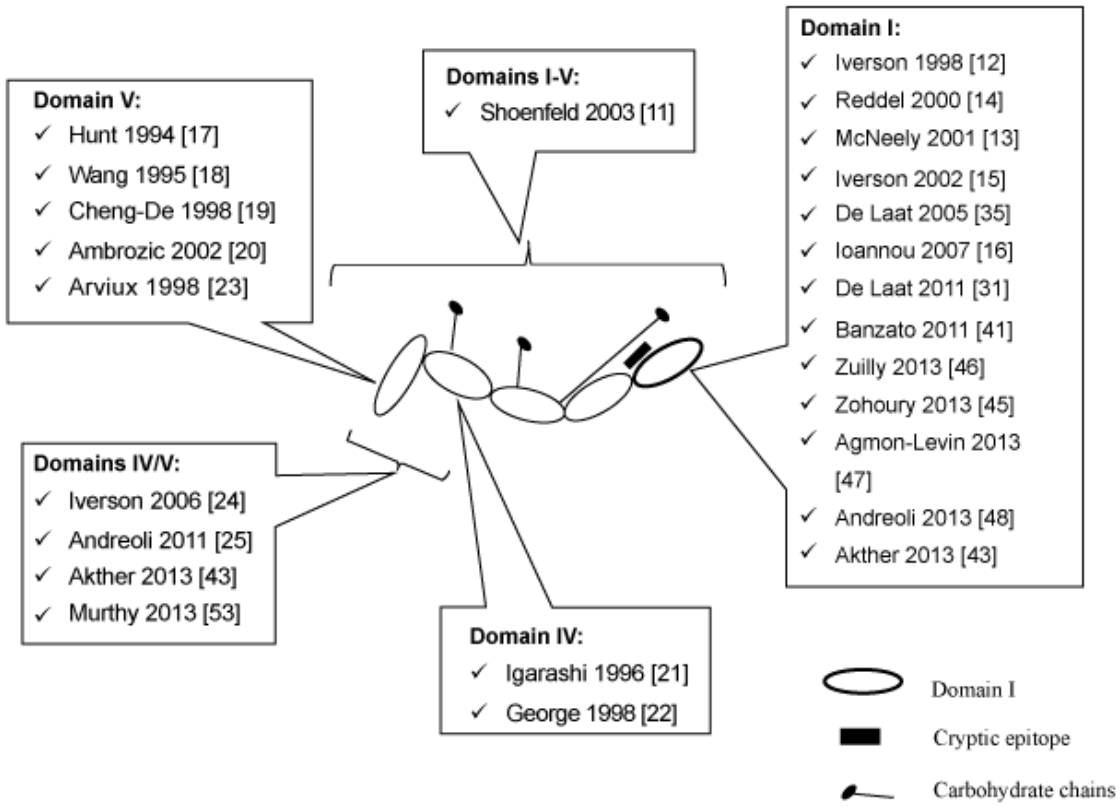

Figure 1: Schematic representation β_2 glycoprotein I, and the studies evaluating reactivities against each of the five domains.

Reactivity towards Domain I of β_2 Glycoprotein I

The earliest hint of DI as the immunodominant epitope of anti-β_2GPI antibodies was provided by Iverson, who in 1998 developed seven domain-deleted mutants of β_2GPI using a baculovirus system. He observed that, in competition assays to inhibit the autoantibodies from binding to immobilized wild-type β_2GPI, only those domain-deleted mutants containing DI inhibited the binding. In direct ELISA assays, a battery of 10 affinity-purified anti-β_2GPI antibodies was shown to bind more robustly to β_2GPI variants containing DI compared to mutants lacking it [12]. The localization of the immunodominant epitope in DI was confirmed few years later employing surface plasmon resonance; 88% of 106 APS patients displayed a selectivity for DI [13]. The binding of human serum anti-β_2GPI antibodies to epitopes located in DI was further supported by evidence raised using his_6-tagged domain mutants I-IV and II-V, three variants with point mutations in DI and wild type molecule. To avoid selecting an artificial subset of anti-β_2GPI antibodies with higher affinity, patients serum samples were used, and almost no binding to deleted mutants II-V was observed [14]. Later on, the development of 10 $glyhis_6$-tagged mutants of whole β_2GPI containing single point mutations in DI allowed to identify the main epitope in the region of aa 40-43. At molecular modeling, the critical mutations were predicted to affect the surface shape and electrostatic charge of a facet of DI. The residues between glycine 40 and arginine 43 (the G40-R43 epitope) were confirmed as contributors of the immunodominant epitope in both a competitive inhibition ELISA assay using affinity-purified anti-β_2GPI antibodies and in a direct binding ELISA test using whole serum [15]. In 2007, Ioannou produced multiple single mutant variants of β_2GPI by an efficient method employing a novel bacterial expression system. In a simple and direct ELISA, the authors demonstrated the discontinuous nature of the main epitope: in addition to G40-R43, it involves also arginine 39, aspartic acid 8-aspartic acid 9, and possibly the interlinker region between DI and DII; R39 appears to be the most important residue [16].

Subequent studies, discussed below, demonstrated DI was not only a discontinuous structure but also a cryptic epitope.

Reactivity towards Domains IV/V of β_2 Glycoprotein I

Since the binding site for PL had been identified in DV, the first studies aimed at characterizing the fine specificities of antibodies reacting against β_2GPI focused on DV as the dominant epitope. Back in 1994, Hunt and Krilis used synthetic peptides spanning DV of β_2GPI in inhibition and direct binding studies to identify the binding site for aCL. The sequence Cys281-Cys288 was the only one to inhibit aCL binding to β_2GPI. In addition, aCL purified from patients were unable to bind to wells coated with β_2GPI cleaved between Lys317 and Thr318. The authors concluded that the epitope for aCL binding was likely located in the DV of β_2GPI [17]. The specificity of aCL for DV emerged also in a subsequent study, using synthetic peptides spanning DV of β_2GPI as well. Indeed, monoclonal aCL purified from APS patients bound to these peptides; further, in inhibition assays the binding to β_2GPI was inhibited in a dose-dependent manner by the same peptides [18]. In 1998, Cheng-De et al. constructed a plasmid

expression vector expressing DV. The purified β_2GPI-DV inhibithed binding of anti-β_2GPI antibodies from APS patients to recombinat whole β_2GPI, in support of the hypothesis of DV as the immunodominant epitope [19].

On the other hand, evidence from other studies pointed towards DIV as the immunodominant epitope in APS patients. Igarashi et al., by expressing mutant β_2GPI genes in Spodoptera frugiperda insect cells infected with recombinant baculoviruses, observed that the critical epitope was located in DI-IV, as aCL binding to β_2GPI occurred upon removal of DV [20]. These authors went further to characterize the conformational epitopes targeted by anti-β_2GPI antibodies from APS patients by screening constrained peptide libraries displayed on filamentous phages. This process lead to the identification of four epitopes, all shaded in the inner side of DIV at N- and C-terminus. Consistent findings were reported by George et al., who suggested that the epitope targeted by aCL might reside in DIV, as no inhibition of aCL binding was observed with β_2GPI mutants I-III and V, while the mutant I-IV inhibited the binding more efficiently than the whole molecule [21].

DIV and DV have also emerged as the main epitopes targeted by anti-β_2GPI from patients with conditions other than APS, whose autoantibodies do not exert a pathogenic potential. Ambrozic et al. evaluated the fine specificities of anti-β_2GPI antibodies isolated from children with atopic dermatitis, observing that antibody binding was abolished upon specific proteolytic cleavage of the PL-binding C-terminal loop in DV [22]. Arvieux et al. observed that anti-β_2GPI antibodies purified from patients with leprosy preferentially target DV, as evaluated by direct binding to DI and DV deleted mutant and by competition assay with a mouse anti-DI monoclonal antibody. Conversely, the main epitope of anti- β_2GPI antibodies from APS patients was shown to reside in the N-terminal of the molecule [23]. In 2006, Iverson developed six different recombinant domain deleted mutants of human β_2GPI. The mutants and the wild-type human molecule were used in competitive assay to inhibit anti-β_2GPI antibody binding. Anti-β_2GPI IgA from patients with atherosclerotic diseases were shown to preferentially target DIV, particularly when DIV was bound to DV. It can be derived that the interaction between DIV and DV may be essential to expose the suitable structure for antibody binding. Conversely, only constructs containing DI were able to inhibit binding of both IgG and IgA antibodies isolated from APS patients [24]. Similar findings were reported in 2011 by Andreoli et al., who examined by ELISA kits the specificities of anti-β_2GPI antibodies in one-year old healthy children born to mothers with systemic autoimmune diseases and in children with atopic dermatitis. Those autoantibodies targeted preferentially DIV/V (41%), whereas sera from APS patients reacted mainly against DI (75%) [25].

The Three Conformations of β_2 Glycoprotein I

There is consistent evidence that the immunogenicity of β_2GPI depends upon its conformation. To date, three configurations of β_2GPI have been described. Circulating plasma β_2GPI exists in a circular form, as observed at electronic microscopy [26]. Upon binding to suitable anionic surfaces as CL, other PL or to LPS, the molecule opens up to a J-shaped fish-hook configuration, as shown by its christal structure [27,28]. An intermediate S-shape of β_2GPI has been recently observed at small-angle X ray scattering [29]. The hypothesis of its conformation-dependent immungenicity is supported by *in vivo* evidence: mice developed antibodies against DI only when injected with misfolded β_2GPI or with β_2GPI-CL [30].

Such finding might be explained by the fact that when β_2GPI adopts the circular conformation, DI interacts with DV and the critical epitope is thus hidden. In the S-shape, the epitope is covered by DIII-IV carbohydrate chains. These residues provide a shield over DI, preventing antibodies from binding β2GPI. Consistently, it has been observed that antibodies against DI were able to bind β_2GPI only upon removal of the carbohydrate chains without showing any reactivity towards the intact molecule [31]. Conversely, upon opening to a J-configuration, the critical epitope arginine 39-glycine 43 is exposed, thus becoming available for antibody binding.

Oxidative stress is among the several factors that might lead to the surface exposition of the critical epitope. In healthy persons, a free thiol form of β_2GPI, characterized by a broken disulfide bridge, predominates in the plasma. Oxidative stress leads to the formation of disulfide bonds at these sites possibly unmasking the critical B-cell epitope [32]. Consistently, APS patients presented an increased rate of oxidized plasma β_2GPI compared to asymptomatic aPL carriers and healthy volunteers. Consistently, anti-β_2GPI antibodies purified from β_2GPI-immunized animals as well as from APS patients displayed a decreased binding to β_2GPI treated with oxidoreductase [33].

Evidences of the Pathogenetic Role of anti-domain β_2 Glycoprotein I Antibodies

Using a hexapeptide phage library, three peptides, corresponding to DI-II, DIII and DIV of the molecule, were identified as interacting specifically with anti-β_2GPI monoclonal antibodies. These peptides were shown to inhibit the binding of monoclonal antibodies to both β_2GPI and endothelial cells and to prevent the upregulation of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin. Moreover, mice infused with anti-β_2GPI monoclonal antibodies and treated with the peptides were protected from the induction of experimental APS [34].

The remaining studies on the pathogenicity of anti-domain antibodies focused on anti-DI antibodies, with evidence coming from both *in vitro* and *in vivo* studies. Firstly, anti-DI antibodies were extensively described to exert an *in vitro* prolongation of clotting time [35]. In *in vivo* experiments, the passive infusion of a synthetic DI peptide in naïve mice abolished in a dose-dependent manner the thrombus enhancement mediated by polyclonal aPL human IgG fractions. The peptide also prevented, even though not completely, the expression of adhesion molecules on aortic endothelial cells and the production of Tissue Factor (TF) by murine macrophages. Noteworthy, DI mutations, associated with either an increase or a decrease in its affinity for IgG purified from APS patients, affected in a corresponding manner the ability of the mutant peptide to reverse the effects mediated by the same aPL fractions [36]. Recently eluted fractions rich in anti-DI antibodies obtained from an APS patient were reported to induce a greater increase in TF activity and significantly larger thrombi than the anti-DI antibody poor serum recollected after affinity-purification [37]. When administered concomitantly with LPS, a monoclonal anti-DI IgG induced clotting and fetal loss in naïve mice. This observation, besides fitting well with the "two-hit" hypothesis, provides the first direct demonstration of the pathogenic effects of anti-DI antibodies [38].

Tests Detecting Anti-Domain I Antibodies

No commercial kit to detect anti-DI antibodies has been yet introduced on the market; however, several research assays have been described, each implying different molecular antigenic targets.

The first assay has been developed by a Dutch group that used a baculovirus expression system to synthetize DI. This is a direct two-step ELISA assay that measures the strength of binding to DI coated at the same density on both hydrophobic and hydrophilic plates. The assay relies on the fact that the criptical epitope is exposed on hydrophobic but not hydrophilic plates. The results are reported in a dichotomous way: when the ratio between the Optical Density (OD) on hydrophobic plate and that on hydrophilic plate exceeds 2, the sample is considered to be anti-DI antibody positive [35]. In the UK, *Escherichia coli* was used as the expressing host to synthetize DI, and a direct ELISA test was then developed [39]. An italian group synthesized DI chemically, exploting it to set up 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 [40,41]. INOVA Diagnostics (San Diego, USA) has developed two tests to detect anti-DI antibodies: a commercial anti-DI assay and a β_2GPI-DI chemiluminescence immunoassay (CIA). The latter uses a recombinant DI coupled to paramagnetic beads exploiting the BIO-FLASH technology (Biokit, Barcelona, Spain). Interestingly, the ELISA and CIA research assays, when directly compared, have been shown to display the same specificity although a different sensitivity. The CIA immunoassay has also been demonstrated to well correlate with the UK in-house ELISA test [6,42]. These preliminary data support a comparability between the solid phase assays and the CIA, however the reproducibility of the different anti-DI antibody assays warrants to be further assessed in prospective studies.

Tests Detecting Anti-Domain IV/V Antibodies

All the studies published to date detected anti-DIV/V antibodies by ELISA assays, either the direct or the inhibition test, using recombinat domain deleted mutants of β_2GPI synthetized using different expression vectors [19,20,23,24]. INOVA Diagnostics (San Diego, CA, USA) has developed a research ELISA kit, not yet commercially available, to detect anti-DIV/V IgA [25,43].

Anti-Domain I β_2 Glycoprotein I Antibodies in APS

Most of the studies addressing the clinical significance of anti-DI antibodies in APS patients focused on the association with thrombosis, since the results presented by De Laat et al. in 2005. In this work, positivity for anti-DI antibodies was associated with a greater risk of thrombosis in a cohort of 198 patients [35]. Such association was later confirmed in the largest multi-centre study to date published, that recruited 442 patients selected upon positivity for anti-β_2GPI antibodies [44]. Later on, many other authors have conducted yet unpublished studies among aPL positive subjects, all concordantly confirming the relationship between anti-DI antibodies and thrombotic events affecting the venous as well as the arterial vascular tree [6,45-47].

It should be noted that also those studies investigating anti-DI antibodies in the context of aPL-related pregnancy morbidity evinced a positive association. This is the case of the above cited multicentre study conducted in the largest cohort, and of an Italian study, yet unpublished [44,48]. These authors identified anti-DI antibodies as the prevalent antibody specificities among APS patients with pure obstetric morbidity as well. Although the positivity rate was slightly lower among women with obstetric APS compared to subjects with vascular APS (61.3% versus 78.2%), no significant differences in anti-DI antibody frequency and titres were observed between the two subgroups of patients [48].

Consistently with the strong association with APS manifestations, antibodies targeting DI, differently from those reacting against the whole molecule, were significantly associated with APS diagnosis in one study [6]. Furthermore, antibodies against DI have been proposed as a marker of severity among APS patients. It is well established that patients with positivity in all the three criteria tests are at the highest risk of developing clinical events [49]. Banzato et al. observed, using an inhibition ELISA test with a synthetic DI, that triple-positive patients carry significantly higher anti-DI antibody titres compared to double- or single-positive subjects or healthy controls [41]. This finding might imply that anti-DI IgG may represent a more predictive aPL profile, and that anti-DI antibodies might be helpful in the risk-stratification of APS subjects.

Anti-Domain IV/V β_2 Glycoprotein I Antibodies in APS

Back in 1998, Cheng-De et al. detected anti-DIV/V antibodies by ELISA test in 27 of 28 APS patients who had positive antibodies against recombinant β_2GPI [19]. More recently, Andreoli et al. described a positivity for anti-DIV/V antibodies in 8 of 64 APS patients (12.5%) [25].

Anti-Domain I Antibodies in Systemic Lupus Erythematosus

Data about anti-DI antibodies among anti-β_2GPI antibody positive SLE patients can be extrapolated from the paper published by De Laat et al. in 2009. Antibodies targeting DI were described in 36 of 93 lupus patients without any clinical feature of APS (38.7%). Among the 128 patients with SAPS included in this study, anti-DI antibodies were detectable in 54 (42.2%). Unfortunately, no subgroup analysis was carried out to specifically investigate the clinical meaning of anti-DI antibodies among subjects with SLE [44].

More recently, Akhter et al. assayed anti-DI antibodies in the Hopkins Lupus Cohort comprising 326 patients, of whom 164 had a history of thrombosis. Twenty per cent of lupus subjects displayed anti-DI antibodies; no association with thrombosis, either venous or arterial, could be reported even though anti-DI antibodies carried an Odds Ratio (OR) for LA of 6 [43].

In a yet unpublished study, Zuily et al. assayed anti-DI antibodies by an ELISA kit manufactured by Inova Diagnostics (San Diego, CA, USA) in a cohort of 92 patients comprising subjects with SLE and aPL, SLE or aPL alone. In this population, highly positive anti-DI antibodies were associated with a 3.6 fold increase in thrombotic risk [46].

The potential clinical meaning of anti-DI antibodies is also suggested by the good correlation with annexin A5 resistance assay [50]. Annexin A5 is a potent anticoagulant protein mainly found in trophoblasts and vascular endothelial cells; β_2GPI-dependent aPL have been shown to interfere with the protective shield that annexin A5 provides over the endothelium, hence favouring thrombosis. Annexin

A5 resistance is a novel 2-stage coagulation assay previously shown to be decreased among aPL-positive patients with a history of thrombosis [51]. In a cohort of 183 children with SLE, anti-DI antibodies were shown to be more frequent among patients than pediatric controls. Moreover, children with lupus presented a significantly reduced Annexin A5 Resistance. Furthermore, children with SLE and positive anti-DI antibodies had significantly lower mean annexin A5 resistance levels compared to those with negative anti- DI antibodies. In multivariate analysis, anti-DI antibodies and LA were both independently associated with reduced annexin A5 resistance [52].

Anti-Domain IV and Anti-Domain V Antibodies in Systemic Lupus Erythematosus

More recently, anti-DIV/V IgA were described in 63 of the 326 (19.3%) SLE patients included in the Hopkins Lupus Cohort; noteworthy, in this work a significant association with stroke -but not with venous thrombosis- emerged (OR 2.2, 95% CI 1.1-4.4, p=0.033). Interestingly, anti-DIV/V antibodies well correlated with a positivity for LA [43].

Murthy et al. evaluated antibodies against DIV/V in a cohort of subjects selected upon positivity for anti-β_2GPI IgA, comprising 80 lupus patients and 35 individuals referred to the Antiphospholipid Standardization Laboratory. 62 of the samples (54%) were found to be positive in this assay. IgA anti-β_2GPI DIV/V titers well correlated with IgA anti- β_2GPI titers ($p<0.0001$). Of the 62 patients whose samples were positive for binding of IgA to anti-β_2GPI DIV/V, 77% had clinical manifestations of APS that included DVT, strokes, myocardial infarction, pulmonary arterial hypertension, seizures, pregnancy losses, skin ulcers, and livedo reticularis [53].

β2 Glycoprotein I Domain Specificities in an Italian Monocentric Cohort of Systemic Lupus Erythematosus Patients

Antibodies against DI and DIV/V of β_2GPI were tested in a monocentric Italian cohort comprising 11 SLE subjects and 62 PAPS patients. Seven lupus patients (64%) had a history of clinical manifestations suggestive of SAPS; in particular, two women presented pregnancy morbidity while the remaining six individuals presented vascular SAPS. 40 PAPS patients (64%) had vascular and/or obstetric manifestations, while 22 women presented pure pregnancy morbidity. LA was performed according to international guidelines [54]. aCL IgG/IgM and anti-β2GPI IgG/IgM were detected by internationally validated in-house ELISA assays [55]. The fine specificities of anti-β2GPI IgG antibodies was investigated using prototype research ELISAs developed by INOVA Diagnostics (San Diego, California, USA). Results were expressed as OD values; the 95th percentile values in a control population of 100 healthy individuals were used as cut-offs: 0.235 OD for anti-DI and 0.405 for anti-DIV/V antibodies. All the clinical and serological details of the recruited patients are presented in Table 1.

In our cohort, anti-DI antibodies were detected in 9 lupus subjects (82%, six in the SLE-SAPS group and 3 in the aPL+ SLE group). There was no statistical significant difference between the two subgroups of lupus patients with respects to anti-DI antibody titers and positivity rate ($p=0.445$ at Mann-Whitney test and chi-squared=0.444, $p=0.505$ at Chi-squared test respectively). 68% of the APS patients (n=39) presented antibodies against DI; in particular, anti-DI antibodies were detected in 65% of the subjects with vascular APS (n=24) and in the 75% of women with pure obstetric APS (n=15). There was no statistical difference in anti-DI antibody titers and positivity rates between patients with vascular and obstetric APS ($p=0.585$ at Mann-Whitney test and chi-squared=0.0331, $p=0.856$ at Chi-squared test respectively). A positivity for anti-DIV/V antibodies was observed in no SLE patient, while 10 APS individuals (18%) beared positive anti-DIV/V antibodies (8 (22%) in the vascular APS group and 2 (10%) in the obstetric APS group). Any significant difference between the two subgroups of lupus patients with respects to anti-DIV/V antibody titers was observed ($p=0.8501$ at Mann-Whitney test). A statistical difference in anti-DIV/V antibody titers and positivity rates emerged between patients with vascular and obstetric APS ($p=0.0238$ at Mann-Whitney test and chi-squared=5.590, $p=0.018$ at Chi-squared test respectively). Two lupus patients (18%) and 20 (35%) APS subjects were double negative, despite in most cases they carried autoantibodies reacting against the whole molecule. The positivity rate of anti-DI antibodies did not present a significantly different distribution between SLE and APS (chi-squared: 0.0204, $p=0.887$ at Chi-squared test). Patients with SLE presented anti-DI and anti-DIV/V antibody titres similar to PAPS patients ($p=0.068$ and $p=0.871$ respectively at Mann-Whitney test). The above presented data clearly show that lupus patients present a similar positivity rates of anti-DI antibodies as compared to APS subjects. DI thus provides the main epitope targeted by anti-β_2GPI antibodies even among SAPS-SLE subjects and aPL positive SLE individuals with no clinical feature suggestive of APS. However, there is a consistent rate of anti-β_2GPI antibody positive subjects displaying reactivity against β_2GPI domains other than DI and DIV/V.

Conclusion

It is well ascertained that anti-β_2GPI antibodies may target multiple epitopes in the same molecule; basic research as well as clinical studies focused on anti-DI and anti-DIV/V antibodies. Over the recent years, a growing body of evidence has been raised in favour of DI as the most relevant epitope targeted by anti-β_2GPI antibodies in patients with APS. Consistently, anti-DI antibodies have been shown to be significantly associated with aPL-related events, of both vascular and obstetric nature. The fact that anti-β_2GPI antibodies specifically reacting against DI are more commonly detected among patients with APS compared to conditions associated with transient aPL positivity due to polyclonal B cell activation implies that anti-DI antibodies provide a higher specificity for APS than antibodies targeting the whole molecule. Indeed, anti-β2GPI IgG isolated from sera of aPL positive asymptomatic carriers, subjects with atherosclerosis, individuals with leprosy or children with atopic dermatitis have been shown to preferentially recognize epitopes on DIV or V [20,23-25]. Further, APS patients at highest risk -those with triple aPL positivity- have been demonstrated to have higher titres of anti-β2GPI-DI antibodies [41,47,48]: therefore, anti-DI IgG have been proposed as an additional tool to risk-stratify APS subjects in order to identify patients with a more aggressive clinical presentation. On the other hand, it should be considered that there are anti- β_2GPI antibody positive patients with a formal APS diagnosis carrying autoantibodies reacting with β2GPI epitopes other than DI. Therefore, anti-DI antibody testing provides a lower sensitivity for APS compared to the assay detecting the antibodies against the whole molecule.

	SAPS-SLE (n=7)	aPL+ SLE (n=4)	Vascular PAPS (n=40)	Obstetric PAPS (n=22)
Female, n (%)	5 (71%)	2 (50%)	27 (68%)	22 (100%)
Thrombosis	6 (85%)	/	40/40 (100%)	/
Obstetric manifestations	2 (29%)	/	7 (17.5%)	22 (100%)
Positive LA	5 (72%)	2 (50%)	29 (73%)	18 (82%)
aCL IgG, GPL (median, IQR)	14 (12-106.5)	58 (27.5-91.25)	82 (46-103)	41 (5-75)
aCL IgM, MPL (median, IQR)	7 (3-8.5)	4 (0.75-7.25)	7 (2-16)	8 (2-17)
Anti-β_2GPI IgG, OD (median, IQR)	0.67 (0.335-1.125)	0.985 (0.76-1.13)	1.24 (0.37-1.55)	0.49 (0.115-1.162)
Anti-β_2GPI IgM, OD (median, IQR)	0.23 (0.17-0.43)	0.16 (0.095-1.2675)	0.25 (0.1-0.89)	0.13 (0.082-0.217)
Anti-D1, OD (median, IQR)	0.7 (0.4-2)	0.980 (0.373-1.541)	0.409 (0.212-0.74)	0.267 (0.191-0.493)
Anti-D4/5, OD (median, IQR)	0.25 (0.13-0.20)	0.226 (0.167-0.267)	0.233 (0.148-0.342)	0.151 (0.134-0.226)

Table 1: Demografic, clinical and laboratory characteristics of 73 subjects with positive anti-β_2-glycoprotein I IgG antibodies.

It is interesting to note anti-DI antibodies provide the main epitopic specificities even in SLE patients with positivity for anti-β_2GPI antibodies. Indeed, aPL positive lupus subjects without any clinical features of APS present a positivity rate of anti-DI antibodies not significantly different from APS patients. However, the association of anti-DI antibodies with aPL related events among SLE subjects is not clear. While anti-DI antibodies were related to thrombosis in the study by de Laat et al. which did not differentiate between APS and lupus patients, the only study on the clinical meaning of anti-DI antibodies conducted specifically in a lupus popupulation did not evince a significant association with aPL-related clinical events [44]. On the other hand, in the same study a significant association emerged between anti-DIV/V IgA and stroke; consistently, Murthy et al. observed that 77% of patients with positive for anti-DIV/V antibodies had clinical manifestations of SAPS [53].

These findings might be explained by the fact that SLE provides a much more complex picture than APS, with many factors contributing to the thrombotic risk. Indeed, positivity for aPL, disease duration, disease activity, nephritis, contribute to the cardiovascular burden. A careful assessment of traditional cardiovascular risk-factors should be accomplished in all SLE individuals: age, diabetes, arterial hypertension, dyslipidemia, obesity, smoking, sedentary lifestyle, hyperhomocysteinemia, Protein C, Protein S and anti-thrombin III deficiency, Factor V Leiden and prothrombin mutations, prolonged immobilization, surgical procedures, oestrogen use. Stratification of the risks of thrombotic events, smoking cessation and the use of protective medications are important elements of thrombosis prevention. At this regard, it should be considered that results might be biased by the fact that SLE patients with aPL positivity routinely receive aspirin and antimalarial medications, which exert a protective anti-thrombotic effect.

Therefore, it clearly emerges how important it would be in SLE to identify a laboratory tool allowing a more accurate estimate of the thrombotic and obstetric risk, possiby leading to a treatment strategy tailored upon the peculiar clinical and laboratory characteristics of each patient.

Anti-domain antibodies surely provide a promising tool in APS although larger and prospective studies are needed to support their diagnostic and prognostic value.

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