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Review Article

News and meta-analysis regarding anti-Beta 2 glycoprotein I antibodies and their determination

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ABSTRACT

Recent advances allow us to propose antibodies targeting beta-2-glycoprotein I (β_2-GPI) as the most specific antibodies associated with anti-phospholipid syndrome (APS). Therefore, there is now a crucial need for powerful biological assays to adequately monitor them. It is well established that these antibodies recognize mainly cryptic epitopes, which requires a great deal of consideration in the choice of laboratory tests to identify these antibodies. To this end, an update on the pathophysiological role of β_2-GPI and a meta-analysis were conducted providing an overview of the current progress towards anti-β2-GPI detection.

1. History and STATE of the art

1.1. From syphilis diagnosis to the anti-cardiolipin autoantibodies

While the definition of the antiphospholipid syndrome (APS) was established in 1999 [1], the biological history of the antiphospholipid antibodies (APLA) started long before that in the first decade of the 20th century with development of assays for the diagnosis of syphilis. Indeed, Wassermann was the first in 1906 to apply the complement fixation reaction, previously described by Bordet and Gengou, in a new test for the diagnosis of syphilis using fetal liver extracts from dead embryos with congenital syphilis as an antigenic source [2]. This antigenic source was shortly replaced with an alcoholic extract from non-syphilis beef heart by Landsteiner who had then developed the Venereal Disease Research Laboratory Test (VDRL). Later on in 1941, Pangborn characterized mitochondrial phospholipids, called cardiolipin because of its cardiac origin, as the main antigenic source from the beef extract [3]. This improvement led to the development of more accurate syphilis tests using purified cardiolipin [4]. It was then shown that cardiolipin was in fact phosphatidylglycerol, though we kept the cardiolipin appellation.

1.2. Associations with thrombosis and/or pregnancy failure

The extensive use of the VDRL assay has led to the observation that a false positive syphilitic serology occurs in two main cases, firstly due to other infectious diseases such as malaria and leprosy, and secondly due to an autoimmune disease that is, in most of the cases, systemic lupus erythematosus (SLE) [5]. In the 1950s, Conley and Hartmann described two patients with SLE yielding both a false-positive test for syphilis and a prolonged blood clotting time [6]. A few years later, in 1963, the improperly named term, Lupus Anticoagulant (LA), [7] was introduced by Bowie et al. based on the triple association observed between SLE, thromboses and an extended coagulation test that was not corrected after mixing the SLE sera with a normal plasma due to the presence of an anticoagulant antibody. Such an observation was followed by the report of an association between circulating anticoagulant antibodies with repeated miscarriages and/or thrombotic events by Soulier and Boffa [8]. This was the first step towards a definition of APS. However, it was the development of a radioimmunoassay (RIA) [9] in 1983 and then two years later of an enzyme-linked immunosorbent assay (ELISA) [10] for the detection of the anticardiolipin (aCL) autoantibodies, that were critical in establishing the first official definition of APS, associating at least one clinical manifestation with APLA detection that could be either a positive aCL and/or the presence of a LA in 1987 [11]. Thanks to Asherson [12], APS is now subdivided into primary and secondary whether it's associated or not with another autoimmune disease, mainly SLE but alternatively it could be rheumatoid arthritis, Sjögren's syndrome, systemic sclerosis, autoimmune thyroid diseases, systemic vasculitis, dermatopolymyositis, primary biliary cirrhosis or autoimmune hepatitis. The catastrophic antiphospholipid (CAPS) was first described by Asherson in 1992 as a widespread coagulopathy related to the APLA [13], highlighting the severity of the disease.

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1.3. Anti-beta 2-glycoprotein I (β2-GPI) autoantibodies

In the 1990's, research focused on characterization of the APLA responsible for APS. It was demonstrated that aCL were not directed against the cardiolipin, as thought for years, but against its main co-factor, beta 2-glycoprotein 1 (β_2-GPI) present in the sera that is used for ELISA plate preparation [14] [15] [16]. In the LA assay, another co-factor has been characterized, in addition to β_2-GPI, as an antigenic target, prothrombin (PT). All these autoantibodies (aCL, LA, anti-(a)β_2-GPI and anti-PT) are part of the APLA family. In 1995, it was discovered that the aβ_2-GPI antibodies can recognize β_2-GPI even in the absence of phospholipids when β_2-GPI is dimerized, which can be achieved by using irradiated ELISA plates [17]. This has led to new specific diagnostic tests using both irradiated ELISA plates and animal β_2-GPI as an antigenic target (first generation assays). Accordingly, and with a large number of publications highlighting the key role of β_2-GPI in APS, the first international definition of APS based on LA and/or aCL positivity was proposed during the 1999 APS symposium in Sapporo [1]. Later on, the APS definition has evolved with the inclusion of the aβ_2-GPI antibodies (IgG and IgM) as the third biological criteria for the diagnosis of APS in 2006 [18].

1.4. Anti- β2-GPI autoantibody determination and limitations

The identification of β_2-GPI as the most important antigen in APS gave hope for an optimized assay with better sensitivity and specificity than LA and aCL. However, this was only partially achieved since the first generation of aβ_2-GPI tests was not as powerful as expected. In order to circumvent these limitations, some efforts have been made and are still ongoing in order to improve the diagnostic performance of the test. For instance, we understand the necessity for using a negatively charged surface that can be attained by irradiation in order to raise the density and antigen dimerization and epitope presentation of coated β_2-GPI on the ELISA plate, allowing for the detection of aβ_2-GPI antibodies without the presence of phospholipids [19]; or else, in the second generation assays, the interest in using human β_2-GPI instead of animal β_2-GPI to avoid heterophilic antibody detection and in turn false positivity [20,21]. In parallel, several attempts to standardize practices were also made in order to provide the most efficient assays in laboratories [21–24].

Characterization of the β_2-GPI epitopes associated with thrombotic/miscarriage events were also conducted, revealing that the main epitopes associated with APS are cryptic, present in the domain 1 (D1) of the glycoprotein, and difficult to access, which is particularly true when using irradiated ELISA plates [25]. As a consequence, new directions were developed towards better sensitivity and specificity for the detection of aβ_2-GPI antibodies correlated with clinical events of thrombosis or miscarriages. The last few years have seen a remarkable expansion of new tests (third generation assays). The main objective of this work is to present an update on the pathophysiological role of β_2-GPI and to conduct a meta-analysis of the main technologies to detect aβ_2-GPI antibodies.

2. Beta 2 glycoprotein I: Pathogenic role

The pathophysiology of the APS is not fully understood [26,27] but it has been proposed that events leading to thrombus formation are based on a two-hit model [28]. Indeed, despite the constant presence of APLA, thrombotic events fortunately remain infrequent, suggesting that APLA presence is necessary but not sufficient for the development of real APS. In fact, APLA-dependent mechanisms are mainly implicated in the break of the cellular anticoagulant state through the activation of cellular actors such as endothelial cells, platelets, monocytes and lymphocytes. Then, a “second event” is mandatory to promote the coagulation and fibrinolysis pathways leading to thrombosis [29,30], such as infections, autoimmune diseases, or other pro-coagulative states (e.g. contraceptive pills, genetic mutations, surgery, traditional cardiovascular risk factors, and smoking). Thrombotic risk factors are found in > 50% of the APLA positive patients that have developed APS [27,31]. As a consequence, all risk factors of thrombosis need to be evaluated in individuals with persistent APLA to prevent dire events.

2.1. β2-GPI and cryptic epitopes

β2-GPI is the main antigenic target of aPL in APS. Even though its pathological implication is key to understand the pathological mechanism of the APS onset, its physiological role is not fully understood [32]. β2-GPI is an anionic phospholipid-binding glycoprotein that belongs to the complement control protein superfamily. This protein is synthesized by the liver and formed by a single polypeptidic chain of 326 amino acids divided in 5 repetitive structures or “sushi domains”, termed domain 1 through to 5, for a combined molecular weight of 54 kDa for the protein [26,32]. Performed in 2002, small-angle X-ray scattering experiments have reported, in solution, an S-shaped or an O-shaped conformation with an additional buckle between domains II and III of β2-GPI [33,34]. These 2 forms are both inactive and present in the circulation at elevated concentrations (200 μg/mL) [35]. The main binding site to phospholipids is located on the positively charged fifth domain, thus electrostatically attracted to the negatively charged membranous phospholipids [26,32]. After binding to phospholipids, β2-GPI unfolds and adopts a J-shaped conformation, unveiling domain 1 and its cryptic epitopes [33,36]. The aβ2-GPI associated with clinical events recognize mostly cryptic epitopes present in the first domain, which induces in turn the dimerization of the β2-GPI on cell surfaces. This dimerization is necessary to promote a pro-thrombotic state, either by the interaction with cells, or by inactivation of some anticoagulant proteins [37,38] (Fig. 1).

2.2. Anti-β2-GPI autoantibodies, a heterogeneous family

APLA represent a large family of autoantibodies that can be found in various situations, and they are not all pro-thrombotic [37,38]. APLA could be transient, usually of the IgM isotype, and independent of the presence of the β2-GPI cofactor [39,40]. These transient IgM APLA are often associated with a history of infections, like hepatitis C virus (HCV), human immunodeficiency virus (HIV), leptospirosis, or malaria to name a few [41]. The prevailing theory to explain the generation of transient APLA in infections is molecular mimicry with microbiological sources [42–45]. It's important to note that these “infectious” APLA are neither accompanied by hematological manifestations nor thrombotic events, which define APS [41]. However and according to the “two hit” model, it should also be noted that infections, generally bacterial, in patients with confirmed APS, can lead to a catastrophic antiphospholipid syndrome with a possible fatal outcome [46,47]. In addition, non-thrombotic aβ2-GPI-dependent APLA have been reported that could be directed to non-human β2-GPI (heterophilic antibodies) or to an epitope that is not involved in the dimerization of β2-GPI such as those targeting domain V (D5) as reported in leprosy [48].

Regarding thrombotic aβ2-GPI-dependent APLA responsible for APS, several studies were performed in order to better characterize them. First, it was observed that β2-GPI binding to the anionic phospholipids present on the plasma membrane triggers a change in the conformation of the β2-GPI, exposing cryptic epitopes present in D1 and enabling the possibility of an autoimmune response [49]. Studies were then initiated in order to characterize more specifically the major epitopes present in D1 of the β2-GPI, as they seemed to be related to clinical events of thrombosis. It appeared from such studies that aβ2-GPI antibodies targeting D1 and in particular those recognizing the cryptic glycine (G)40-arginine (R)43 epitope covered by a carbohydrate chain are strongly associated with thrombosis [50,51]. Variations of the carbohydrate chains of β2-GPI correlate to some clinical manifestations [52,53]. The pathogenic key role of the β2-GPI D1 is further highlighted

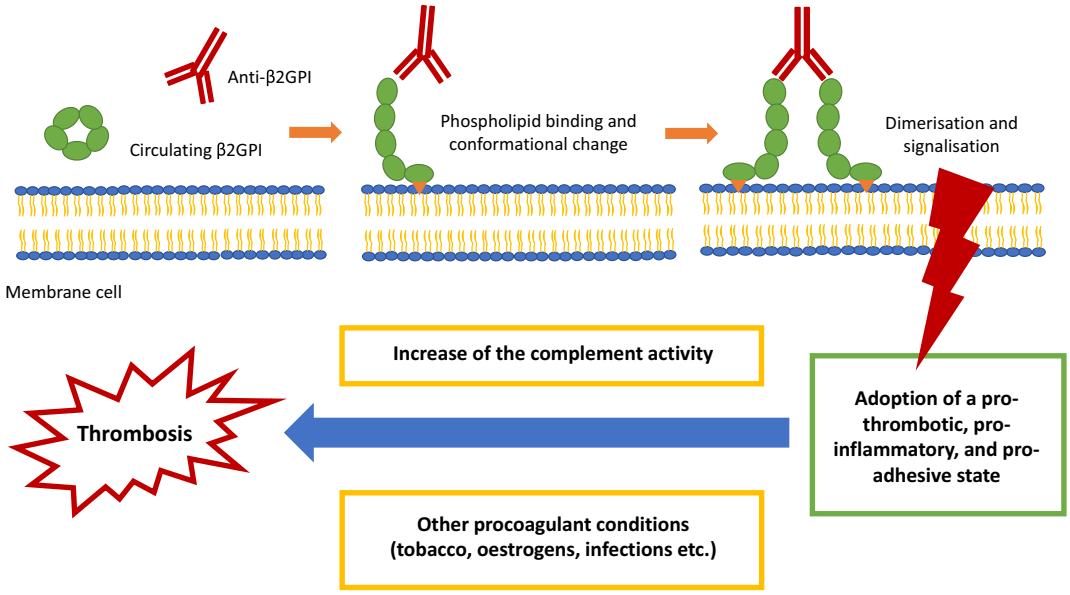

Fig. 1. Pathogenic role of anti-β2GPI autoantibodies. In the physiological state, β2GPI circulates freely either in an S-shaped or an O-shaped conformation. After binding of the fifth domain to phospholipids, β2GPI unfolds and adopts a J-shaped conformation, uncovering the D1 and its cryptic epitopes. When present, pathogenic anti-(a)β2GPI autoantibodies from patients with anti-phospholipid syndrome recognize cryptic epitopes located on the first domain, which induces the dimerization of the β2GPI at the cell surface. This cross-linking of β2GPI is mandatory to elicit a pro-thrombotic state, pro-inflammatory and pro-adhesive state. Moreover and according to the two-hit model, a « second trigger » is mandatory to push the haemostatic balance in favor of thrombosis. This « second trigger » corresponds to other current procoagulant conditions such as smoking, contraceptive pills, infections, surgery or congenital thrombophilia.

by the report, in a β2-GPI immunized mouse-model of APS, of a better oral tolerance when mice were fed with β2-GPI-D1 instead of complete β2-GPI or with β2-GPI-D5 [54].

In addition to the autoantibodies, β2-GPI specific CD4+ T cells are effective in recognizing cryptic β2-GPI peptides when presented by dendritic cells [55]. The predominant β2-GPI specific T cell clone was reported to be associated with HLA DRB4*0103 (DR53) when using peptide p276–290 located in the major phospholipid binding site (D5) [56,57] Minor HLA-dependant restrictions were reported between HLA-DP and β2-GPI D1/2, as well as between HLA-DR and β2-GPI D4/5. During APS, quantitative and qualitative modifications are observed with regards to lymphocytes with total CD4+ T cell reduction, a lower CD4/CD8 ratio, and disturbance in B cell homeostasis [58,59].

2.3. Anti-β2-GPI autoantibodies and cellular activation

In the case of pathogenic APLA, aβ2-GPI binding leads to cellular activation through the interaction of β2-GPI with different cell surface receptors that are annexin A2 and TLRs for endothelial cells and monocytes [60–63]; and apoE2 and GPIb receptors for platelets [64–67]. APLA-activated monocytes and endothelial cells produce pro-thrombotic molecules and in particular the tissue factor, the main activator of the extrinsic pathway of coagulation [68,69]. In addition, endothelial cells take on a pro-inflammatory, pro-adhesive phenotype with the expression of adhesion molecules like *E*-selectin, VCAM-1 and ICAM-1 [69], and they become capable of producing chemokines involved in monocyte recruitment [70]. Regarding the platelets, aβ2-GPI autoantibody binding affects the β2-GPI-GPIIb/IIIa complex, and leads to a higher production of thromboxane (Tx)A2/TxB2 and an increase in platelet aggregation, leading to a vasoconstrictive state [71,72].

2.4. Anti-β2-GPI autoantibodies and obstetric APS

The essential role played by complement activation in obstetric APS was first demonstrated in murine APS models [73]. Indeed, mice deficient in C3, C5 or C5a receptors are protected from fetal loss induced by the injection of APLA [74]. This observation was further confirmed, in other APS mouse models, by using a specific anti-C5 monoclonal antibody capable of inhibiting the complement cascade [75–77]. In addition, the release of pro-inflammatory anaphylatoxin (C3a, C5a) during the complement activation is also implicated in placental lesions [78]. When recruited and activated by C5a, neutrophils become responsible for trophoblast lesions, inhibition of trophoblast growth and differentiation, and, in the end, fetal loss [79]. In humans, several studies have shown consumption of complement proteins during and out of the thrombotic processes in APS [80]. Complement activation is also responsible for activation of endothelial cells, tissue factor expression, leading to a positive feedback loop for continuation of the pro-coagulative state in APS [73,81].

A reduction of annexin V on placental trophoblasts and endothelial cells induced by APLA may be another mechanism leading to fetal loss in APS [82]. Indeed, annexin V is a concave disc shaped protein with a binding domain for phospholipids that blocks the interactions with proteins of coagulation and fibrinolysis on the cell surfaces. In the presence of aβ_2-GPI autoantibodies, the annexin V network is disrupted [83], which exposes the tissue factor and the phosphatidylserine, since the affinity of annexin V for PL is weaker than the affinity of the β_2-GPI/aβ_2-GPI antibody complex at the cell surface. This obviously leads to activation of the coagulation cascade and ultimately to the occurrence of thrombotic events.

2.5. Anti-β2-GPI autoantibodies and natural coagulant activity

APLA exert their anticoagulant activity not only on cells but also through the inhibition of natural anticoagulant activity. Indeed, the APLA alter the activation of protein C as well as its capacity to cleave coagulation factors V and VIII when activated [84]. The other regulation factors (heparin cofactor II, ATIII, TFPI) which are responsible for the suppression of negative feedback on thrombin, coagulation factor X and tissue factor, are also inhibited [85] [86]. These activities are mediated by APLA directed against β_2-GPI and/or prothrombin [37].

Moreover, by blocking the interaction between β2GPI and the tissue-type plasminogen activator (t-PA), the main activator of fibrinolysis, APLA and in particular those directed against β2-GPI

interfere with plasminogen transformation into plasmin that promotes again the maintenance of a prothrombotic state [87]. Other autoantibodies such as anti-annexin 2, and anti-t-PA receptor have been described in APS and can also contribute to the inhibition of fibrinolysis and persistence of a prothrombotic state [88] [89].

2.6. Anti-β2-GPI autoantibodies and atherosclerosis

Atherosclerosis is a systemic inflammatory disease characterized by lipoprotein metabolism alterations that lead to activation of the immune and inflammatory systems, inducing proliferation of smooth-muscle cells, narrowing of arteries and atheroma formation [90]. The main targeted antigens in atherosclerosis are oxidized low density lipoprotein (oxLDL), heat shock proteins (HSP) and β_2-GPI [91]. The occurrence of anti-β_2-GPI in APS and atherosclerosis reveals that these two diseases are close, and it has been shown in mice that APLA are associated with early atherosclerosis and progression of atheromatous plaques [92,93]. Clinical observations also indicate a strong link between the two diseases [94].

3. Anti-BETA 2 glycoprotein I detection and recommendations

The ELISA assay is the standard method for aβ_2-GPI antibody detection and quantification, however new assays are emerging such as chemiluminescence [95,96] as well as immunoassays based on fluorescence or multiplex flow, permitting in the latter the detection of β_2-GPI IgG, IgM and IgA in a single tube [97]. Less sensitive techniques are also used such as line immunoassays.

3.1. Principles of the technologies

The ELISA assay is a solid-phase enzyme immunoassay [98] that uses β2-GPI coated in each of the 96 wells of a microtiter plate. Samples are added and any aβ2-GPI antibodies present can bind specifically to β2-GPI, while the other blood substances remain free. Afterwards, a secondary antibody linked to an enzyme is added in order to bind to the complex aβ2-GPI/β2-GPI. Between each step, the plate is washed with a mild detergent solution allowing removal of non-specifically bound proteins and antibodies. Finally, the plate is developed with addition of a chromogenic substrate that is cleaved by the enzyme linked to the secondary antibody, thus producing a visible signal. The signal is proportional to the quantity of aβ2-GPI antibodies present in the sample. In an adaptation of the ELISA assay, a fluorometric immunoassay is used with a fluorescent molecule instead of a chromogenic substrate. Once the reaction is stopped, the fluorescent signal is measured using an automated immunoanalyzer [99,100].

In the chemiluminescence immunoassay (CLIA), aβ2-GPI antibodies, when present in the sample, bind to magnetic beads coated with human β_2-GPI while any unbound material is removed by washing. The next step is addition of an antiglobulin coupled with a tracer, followed by incubation and wash steps. At last, a trigger solution is added, that causes luminescence from the tracer [101]. Luminescence is proportional to the quantity of aβ2-GPI antibodies and reflects the emission of a visible/nearly visible radiation generated when an electron goes back from an excited state to ground state [101].

The principle of multiplex technology is based on the use of dyed microspheres of polystyrene due to the incorporation of two fluorescent markers: one red and another one orange. These two colors, merged in various amounts, can provide one hundred different shades from the combined microspheres, characterized by a color code [102]. On each type of microsphere, an antigen can be coated through carboxyl, amine/hydrazide, and maleimide groups, creating a covalent link [103]. A mix of various microspheres is exposed to human sera in which the presence of an antibody is to be determined. After incubation, a phycoerythrin-labeled monoclonal anti-human IgG/M conjugate is next added to reveal and quantify the presence of the antibody of interest. Thus, each microsphere will go through two laser beams of a flow cytometer. The red laser (635 nm) identifies the color code of the microsphere, thus the autoantigen coated, while the green laser (532 nm) measures the quantity of conjugate corresponding to the antibodies fixed on the surface of the microsphere [104,105].

At last, an immunodot-based assay uses a hydrophobic membrane providing the reactive environment for the detection of several aPL antibodies within one single test. After an incubation step with the patient's serum, the immobilized autoantibody will be revealed by a secondary labeled antibody. This technique provides the benefit of simple handling along with the advantages of multiplexing, but it lacks power in quantification (results are assessed semi-quantitatively) which, as a result, yields in a large variation coefficient [106,107]. As a consequence, immunodot is not recommended as a first pass but rather as a confirmatory technique.

3.2. General recommendations

It is well established that the specificity of aβ_2GPI antibody detection for APS diagnosis has improved in contrast to the aCL, mainly because of the interference with infectious antibodies in the aCL assay. However, aβ_2GPI antibody detection is unfortunately associated with less sensitivity [108]. In order to improve the detection of aβ2GPI antibodies several recommendations have been made by the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) [21]. Accordingly, one of the critical general points to consider concerns the individuals used as controls. Indeed, the selection of the tested controls should be focused on younger patients with unprovoked venous/arterial thromboembolism, thrombosis at unusual sites or thrombotic-pregnancy complications associated with AID, in order to prevent incidental findings. Beyond these clinical recommendations, the chosen assays must include the use of standards, calibration curves and positive/negative controls at each run. Other parameters must also be under control and are developed in the following paragraphs.

3.3. Animal versus human β2GPI

It is important to use a human antigenic β_2GPI, since not all human anti-β_2GPI bind to β_2GPI from other species, and also to avoid the detection of heterophilic antibodies [21,109]. In addition, the use of human β_2GPI obtained from a purified source is preferred over a recombination process. In spite of these precautions, some great variations of the reagents still remain due to the different techniques to purify β_2-GPI, since, for example, structural modifications can have an impact on the epitope's accessibility to the antibodies.

3.4. Support

The aβ_2-GPI test sensitivity can be compensated for by increasing the density of β_2-GPI coated on the ELISA plate. This requirement for a high density of coated β2-GPI can be explained by the in vivo necessity for the antibody to bind to two different β_2GPI and replicate the scheme of the dimerization of β_2GPI to transmit the signal [52]. Many recommendations emphasize this particular point. This can be achieved by the use of irradiated ELISA plates, as suggested at first by Matsuura et al. [19], and corroborated by several studies [21,110].

In reference to other existing techniques, support for these may differ slightly. The chemiluminescence immunoassays use coated high density magnetic particles, which provides for a better presentation of the β_2-GPI antigens than with the ELISA assay [111], and provides a reduced background signal without loss of sensitivity since the use of magnetic microspheres allows superior washing capabilities [112]. Similarly the multiplex system that uses medium density beads presents a better capacity to detect aβ_2GPI antibodies along with better correlation with clinical manifestations of APS as reported for ELISA

[112–115].

3.5. Antibody avidity

It should be interesting to highlight the differences between the low avidity antibodies and the high avidity antibodies as the latter are suspected to have a higher impact on clinical manifestations [116,117]. In contrast to high avidity aβ_2-GPI autoantibodies that recognize only β_2-GPI when coated onto a negatively charged plate, low avidity aβ_2-GPI autoantibodies are also able to recognize both plasma-purified β_2-GPI when present in solution or when coated onto a neutrally charged plate [52]. This possibility, fortunately rare, can explain some false positivity of the β_2-GPI tests. In addition, and to avoid another source of false positive results due to the presence of antibodies recognizing the plastic of the ELISA plate, the use of non-β_2-GPI coated wells is recommended as well as the use of duplicates, especially for ELISA, in order to achieve a low coefficient of variation (< 10%) [21–24]. In the case of automated systems, harsher conditions can be used easily, leading to a better coefficient of variation (< 10%), allowing one to perform singlet testing.

3.6. Antibody isotype

Currently, only IgG and IgM are part of the diagnostic criteria for APS [18]. It is well documented however that IgG is the best isotype to study in the diagnostic approach. When comparing the odd ratios for thrombosis and the percentage of aβ_2-GPI IgG and aβ_2-GPI IgM positivity, the authors from a meta-analysis conducted between 2001 and 2014 have concluded that aβ_2-GPI IgG autoantibodies show a stronger correlation with thrombosis than with the IgM isotype [118].

The role of IgM is currently debated, especially in case of single positivity, and some authors suggest to use only the presence of the aβ_2-GPI IgG autoantibody to characterize true APS patients [119]. For the time being, it is thought to be too early to remove IgM from the diagnostic criteria of APS [18] [120,121].

Regarding the isotype IgA, it appears that β2-GPI IgA can be observed in thrombotic mice, directed against D1 and D4/5 [122]. Nevertheless, discrepancies between the different tests available on the market to detect aβ2-GPI IgA are important and, in part, related to the choice of the antiglobulin and technologies selected to detect aβ2-GPI IgA [123,124]. As a consequence, IgA are not part of the current criteria for the diagnosis of APS [18].

3.7. Association with aCL and LA

Despite the fact that the aβ2-GPI are superior to the aCL in terms of specificity for APS, a higher association with thrombosis and miscarriages has been noted between positivity on multiple assay (aβ2-GPI, aCL and LA) compared with patients who were positive on one or two assays. One explanation is the observation that those patients with triple positivity (aβ2-GPI, aCL and LA) have usually higher levels of aβ2-GPI antibodies than patients with 2 or 1 positive assay [125]. In addition such a group with multi-reactivity is suspected of having higher levels of high avidity aβ2-GPI able to recognize the G40–44 epitope present in β2-GPI D1 [126]. Counter to this, association of clinical APS with an exclusive positivity is higher for LA, followed by the aβ2-GPI positivity and then by the aCL positivity [127]. The cases of exclusive aB2-GPI positivity are still exceptional though [128].

3.8. Anti-Beta 2 glycoprotein I domain I detection

The different spatial conformation of β_2-GPI explains the observation that aβ_2-GPI autoantibodies could only recognize β_2-GPI when it is coated on a surface [52]. In addition, it is essential to note that exposure of the main epitope G40-R43 on β2-GPI associated with APS is highly variable between commercial aβ2-GPI assays and this is mainly explained by the density of the negative charges available on the support used by the manufacturers [25]. As a consequence, incomplete binding would affect exposure of the epitope G40-R43. One way to avoid such effect is to coat the D1 of β_2-GPI on a hydrophobic surface (ELISA) or magnetic beads (chemiluminescence), allowing the G40-R43 epitope to be available for aβ_2-GPI autoantibodies [50] [51].

aβ2-GPI-D1 IgG assays are currently showing strong correlations with clinical manifestations of APS, leading to great hopes in their use for a better diagnosis of APS 1. It is also important to notice that aβ_2-GPI-D1 IgG antibodies do not seem to be transient when detected in APS, but rather persist 12 weeks after the initial detection, as required by the diagnostic criteria, which corroborates their implication in APS [18,129,130].

4. Practical considerations

In clinical practice, several technical questions are emerging regarding aβ_2-GPI autoantibody detection based on the method of detection, the isotype tested, and the possibility of using specific β_2-GPI domains assays.

4.1. Regarding aβ_2-GPI IgG and available methods for detection

It appears that automated tests represent attractive alternatives to ELISA for the detection of aCL and aβ_2-GPI IgG autoantibodies based on: (i) better reproducibility when using an automated solution; (ii) a higher correlation between aβ_2-GPI and aCL autoantibody levels; (iii) the possibility of not having to use duplicates to test patients; (iv) lower consumption of reagents by the automated systems; and (v) faster acquisition of the analytical signal allowing the laboratory to gain time. Some limitations are to be considered including a lower proportionality between ELISA and CLIA as the concentration of the autoantibodies increase, as well as the cost of the dosages.

In addition, from the first reports that compared automated solutions with ELISA, it was claimed that these methods present better specificity and correlation to the clinical manifestations [131–133], while some authors have reported a loss of sensitivity [134–135]. With regards to automated solutions, first, fluorescence-based enzyme immunoassays haven't brought any superiority to ELISA assays [100,136]; second, multiplex assays present higher odds ratio than ELISA for thrombosis but still appear to be rather complicated for practical routine use in laboratories [137,138]; and third, chemiluminescence (CLIA) has given some encouraging results when comparing both ELISA and CLIA [96,111,131,132,139,140,141].

To proceed with the discussion, we collected data from 8 studies and conducted a meta-analysis [96,140,141,131,132,139,111]. From this meta-analysis, the odds ratios obtained for 10 ELISA assays were compared with 10 CLIA assays revealing the superiority of CLIA (OR = 16.4, IC95 [3.1–34.9]) over ELISA (OR = 8.8 IC95 [2.9–27.9]) for the detection of aβ_2-GPI IgG (Fig. 2).

4.2. Regarding the aβ_2-GPI IgM isotype

Actually, the interest to keep aCL/aβ_2-GPI IgM as a diagnostic criterion of APS is debated as it was initially introduced to report the few cases of seronegative aCL/aβ_2-GPI IgG with a positive IgM isotype detected by ELISA [18] [120,121]. However, some authors have suggested to phase out the aCL IgM and aβ_2-GPI IgM tests in the future [119], based on a long list of studies confirming the absence of correlation between a unique positivity of IgM and the occurrence of thromboembolic events [51,95,96,111,129,139,140,142,143]. As a consequence, an isolated positivity for aβ_2-GPI IgM, which is confirmed in a second sample tested at least 12 weeks later, should be considered with caution and only in case of serious clinical evidence for APS.

To corroborate this affirmation and to compare the interest of CLIA over ELISA assays, the 8 studies previously used for aβ_2-GPI IgG meta-

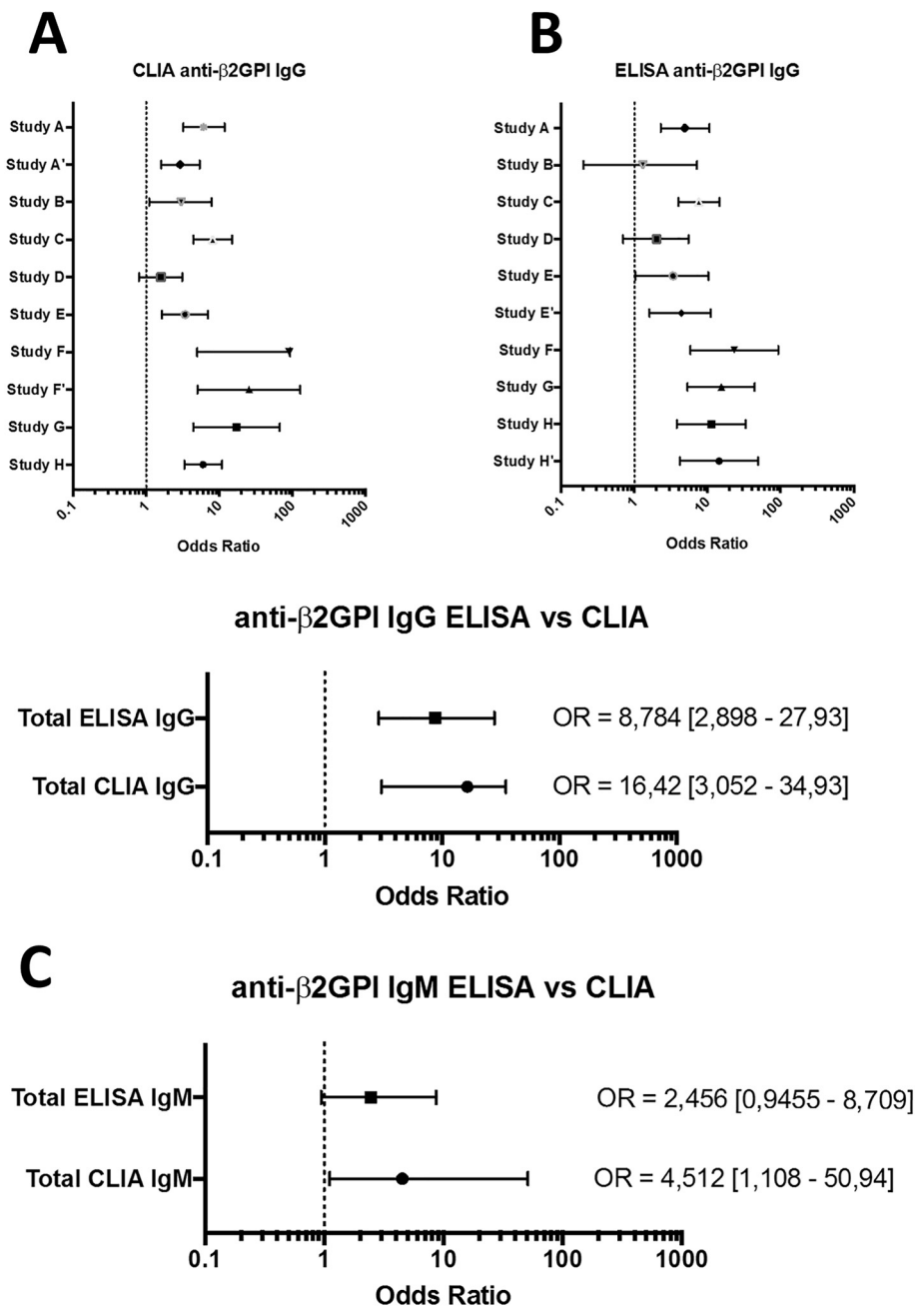

Fig. 2. Meta-analysis of case-control studies to compare chemiluminescence (CLIA) and enzyme linked immunosorbent assays (ELISA): Odds ratio (OR) for the occurrence of anti-β2GPI autoantibodies (Study A: [139]; Study B: [132]; Study C: [131]; Study D: [152]; Study E: [111]; Study F: [96]; Study G: [140]; Study H: [95]. A-C: OR for clinical manifestations of APS obtained for anti-(a)β2GPI IgG autoantibodies measured with CLIA (A) and ELISA (B) for each study. Studies mentioned twice presented results with different CLIA technologies. Studies mentioned twice presented results with different ELISA technologies and these results have been included Means of odds ratio obtained through Fig. 2 *A. means* of odds ratio obtained for aB2GPI IgM (C) after an analysis on the same studies presented above. Ten CLIA measurements were compared to ten ELISA measurements in order to provide these odds ratio means.

analysis were extended for aβ_2-GPI IgM meta-analysis revealing first, that odds ratios obtained for aβ_2-GPI IgM were close to 2; second, the superiority of CLIA over ELISA assays for aβ_2-GPI IgM showing a two-fold ratio in favor of CLIA; and third, that aβ_2-GPI IgG were 4 times higher than aβ_2-GPI IgM (Fig. 2). Altogether, those results obtained with comparative studies emphasize the poor interest in aβ_2-GPI IgM autoantibodies, a slight superiority of the CLIA assay to ELISA for such determination, but without particular interest when IgG are measured.

4.3. Regarding the aβ2-GPI IgA isotype

A growing interest in aβ_2-GPI IgA was observed over the last decade supporting: (i) that aβ2-GPI IgA are associated with APS and with a likelihood ratio that falls between IgM and IgG, indicating that aβ2-GPI IgG remains, without any doubt, the best isotype for the diagnosis of APS [123] [137] [144] [145]; (ii) that a unique positivity of IgA represents the main independent predictive marker for thrombosis in patients who never had a history of APS (OR = 5,64 [2,46–12,91]), before age, sex, diabetes and arterial hypertension in APS [146]; and (iii) that aβ2-GPI IgA determination already represents a validated immunological marker useful for the diagnosis of SLE regardless of the titers [147].

Accordingly, aβ_2-GPI IgA autoantibodies appear to be an alternative determination to conduct in second intention as a single positivity of aβ_2-GPI IgA possesses the greatest sensitivity for APS (offset by a loss of specificity) and/or pregnancy morbidity, when APS biological markers are negative despite a clinical context strongly in favor of an APS [21] [137] [146] [148]. However, the actual limitations to its introduction in routine analysis appear to be mainly technological as recommendations in terms of standardization are lacking. More studies are then required to strengthen the position of IgA and for a switch with IgM in the matter of diagnosis of APS.

4.4. Anti-domain 1 antibodies

Delving into aβ2-GPI-D1 IgG autoantibodies, recent studies support a strong link of aβ2-GPI-D1 IgG with clinical manifestations of APS [51] [144] [149]. In comparison with aβ_2-GPI IgG autoantibodies directed against other domains of the protein, aβ2-GPI-D1 IgG autoantibodies present the higher odds ratio to predict the occurrence of thrombotic events and obstetrical complications [50].

However, aβ2-GPI IgG and aβ2-GPI-D1 IgG are strongly correlated and aβ2-GPI-D1 IgG do not appear to provide any more information than the classical panel of antibodies tested for APS (aCL/aβ2-GPI IgG/M and LAC), especially in cases of triple positivity [129] [150]. In addition, their superiority over aβ2-GPI IgG autoantibody measurements is still to be proven and is controversial [51,150], which tempers the superiority of the aβ_2-GPI-D1 assay compared to the classical aβ_2-GPI assay. For the moment, it could be interesting to search for aβ_2-GPI-D1 IgG autoantibodies in situations of obvious manifestations of APS but with negative biological markers (aCL/aβ2-GPI IgG/M and LAC), as suggested by some authors [142] [148]. Unfortunately, this concerns only rare cases [144] [149]. Regarding the IgA and IgM isotypes, aβ_2-GPI-D1 IgA and IgM have also been tested with opposite results as Despierres et al. reported that there was no relationship between aβ_2-GPI-D1 IgA and APS [151], while others [144] found a connection with APS for all three isotypes (but not as much as when using total β_2-GPI).

To conclude, there are still insufficient demonstrations to make aβ2-GPI IgG part of the diagnostic criteria of APS, since there is no consensus between the available studies, and the discrepancy between available tests impose a need for work on standardization. Moreover, it is not clearly established that there are no other epitopes identified related to APS, and this test cannot replace the classical aβ2-GPI test exposing all the β2-GPI epitopes [25]. From now and given their central role played in pathogenesis, we could consider using aβ2-GPI-D1 IgG as a risk factor for stratification based on their positivity, along with the measurement of the other classical biological markers, as an additional test.

4.5. Anti-domains 4–5 antibodies

Apart from D1, other domains have been explored to identify specific targets of particular interest linked to clinical manifestations. In this context, D4/5 were thought to be the target of transient antibodies occurring during infections and without clinical manifestations [41]. However, it has also been reported that there is an association between aβ_2-GPI-D4/5 IgG positivity and APS events with the limitation that the two tests aβ_2-GPI-D1 and aβ_2-GPI-D4/5 are positive in a majority of cases suggesting that more than one domain could be behind the overall history of APS [144].

Such an observation does not appear to apply to aβ_2-GPI-D4/5 IgA autoantibodies as reported by Despierres et al. [151] who have surprisingly highlighted a significant association between aβ_2-GPI-D4/5 IgA autoantibodies and thrombotic events in SLE patients, whereas aβ_2-GPI-D1 IgA autoantibodies did not provide any association with thrombotic incidents. Accordingly, it was proposed that the targeted epitope depends on the immunoglobulin's isotype, with the importance of D1 targeted by IgG and the D4/5 targeted by IgA.

5. Conclusion

In conclusion, several advancements were made in the biological diagnosis of APS over the last thirty years and the discovery of the central role played by aβ_2-GPI in APS. This has led to the inclusion of aβ_2-GPI G/M autoantibodies in the APS diagnostic criteria of 2006 with high hopes, but those hopes have been incompletely satisfied while some improvements were made since that time.

To this end and in order to achieve a better biological diagnosis, some researchers have tried to target more specific antibodies, known to be involved in the APS. Assays to highlight aβ_2-GPI-D1 IgG were designed and showed a significant correlation with clinical manifestations, thanks to a greater specificity. However, their superiority over the traditional aβ_2-GPI IgG remains unclear, as we may miss a lot of diagnoses by neglecting other pathogenically relevant aβ_2-GPI epitopes. This observation can be transposed for other anti-domain antibodies like aβ_2-GPI-D4/5 IgG.

Moreover, a greater focus was placed on the isotype of the aβ_2-GPI, and it led to surprising conclusions. As it was suspected, IgM antibodies don't seem to provide as much as the IgG. Some authors even suggest abandoning them in regular dosages. On the other hand, the testing of IgA showed an attractive link with the occurrence of thrombosis, thanks to a wider sensitivity amongst patients. As a consequence, β_2-GPI IgA measurement in the diagnostic strategy for APS should be considered by each laboratory, at least as a second effort.

Last but not least, several techniques were also developed to provide concurrence with the inescapable ELISA assay. CLIA and multiplex assays were popularized and recent studies strongly suggest adopting them for routine measurements. They provide a better sensitivity as well as specificity for the occurrence of thromboembolic manifestations. The CLIA assay especially gives us promising results along with compelling hindsight.

More studies are obviously needed for each of the above-mentioned points, which may lead to modifications of diagnostic criteria of APS, in effect since 2006.

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