Anti-lysobisphosphatidic acid antibodies in patients with antiphospholipid syndrome and systemic lupus erythematosus

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Accepted for publication 2 December 2004
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Summary
Lyso(bis)phosphatidic acid (LBPA) is a novel antigenic target in antiphospholipid syndrome (APS) and antibodies directed against LBPA (aLBPA) have been detected in sera from APS patients. In this study we first evaluated aLBPA in comparison with the most widely used methods (i.e. anticardiolipin [(aCL)-enzyme-linked immunosorbent assay (ELISA)] and antibeta-2-glycoprotein-I antibodies (aβ2-GPI-ELISA) utilized to detect antiphospholipid antibodies in patients with primary or secondary APS, systemic lupus erythematosus, chronic HCV infection and healthy subjects. We then assessed the relationship between aLBPA, lupus anticoagulant (LAC) and the main clinical manifestations of APS. Finally, we evaluated the presence of ‘pure’ (i.e. β2-GPI-independent) aLBPA in patients with APS and controls. The results indicate that aLBPA as well as aβ2-GPI display higher specificity but lower sensitivity for APS compared to aCL. Moreover, serum aLBPA correlate closely with aCL and aβ2-GPI in APS patients and are strictly associated with LAC positivity. We demonstrate that β2-GPI binds to LBPA with affinity similar to CL, and antibodies able to react with phospholipid-protein complex exist; however, ‘pure’ aLBPA can also be detected in sera of APS patients. Altogether these data confirm that LBPA may be an antigenic target in APS and that aLBPA are serological markers of APS with similar sensitivity and specificity compared to aβ2-GPI. However, the clinical utility of aLBPA detection alone or in combination with aCL and/or aβ2-GPI remains to be elucidated in larger and longitudinal studies.

Keywords: antiphospholipid syndrome, autoantibodies, cardiolipin, lyso(bis)phosphatidic acid, systemic lupus erythematosus

Introduction
Antiphospholipid syndrome (APS) is a disease characterized by arterial and venous thrombosis, recurrent miscarriages or fetal loss and circulating antiphospholipid antibodies (aPL) directed against different anionic phospholipids, mainly cardiolipin (CL) [1,2]. In the last two decades several reports have indicated that the main antigenic target of these autoantibodies are phospholipid binding proteins, most notably beta-2-glycoprotein I (β2-GPI) [3–5], or a complex of these proteins with phospholipids [6–9]. Nevertheless, in this context several contradictory results have been reported and the exact nature of the aPL antigens is still a matter of controversy [3–10]. Recently, it has been described that a phospholipid restricted to the late endosome, the lyso(bis)phosphatidic acid (LBPA), could represent a novel antigenic target in the course of APS [11].

LBPA is a hydrophobic isomer of phosphatidyglycerol, related structurally to cardiolipin, localized in specialized domains within the internal membranes of multi-vesicular late endosomes. These organelles are constituted by a limiting membrane and an internal membrane, the latter containing triglycerides, cholesterol esters and a subset of phospholipids forming particular domains involved in protein-sorting steps [11–13]. Interestingly, human sera from patients with APS label late endosomes containing LBPA and induce a redistribution of intracellular β2-GPI, a protein involved in the pathogenesis of APS [14]. Moreover, we have reported that anti-LBPA antibodies (aLBPA) are able to exert lupus anticoagulant activity in vitro, supporting the view
that aLBPA may have a pathogenic role in APS [14]. Nevertheless, a few studies, restricted to a low number of patients, focused on the association between the presence of aLBPA and clinical manifestations in APS patients [11,14,15].

In the present study we evaluated serum aLBPA in patients with primary or secondary APS, systemic lupus erythematosus (SLE), chronic HCV infection and healthy controls. The serum levels of aLBPA were correlated to the clinical manifestations and compared to the levels of anti-CL antibodies (aCL) and antiβ2-GPI antibodies (aβ2-GPI) in all patient groups.

Patients, materials and methods

Subjects

Seventy-three consecutive out-patients, attending the Rheumatology Division of the University of Rome ‘La Sapienza’, were enrolled. Thirty patients had APS, diagnosed according to the Sapporo criteria [2], primary (N = 15) or secondary (N = 15) to other diseases (13 SLE, one sarcoidosis, one mixed connective tissue disease); 43 patients had SLE fulfilling the ACR revised criteria for the classification of SLE [16]. We also enrolled 37 patients with chronic HCV infection and 40 healthy subjects (normal blood donors) matched for age and sex as controls.

After informed consent was obtained, each subject underwent peripheral blood sample collection. The serum recovered was then stored at −20°C until assayed.

Materials

CL (bovine heart) was obtained from Sigma Chemical Co. (St Louis, MO, USA). LBPA and hydrocardiolipin (HCL) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). High performance thin layer chromatography (HPTLC) was performed as reported previously [17] to assess the presence of cross-contamination between phospholipid preparations. Human β2-GPI was obtained from Chemicon International (Temecula, CA, USA).

The following antibodies were used: rabbit polyclonal antihuman β2-GPI (Chemicon International); goat polyclonal antihuman IgG, IgA, IgM alkaline phosphatase conjugate (Sigma); and mouse antirabbit IgG alkaline phosphatase conjugate (Sigma).

Human IgG fractions were first isolated with 33% ammonium sulphate fractionation from plasma of patients with APS and from healthy donors; the enriched fractions were then centrifuged at 10 000 r.p.m. for 30 min and resuspended in one-fourth of the original volume of distilled water. Samples were dialysed overnight against 0·1 M ammonium carbonate, and then separated using a Progel TSK G3000 column (Supelco, Bellefonte, PA, USA). IgG fractions were obtained eluted with 0·2 M phosphate buffer and subsequently dialysed against 5 l of distilled water. Protein concentration was measured with the Lowry method [18] and the purity of the IgG preparations was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme-linked immunosorbent assay (ELISA) for aLBPA, aCL and antiβ2-GPI IgG

Serum aLBPA and aCL were detected by means of an ELISA. Phospholipids at 50 µg/ml concentration in ethanol were used to coat microtitre plates overnight at 4°C. After four washes with phosphate buffered saline (PBS), plates were blocked for 1 h at room temperature (RT) with 10% fetal calf serum (FCS) in PBS (PBS-F) or 0·25% gelatine (PBS-G) to assess the binding to LBPA and CL in the absence of β2-GPI in indicated experiments. After four washes with PBS-F (or PBS-G), plates were incubated for 90 min at RT with sera diluted at 1:50 or human IgG (100 µl of concentrated solutions of 4·8 mg/ml) in PBS-F (or PBS-G). Subsequently, after four washes, plates were incubated for 90 min at RT with goat polyclonal antihuman IgG-IgA-IgM alkaline phosphatase conjugated antibodies (Sigma) diluted at 1:1000 in PBS-F (or PBS-G). After four washes, a solution of paranitrophenyl phosphate tablets in ethanolamine was used for the enzyme reaction and the plates were read at a 405 nm wavelength. All assays were performed in duplicate and the absorbance of control wells was subtracted to account for non-specific binding. A titration curve of two positive reference sera (with medium–high ELISA immunoreactivity for aLBPA and aCL, respectively) was performed to show the performance of the tests.

In order to investigate the specificity of the assay, absorption tests were performed according to techniques described elsewhere [19,20]. Briefly, three aLBPA/aCL-positive sera (1/50 in 1% PBS-BSA) were preincubated for 60 min at 37°C with increasing amounts of LBPA and CL dried onto the surface of glass tubes. Subsequently, after a centrifugation step (10 000 g for 30 min) the supernatants were tested for autoantibody reactivity, as above. As control, in these experiments we further used a fully hydrogenated CL derivative (HCL) that does not occur naturally, but can provide a reference structure for basal immunorecognition of the lipid [20].

Intra- and interassay variations were determined by assaying two samples (one APS patient and one SLE patient) 16 times in one assay (intra-assay) and assaying the two samples in quadruplicate in nine consecutive assays (interassay). Finally, to establish the clinical significance of aLBPA and aCL we considered these autoantibodies as positive when the OD was higher than the cut-off value corresponding to the mean plus 5 s.d. of 40 healthy controls matched for sex and age.

Anti-β2-GPI IgG ELISA kits were obtained from Diamedix (Miami, FL, USA) and performed according to the manufacturer’s instructions. To evaluate the binding of β2-GPI to
LBPA and CL, plates were coated overnight with an increasing amount of these lipids and then reacted with PBS-F, containing an excess of β2-GPI, for 2 h at RT. A rabbit polyclonal antiβ2-GPI was used to detect the binding of β2-GPI to lipids.

Immunostaining on TLC plates for aLBPA and aCL

The immunostaining of TLC plates (Merck, Darmstadt, Germany) was performed as described previously [21], using 2·5 µg of LBPA or CL. Affinity-purified IgG fractions (2-4 mg/ml) from APS sera (which had been deemed aLBPA and aCL-positive by ELISA screening) and normal human sera were diluted 1:100 in PBS containing 0·5% (w/v) gelatine. Parallel blots were processed without primary antibody or without antigen as control for non-specific reactivity.

Statistical analysis

Statistical analysis was carried out using the Mann–Whitney U-test for comparison of means between different groups of patients and controls. Wilcoxon’s paired test was used to compare differences between autoantibody profiles in each group. Qualitative differences between subgroups were analysed by χ² or Fisher’s exact test when appropriate. Correlation analysis was carried out by the Spearman test. A P-value <0·05 was considered statistically significant.

Results

Patients clinical details

The demographic and clinical features of the patients and controls enrolled in this study are reported in Table 1. SLE patients were used as diseased controls compared to APS (primary or secondary). All SLE patients were consecutive out-patients attending the rheumatology clinic and met the ACR criteria for SLE. None of the 43 patients in the SLE without APS group fulfilled the Sapporo criteria for APS. None of the healthy subjects experienced arterial or venous thrombosis, nor recurrent fetal loss. The lupus anticoagulant (LAC) positivity was deduced in each case from recent clinical records and measured following the guidelines proposed by the Subcommittee for Standardization of Lupus Anticoagulants (SSC) of the Scientific and Standardization Committee of the ISTH [22]. In particular, at our institution LAC was determined by kaolin-clotting time, the textarin time, a modified dilute Russell’s viper venom time (DRVVT) and confirmed by the Staclot LA test.

aLBPA and aCL ELISA

To compare immunoreactivity of LBPA and CL, it was important to ascertain that the lipid preparations were not cross-contaminated. By means of HPTLC, we could not observe any detectable contamination in lipid preparations (data not shown).

Serum levels of aLBPA and aCL IgG in patients and controls were quantified by ELISA (Fig. 1, Table 2). Similar results were obtained in selected patients when purified IgGs instead of sera were used (data not shown). In the present study immunoreactivity to LBPA and CL was significantly higher in APS and SLE patients with respect to controls (see Fig. 1 and Table 2 for statistical significance). According to our cut-off (mean plus 5 s.d. of 40 normal controls) the prevalence of aLBPA IgG was 53·3% (16 of 30) in APS, 4·6% (two of 43) in SLE and 2·7% (one of 37) in HCV patients, sensitivity (95% CI) 0·53 (0·45–0·61), specificity (95% CI) 0·97 (0·95–1·00). The prevalence of aCL IgG was 80% (24 of 30) in APS, 16·3% (seven of 43) in SLE and 8·1% (three of 37) in HCV patients, sensitivity (95% CI) 0·8 (0·73–0·86), specificity (95% CI) 0·92 (0·89–0·94). In Table 2 the isotype distribution of aLBPA and aCL-positive sera in patients groups is reported. Finally, no difference in immunoreactivity to LBPA (as well as CL and β2-GPI) was observed in APS patients divided into primary and secondary APS. This, together with

<table>
<thead>
<tr>
<th>Variable</th>
<th>APS (N = 30)</th>
<th>SLE (N = 43)</th>
<th>HCV (N = 37)</th>
<th>NHS (N = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females/males</td>
<td>23/7</td>
<td>38/5</td>
<td>14/23</td>
<td>22/18</td>
</tr>
<tr>
<td>Ethnicities</td>
<td>Caucasian</td>
<td>Caucasian</td>
<td>Caucasian</td>
<td>Caucasian</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (range)</td>
<td>34 (7–68)</td>
<td>36·8 (14–70)</td>
<td>55·4 (20–74)</td>
<td>53 (32–70)</td>
</tr>
<tr>
<td>Disease duration, months</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (range)</td>
<td>143 (1–322)</td>
<td>107 (6–312)</td>
<td>41·2 (3–132)</td>
<td>0</td>
</tr>
<tr>
<td>Venous thromboses (%)</td>
<td>18 (60)</td>
<td>2 (4·6)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Arterial thromboses (%)</td>
<td>13 (43·3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Recurrent thromboses (%)</td>
<td>11 (36·6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Miscarriages/pregnancy (%)</td>
<td>11/14 (78·5)</td>
<td>5/14 (35·7)</td>
<td>2/11 (18·1)</td>
<td>0</td>
</tr>
<tr>
<td>Lupus anticoagulant activity (%)</td>
<td>21 (70)</td>
<td>10 (23·2)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d. = not done.
the very low prevalence of anti-LBPA in patients with SLE without APS, confirms that anti-LBPA are closely associated with APS independently in the presence of an underlying connective tissue disease.

The specificity of the assays was examined by dilution and absorption experiments. Serial dilutions of positive aLBPA and aCL sera showed a progressive reduction of the antibodies binding, while this reactivity was enhanced as concentration of lipids on coated wells was raised from 1 to 50 μg/ml (Fig. 2a). Conversely, binding of both positive sera was significantly inhibited by preincubation with increasing amounts of LBPA and CL, respectively (Fig. 2b). None of the sera showed reactivity to HCL and absorption with HCL did not influence aLBPA and aCL reactivity. Finally, the coefficients of intra-assay and inter assay variation of the two samples assayed were 4-6%, 5-2% and 12-6%, 13-6%, respectively.

To establish the clinical significance of aLBPA we grouped APS patients according to serological and clinical findings. As expected, we found a significantly increased prevalence of aCL and αβ₃-GPI IgG in patients who presented LAC posi-

Table 2. IgG, IgM and IgA isotype distribution of aCL and aLBPA in the different populations studied; the numbers represent number of patients with percentages in brackets

<table>
<thead>
<tr>
<th></th>
<th>APS (n = 30)</th>
<th>SLE (n = 43)</th>
<th>HCV (n = 37)</th>
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</thead>
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<tr>
<td>aCL (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>24 (80)</td>
<td>7 (16-2)</td>
<td>3 (8-1)</td>
</tr>
<tr>
<td>IgM</td>
<td>14 (46-6)</td>
<td>3 (6-9)</td>
<td>4 (10-8)</td>
</tr>
<tr>
<td>IgA</td>
<td>4 (13-3)</td>
<td>2 (4-6)</td>
<td>1 (2-7)</td>
</tr>
<tr>
<td>Total (anti-human Ig)</td>
<td>24 (80)</td>
<td>10 (23-2)</td>
<td>7 (18-9)</td>
</tr>
<tr>
<td>aLBPA (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>16 (53-3)</td>
<td>2 (4-6)</td>
<td>1 (2-7)</td>
</tr>
<tr>
<td>IgM</td>
<td>8 (26-6)</td>
<td>1 (2-3)</td>
<td>3 (8-1)</td>
</tr>
<tr>
<td>IgA</td>
<td>7 (23-3)</td>
<td>2 (4-6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total (anti-human Ig)</td>
<td>19 (63-3)</td>
<td>3 (6-9)</td>
<td>4 (10-8)</td>
</tr>
</tbody>
</table>

Fig. 2. The specificity of the ELISA was examined by dilution and absorption experiments. (a) A titration curve of two positive reference sera (aLBPA and aCL-positive sera, respectively) was performed by ELISA to show the performance of the tests. IgG binding was dose-dependently enhanced by increasing the concentration of phospholipids coated on the ELISA plate wells. Hydrogenated CL derivative (HydroCL) provided a reference structure for basal immunorecognition of the lipid. (b) To further characterize the Ag specificity, absorption tests were performed with three aLBPA/aCL-positive sera by ELISA performed as described in Methods’ section. A significant decrease in aLBPA and aCL binding was observed. Each data point represents the mean of triplicate determinations and the figure is representative of all sera tested.
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Fig. 3. Box-and-whisker plot of aCL, aLBPA and αβ2-GPI autoantibodies detected by ELISA, in patients grouped according to LAC positivity deduced in each case from recent clinical records. Median, quartiles, range and possibly extreme values are shown. A significantly increased prevalence of aCL, αβ2-GPI and aLBPA IgG in patients who presented LAC positivity was observed (P = 0.0027, P = 0.031 and P = 0.0018, respectively).

αβ2-GPI IgG ELISA

The prevalence of αβ2-GPI IgG was 56.6% (17 of 30) in APS and 6.9% (three of 43) in SLE while none of the control sera resulted positive for αβ2-GPI, sensitivity (95% CI) 0.57 (0.49–0.65), specificity (95% CI) 0.97 (0.95–1.00). As reported previously [15], we found a significant positive correlation between αβ2-GPI, aCL and aLBPA IgG reactivity (Fig. 4). According to the prevalence of aCL and αβ2-GPI IgG in APS patients, concordance between the two antibodies was 62.5% (15/24) while concordance between aLBPA and αβ2-GPI IgG was 88.2% (15/17), confirming that aCL, aLBPA and αβ2-GPI represent populations of autoantibodies largely but not completely overlapping.

β2-GPI dependency of aLBPA and aCL reactivity

Because it is known that CL and LBPA are able to complex with β2-GPI [15], but it is also possible to detect serum aCL and aLBPA in the absence of αβ2-GPI in APS patients, we investigated whether aCL and aLBPA antibodies from APS could bind to lipids alone. For this purpose we used two different approaches: ELISA, both in the presence or in the absence (i.e. gelatine) of serum-derived co-factor (blocking buffer, PBS-gelatine) and TLC immunostaining. Interestingly, a reduction, but not reaching a significant difference, was observed in mean reactivity of aLBPA and aCL IgG-positive sera regarding to the presence of β2-GPI in the

Fig. 4. Correlation analysis between αβ2-GPI, aCL and aLBPA IgG ELISA reactivity in the patients studied was carried out by the Spearman test. As expected, we found a significant correlation between aCL, αβ2-GPI and aLBPA IgG ELISA reactivity.

ELISA (Fig. 5a). On the other hand, β2-GPI binding to LBPA was comparable to that of CL in solid phase (Fig. 5b). Moreover, the partial β2-GPI independence of aLBPA/aCL-positive sera was confirmed by TLC immunostaining which, in
b2-GPI-free conditions, showed a specific reactivity to CL and LBPA in several representative sera (Fig. 5c).

Discussion

This study provides evidence that a high percentage of sera from patients with APS show immunoreactivity in a solid phase assay towards LBPA, an anionic phospholipid present in the late endosomes. In addition, a strict correlation between the serum aLBPA and aCL was observed. The HPTLC analysis of the two phospholipid preparations ruled out the possibility that the significant correlation was due to cross-contamination between the two phospholipid samples. Thus, our results show that most sera that bind to cardiolipin also bind to LBPA.

To verify whether the protein co-factor b2-GPI may mediate the binding of antibodies to LBPA, we compared aLBPA to a b2-GPI and a significant association was observed, although to a lesser extent than that between aCL and aLBPA. These findings are in agreement with those by Galve-de Rochemonteix and colleagues [15], who previously reported the reactivity to LBPA of sera from patients with APS. Nevertheless, we clearly demonstrated by means of solid phase ELISA in b2-GPI-free conditions and TLC that a population of aCL and aLBPA also can bind directly to lipids in the absence of b2-GPI. These findings confirm that at least in autoimmune diseases such as APS and SLE, aPL reactivity reveals high antibody heterogeneity [23–25].

To address the diagnostic potential of aLBPA in identifying APS patients, in the present study we further analysed the presence of these antibodies in sera of SLE patients without clinical manifestations of APS, in chronic HCV hepatitis, an infectious disease in which aCL have been previously detected [26,27] and in healthy controls. Our results revealed a highly significant difference in the occurrence of aLBPA antibodies comparing patients with APS and SLE to chronic...
HCV infection or healthy subjects. The overall sensitivity and specificity of aLBPA for APS in our study showed values almost identical to those observed using the \( \alpha_\beta_2 \)-GPI test, with a very high specificity (>97%) but a rather lower sensitivity compared to aCL (~55% versus 80%). Our results confirmed the recent observation that aCL are present in about 25% of APS patients fulfilling the Sapporo clinical criteria who are negative for \( \alpha_\beta_2 \)-GPI or LAC [28]. Although our results suggest that anti-LBPA may not represent an improvement in testing for APS in comparison with anti-CL and \( \alpha_\beta_2 \)-GPI, this is the first study to demonstrate that anti-LBPA are present in a large number of sera of APS patients but virtually absent in SLE or controls, showing almost identical sensitivity and specificity for APS compared to \( \alpha_\beta_2 \)-GPI (and are slightly more specific although less sensitive than aCL) and close association with LAC.

The possibility that aLBPA antibodies may be considered as a novel and reliable serum marker of APS prompted us to hypothesize that these antibodies may also represent a useful tool for clinical monitoring the disease and/or may be related to the clinical features of the syndrome. Thus, we first evaluated the relationship between the presence of aLBPA antibodies and LAC positivity and a very significant association was found. This finding strongly supports the previous observation that aLBPA antibodies display dose-dependent anticoagulant properties in vitro, as they were shown to induce a prolongation of both activated partial thromboplastin time and dilute Russell’s viper venom time [14]. This effect was shown to be highly specific and significantly higher than that due to \( \alpha_\beta_2 \)-GPI antibodies alone. We then correlated aLBPA, aCL and \( \alpha_\beta_2 \)-GPI positivity with the main clinical features of the APS syndrome separately, such as arterial and venous thrombosis or fetal loss, but no significant association was found. Nevertheless, this finding is not surprising, because the association of aPL and antico-factor antibodies with clinical manifestation in APS has been long debated (reviewed in [29]). Interestingly, patients with low titres of aCL may have a significant disease and the aCL titre may also fluctuate into normal range at the time of thrombosis [30]. Finally, factors associated with higher risk of thrombosis include a previous history of thrombosis [31,32] and LAC positivity [33]. Longitudinal studies are currently under investigation to clarify the possible predictive value of these antibodies regarding the characteristic clinical features of the syndrome. Interestingly, preliminary results revealed the presence of aLBPA antibodies in two of five sera from patients with the clinical features of the syndrome, but without detectable titres of aCL or \( \alpha_\beta_2 \)-GPI (data not shown).

Remarkably, our findings demonstrate that LBPA may be an important lipid target for aPL antibodies with potential implications in the pathogenesis of APS. It remains unclear how an antigen which is normally confined in late endosomes becomes a target for these antibodies. Although we cannot exclude the possibility that antibodies may enter target cells [34], the recent demonstration of LBPA in exosomes shed by the cell supports the view that LBPA, possibly complexed with protein co-factors in these small vesicles, may be the direct target of the antibodies [36]. Alternatively, apoptosis may represent a possible trigger for LBPA exposure on the cell surface. This hypothesis is supported by our recent observation that cardiolipin and its metabolites move to other cellular membranes during death receptor-mediated apoptosis [37].

In conclusion, aLBPA antibodies appear to represent a marker of APS with similar sensitivity and specificity compared to \( \alpha_\beta_2 \)-GPI and display a slightly higher specificity despite a lower sensitivity compared to aCL antibodies. In addition, aLBPA are strongly associated with the presence of LAC, suggesting their pathogenic potential. However, the assessment of their clinical utility as diagnostic and predictive tools as a single test or in combination with aCL and/or \( \alpha_\beta_2 \)-GPI in patients with APS deserves further investigation in longitudinal studies.

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