Resistance to annexin A5 anticoagulant activity in women with histories for obstetric antiphospholipid syndrome

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OBJECTIVE: The objective of the study was to investigate whether resistance to annexin A5 anticoagulant activity (AnxA5) occurs in women with histories for obstetric complications of antiphospholipid syndrome (Obst-APS) and whether this correlates with antibody recognition of domain 1 of β2-glycoprotein.

STUDY DESIGN: One hundred thirty-six women with antiphospholipid antibodies, including 70 with histories for Obst-APS and 30 controls, were investigated.

RESULTS: Women with Obst-APS showed resistance to AnxA5 activity (median, 216%; range, 130–282% vs controls; median, 247%; range, 217–283%; P < .0001) and elevated levels of anti-domain I immunoglobulin (Ig) G (optical density: median, 0.056; range, 0.021–0.489 vs median, 0.042; range, 0.020–0.323; P = .002). Those in the lowest tertile of AnxA5 anticoagulant ratios had an odds ratio for Obst-APS of 58.0 (95% confidence interval, 3.3–1021.5). There was an inverse correlation between levels of annexin A5 anticoagulant activity and anti-domain I IgG.

CONCLUSION: Resistance to AnxA5 anticoagulant activity is associated with antibody recognition of domain I of β2-glycoprotein I and identifies a subset of women with histories for Obst-APS.

Key words: annexin A5, antiphospholipid antibodies, antiphospholipid syndrome, obstetric, β2-glycoprotein I, pregnancy loss


The antiphospholipid syndrome (APS) is defined by the association of a persistently abnormal antiphospholipid antibody (aPL) assays (ie, elevated immunoassays anticardiolipin and/or anti-β2glycoprotein I immunoglobulin (Ig) G or IgM antibodies or a positive lupus anticoagulant test) with a history of thrombosis or specific pregnancy complications.1

The currently available antiphospholipid assays are empirically derived tests that do not measure a disease mechanism; the immunoassays were derived from the biological false-positive syphilis phenomenon and the lupus anticoagulant from the observation an inhibitor to the activated partial thromboplastin time, both described more than 50 years ago. The pathogenic mechanism for obstetric APS has remained enigmatic.

The syndrome is referred to as primary APS (PAPS) when it occurs without other autoimmune disease and secondary APS when it is associated with another autoimmune disease, usually systemic lupus erythematosus. In this paper, the term obstetric APS applies to aPL associated with the pregnancy complications that were defined by consensus diagnostic criteria; these include a previous unexplained recurrent first trimester loss and/or midtrimester and third-trimester intrauterine death and/or severe preeclampsia, placental abruption, or intrauterine growth retardation.1

The purpose of this study was to investigate whether women with histories of obstetric APS might have evidence for resistance to annexin A5 (AnxA5) anticoagulant activity in their blood. AnxA5 is a placent al anticoagulant protein that is highly expressed on the apical surfaces of syncytiotrophoblasts2 in which the protein is in an anatomic position to play a thrombomodulatory role and contribute to the fluidity of the maternal circulation through the intervillous space.

The protein is also expressed in a number of other cell types including, among others, vascular endothelial cells, renal tubular epithelial cells, and bile duct epithelial cells. The protein’s potent anticoagulant activities result from its forming 2-dimensional crystals over anionic phospholipids that shield the phospholipids from contributing to critical phospholipid-dependent coagulation enzyme reactions. The aPL an-
tibodies have been shown to reduce the quantity of AnxA5 on cultured placental trophoblasts and accelerate the coagulation of plasma that is exposed to these cells. Furthermore, aPL antibodies reduce the binding of AnxA5 to phospholipid bilayers and create significant defects in the ordered crystallization of this protein that expose unshielded phospholipids, thereby accelerating coagulation enzyme reactions.

We previously reported that patients with APS-associated vascular thrombosis had resistance to AnxA5 anticoagulant activity and that this reduced AnxA5 anticoagulant activity correlated strongly with antibody-mediated displacement of AnxA5 from binding to phospholipids and with antibody recognition of a specific epitope on domain 1 of β2-glycoprotein I (β2GPI).

We also previously reported that women with a history of recurrent spontaneous pregnancy losses, not screened for aPL antibodies, had reduced AnxA5 anticoagulant activity. However, the specific question of whether there may be evidence for resistance to annexin A5 anticoagulant activity in the blood of women with aPL-associated pregnancy complications has never been previously investigated. Nor has the question of whether anti-domain I IgG antibodies might correlate with obstetric APS been previously investigated.

Therefore, the aim of this study was to measure these specific parameters in women with histories of obstetric APS. Because of the inflammatory state induced by systemic lupus erythematosus, the study was confined to patients with PAPS.

**Materials and Methods**

**Patients**

After obtaining local ethical committee approval at Guy’s and St Thomas’s Trust, blood specimens were collected with informed consent from healthy, nonpregnant women who had a history of obstetric PAPS and both men and women with a diagnosis of thrombotic PAPS or isolated aPL antibodies. All PAPS patients satisfied the Miyakakis criteria for the diagnosis of aPL and APS.

In total, 136 patients with aPL antibodies were classified into 3 groups: (1) women, not currently pregnant but with a past history of obstetric PAPS (n = 70); (2) subjects without obstetric APS but with a history of thrombotic PAPS, with their last thrombotic event more than 6 months previously (n = 50); and (3) subjects with isolated aPL antibodies who had not sustained any thrombotic or pregnancy events (n = 16).

The demographic, aPL characteristics including types of obstetric APS and treatment details are summarized in Table 1. There was no significant difference in ages between groups, but obviously, those with obstetric PAPS were all female, and the majority of the other groups were also female. As described in Table 1, 29 of the 70 women with obstetric APS had histories for 3 or more spontaneous first-trimester losses, 39 of the women had a history for intrauterine fetal demise, and 26 had histories for placental insufficiency.

A minority of the obstetric PAPS group (n = 23, 30%) also had a thrombotic history. Those in the thrombotic PAPS group had similar rates of venous and arterial previous events with 9 (18%) having had both venous and arterial thrombotic events.

In addition, 30 plasmas from disease-free, nonpregnant women (group D) were obtained from a commercial vendor (George King Bio-Medical Inc, Overland Park, KS) as normal healthy controls. The plasma samples were sent as coded samples to the Pathology Department of the Montefiore Medical Center for AnxA5 resistance assay and to the Hematology Department of Utrecht University Hospital for the anti-domain I immunoassays.

**Annexin A5 resistance assay**

AnxA5 was purified from human placenta as previously described. The effects of patient plasmas on AnxA5 anticoagulant activity were determined using a 2-stage assay as previously described. Briefly, ethylenediaminetetraacetic acid (EDTA) (0.5 M) was added to recombinant human tissue factor (Innovin; Dade Behring Inc, Newark, DE) to a final concentration of 10 mM. The Innovin-EDTA was then mixed with activated partial thromboplastin time reagent-phospholipids (Actin FSL; Dade Behring Inc) at 1:1 ratio. The mixture of Innovin-EDTA–actin FSL (200 μL) was incubated with citrated test plasma (50 μL) for 5 minutes at room temperature.

The plasma-treated mixture was then centrifuged with a microcentrifuge (Eppendorf centrifuge 5417R; Brinkmann Instruments, Westbury, NY) for 15 minutes at 20,800 × g at 25°C. The pellets were washed once in N-2-hydroxyethylpiperazine-N-2-thane sulfonic acid (HEPES) buffer saline (HBS; 0.01 M HEPES, 0.14 M NaCl [pH 7.5]) and resuspended in HBS (220 μL). The suspension (50 μL) was incubated with pooled normal plasma (50 μL) at 37°C in a ST4 coagulation instrument (American Bioproducts, Parsippany, NJ) for 30 seconds.

The plasma was then recalcified with 50 μL of 0.02 M calcium or 0.02 M calcium containing AnxA5 (30 μg/mL). The coagulation times, in the presence and absence of AnxA5, were determined and the mean times of duplicate tests were recorded. The anticoagulant activity of AnxA5 was calculated as follows: AnxA5 anticoagulant ratio = (coagulation time in the presence of AnxA5/coagulation time in the absence of AnxA5) × 100%.

Plasma samples were considered to demonstrate resistance to AnxA5 anticoagulant activity when the ratios were below the mean minus 2 SD of the 30 normal healthy controls.

**Anti-domain I IgG enzyme-linked immunosorbent assay (ELISA)**

Anti-β2GPI IgG antibodies with reactivity toward domain I were assayed as previously described. Briefly, hydrophobic microtiter plates (catalog no. 2595; Costar, New York, NY) were coated with domain I IgG of β2GPI (10 μg/ml in Tris-buffered saline [TBS] consisting of 50 mM Tris and 100 mM NaCl) for 1 hour at 37°C. The plates were blocked with 150 μL of blocking solution (4% bovine serum albumin/TBS/0.1% Tween) for 1 hour at 37°C and subsequently incubated with patient plasma (diluted 1:100 in the...
blocking solution) containing anti-
β2GPI IgG antibodies for 1 hour.

After every incubation step, the plates
were washed 4 times with washing solu-
tion (0.1% Tween/TBS). The bound IgG
antibodies were detected by a goat-anti-
human IgG alkaline-phosphatase-labeled an-
tibody (Invitrogen Corp, Carlsbad, CA),
followed by staining with paranitrophenyl
phosphatase (Sigma, St Louis, MO).
The reaction was stopped by 2.4 M
NaOH, and absorbance was measured at
405 nm with a microtiter plate reader.
Plasma samples were regarded as having
levels of antiantidomain I IgG when
the absorbed value exceeded the cutoff
value (mean + 3 SD of the 30 normal
healthy controls).

**Study and statistical analysis**

Differences between the 4 groups of
patients were analyzed with the non-
parametric Mann-Whitney test (www.
graplhpad.com; GraphPad Software, San
Diego, CA) because of departures of data
from normal distribution.

To compute odds ratios and 95% con-
fidence intervals (CIs) for those with ob-
stetric PAPS, data were categorized into
terziles, using the frequency distribution
of patients and controls combined. The
data were analyzed using uncondition-
all logistic regression, and odds ratios were
computed relative to the highest tertile
for AnxA5 resistance and to the lowest
terzile for antiantidomain I IgG. Likelihood
ratio tests were used to calculate P values
for trends. All reported P values were 2
tailed, and P < .05 was considered statisti-
cally significant. Correlation coeffi-
cients between AnxA5 resistance assay
and domain 1 IgG assay among the
groups were calculated using the Spear-
man rank correlation and the odds ratio
among the 2 assays were analyzed using
Fisher's exact test.

**Results**

**Characteristics of subjects**
The demographics, aPL characteristics,
and anticoagulant treatment details of
the subjects are shown in Table 1.

**Annexin A5 resistance assay**

Patients with a history of obstetric PAPS
(Figure 1, group A, n = 70) had a signif-
ican reduction of AnxA5 anticoagulant
ratios (mean ± SD 211 ± 35%; median,
216%, range, 130–282%) compared
with the normal healthy controls (group
D, n = 30; 247 ± 19%; median, 247%,
range, 217–283%, P < .0001). It is nota-
table that 41% of the patients within group
A (29 of 70) had resistance to AnxA5 an-
ticoagulant activity (ie, the AnxA5 anti-
coagulant ratios were below 2 SD of the
mean of normal healthy controls).
The patients in the middle and lowest tertile
of AnxA5 anticoagulant ratios had an
ods ratio for obstetric APS of 2.4 (95% CI,
0.9–6.3) and 58.0 (95% CI, 3.3–
1021.5), respectively (Table 2).

Patients with thrombotic PAPS (Fig-
ure 1, group B) also showed significant
reduction of AnxA5 anticoagulant ratios
compared with the normal healthy con-
trols (mean ± SD 206 ± 37%; median,
208%, range, 132–269%; P < .0001);
52% of the patients within group B (26 of
50) had reduced AnxA5 anticoagulant
ratios. Plasma from those with isolated
aPL (Figure 1, group C, n = 16) also
showed significant reduction of AnxA5
anticoagulant ratios (mean 218 ± 36%;
median, 225%, range, 146–270%) com-
pared with healthy controls (P = .007);
38% (6 of 16) of the patients in this
group had reduced AnxA5 anticoagu-
Obstetric PAPS patients (group A) and the thrombotic PAPS patients (group B) showed significant reduction of AnxA5 anticoagulant ratios as compared with the normal healthy controls (both \( P < .0001 \)). The patients with isolated aPL antibodies (group C) also showed significant reduction of AnxA5 anticoagulant ratios compared with the normal controls (\( P = .007 \)). There were no significant differences in AnxA5 anticoagulant ratio between the groups A and B and between the groups A and C and between the groups B and C. The horizontal lines show the mean of each group; the dashed lines show the mean \( \pm 2 \) SD of the 30 normal healthy controls.

AnxA5, annexin A5 anticoagulant activity; APS, antiphospholipid syndrome; PAPS, primary antiphospholipid syndrome.


### TABLE 2

Resistance to AnxA5 anticoagulant activity and ORs of women experiencing obstetric PAPS (n = 70 cases vs n = 30 normal healthy controls) (relative to the highest tertile of AnxA5 anticoagulant activity values)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases, n (%)</th>
<th>Controls, n (%)</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>AnxA5 anticoagulant ratio, % tertile</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1 (&gt;233)</td>
<td>18 (26)</td>
<td>19 (63)</td>
<td>1.0</td>
<td>Reference</td>
</tr>
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<td>2 (205-233)</td>
<td>25 (36)</td>
<td>11 (37)</td>
<td>2.4</td>
<td>0.9–6.3</td>
</tr>
<tr>
<td>3 (&lt;205)</td>
<td>27 (39)</td>
<td>0 (0)</td>
<td>58.0</td>
<td>3.3–1021.5</td>
</tr>
<tr>
<td>( P ) value for trend</td>
<td>&lt; .0001</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AnxA5, annexin A5 anticoagulant activity; CI, confidence interval; OR, odds ratio; PAPS, primary antiphospholipid syndrome.


### Anti-domain I Assay

The women with obstetric PAPS (Figure 2, group A, n = 50) showed significantly elevated levels of anti-domain I IgG (optical density [OD]: mean \( \pm SD: 0.122 \pm 0.131 \); median, 0.056, range, 0.021–0.498) compared with the normal healthy controls (Figure 2, group D; OD: mean \( \pm SD: 0.058 \pm 0.057 \); median, 0.042; range, 0.020–0.323; \( P = .002 \)). 20% of the patients in this group (14 of 70) had the IgG levels that were above 2 SD (ie, the OD >0.172) of the mean of normal healthy controls. The patients with thrombotic PAPS (Figure 2, group B) also showed significantly elevated levels of the anti-domain IgG (0.142 ± 0.135; median, 0.073, range, 0.018–0.474) compared with the normal healthy controls (\( P = .002 \)); 30% of the patients in group B (15 of 50) had elevated levels of anti-domain I IgG. The patients with isolated aPL antibodies (Figure 2, group C) also showed significantly elevated levels of anti-domain I IgG (0.092 ± 0.081; median, 0.068, range, 0.028–0.345) compared with the normal healthy controls (group D; \( P = .03 \)); 2 patients (in this group 13%) had elevated levels of the IgG that were above 2 SD of the normal healthy controls. The patients with obstetric PAPS in the middle and highest tertile of anti-domain I IgG had an odds ratio for obstetric PAPS of 3.4 (95% CI, 1.2–9.4) and 6.3 (95% CI, 1.8–21.6), respectively (Table 3). There were no significant differences in the levels of anti-domain I IgG between the groups A, B, and C (Figure 2).

### Correlation

Analyses were performed to determine whether there might be a correlation between the assays for AnxA5 anticoagulant activity and anti-domain I IgG within the groups (Table 4). There was a significant inverse correlation between AnxA5 resistance and anti-domain I IgG within group A, those with obstetric PAPS (\( r = -0.49 \)), and within group B, those with thrombotic PAPS (\( r = -0.60; P < .0001 \) for both groups). Within group C, the isolated aPL antibodies, the inverse correlation between the two assays was weaker and was not statistically significant (\( r = -0.23; P = .40 \)), although it was based on only 16 subjects. Similarly, group D, the normal healthy controls, showed a weak inverse correlation between these 2 parameters that was not statistically significant (\( r = -0.18; P = .35 \)). Interestingly, 32 of 166 tested plasmas had elevated anti-domain IgG, of which,
27 (27 of 32; 84%) had significantly reduced AnxA5 anticoagulant ratio (Figure 3). One hundred thirty-four of 166 tested plasmas had anti-domain I IgG levels that were within the mean ± 2SD, of which 33 (33 of 134; 25%) had a significant reduced AnxA5 anticoagulant ratio (Figure 3).

**Comment**

Although it was previously reported that a significant proportion of otherwise unselected women with histories of unexplained recurrent spontaneous pregnancy losses have reduced AnxA5 anticoagulant activity, the prevalence of resistance to AnxA5 anticoagulant activity in women who met the current criteria for antiphospholipid syndrome by having had obstetric complications has not been established. We also compared the results with individuals with antiphospholipid antibodies and previous vascular thrombosis (thrombotic APS) and individuals with isolated aPL antibodies who did not have histories for obstetrical or thrombotic complications and normal healthy controls.

In addition, because a previous study had reported a negative correlation between AnxA5 anticoagulant activity and elevated anti-domain I β2GPI IgG levels in patients with thrombotic APS, we investigated there might be a similar correlation in women with histories for obstetric APS.

We found that women with obstetric APS showed a significant reduction in mean AnxA5 anticoagulant activity, compared with controls and patients with isolated aPL. In view of our finding that about 41% of the patients with obstetric APS had AnxA5 anticoagulant ratios that were below 2 SD of the mean of normal healthy controls, we hypothesize that resistance to AnxA5 may be a mechanism for disease in this subset of APS patients. Although these results are consistent with the hypothesis that antibody-mediated disruption of annexin A5 function may directly affect pregnancy, the validity of this idea would need to be tested by direct experimentation (eg, evidence that the same antibodies that are yielding AnxA5 resistance in a patient’s blood test are having a similar effect on AnxA5 function in their placenta).

There is strong evidence that AnxA5 does indeed have a significant role in maintaining placental function. AnxA5 is highly expressed by syncytiotrophoblasts in an apparently constitutive manner and is localized on the apical membranes of syncytiotrophoblasts in which it faces the maternal blood circulating through the intervillus space. A common haplotype in the promoter region

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**TABLE 3**

Levels of anti-domain I IgG and ORs of women experiencing obstetric APS

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases, n (%)</th>
<th>Controls, n (%)</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
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<tr>
<td>Anti-domain I IgG, optical density</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Tertile</td>
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<tr>
<td>1 (&lt;0.041)</td>
<td>18 (26)</td>
<td>18 (60)</td>
<td>1.0</td>
<td>Reference</td>
</tr>
<tr>
<td>2 (0.041–0.081)</td>
<td>27 (39)</td>
<td>8 (27)</td>
<td>3.4</td>
<td>1.2–9.4</td>
</tr>
<tr>
<td>3 (&gt;0.081)</td>
<td>25 (36)</td>
<td>4 (13)</td>
<td>6.3</td>
<td>1.8–21.6</td>
</tr>
<tr>
<td>P value for trend</td>
<td></td>
<td></td>
<td>.001</td>
<td></td>
</tr>
</tbody>
</table>

APS, antiphospholipid syndrome; CI, confidence interval; IgG, immunoglobulin G; OR, odds ratio.


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**FIGURE 2**

Anti-domain I IgG ELISA

Obstetric PAPS patients (group A) and the thrombotic PAPS patients (group B) had significantly elevated levels of anti-domain I IgG compared with the normal healthy controls (both P = .002). Those with isolated aPL antibodies (group C) also showed significantly elevated levels of anti-domain I IgG compared with the normal nonpregnant controls (P = .03). There were no significant differences in the levels of anti-domain I IgG between the groups A and B, between the groups A and C, and between the groups B and C. The horizontal lines show median values for each group. The dashed line shows the mean ± 2 SD of the 30 normal healthy controls.

aPL, antiphospholipid antibody; APS, antiphospholipid syndrome; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; PAPS, primary antiphospholipid syndrome.

of the AnxA5 gene, designated M2, results in reduced placental expression of AnxA5, and is associated with increased risk for recurrent spontaneous pregnancy losses. This haplotype also appears to have a prothrombotic effect in the systemic vasculature because it is also associated with an increased risk of pregnancy-related venous thromboembolism. Furthermore, in an animal model, AnxA5 was shown to be necessary for preserving placental integrity; infusion of pregnant mice with anti-AnxA5 antibodies resulted in placental infarction and pregnancy losses.

There has been a question about whether the aPL-mediated mechanism for pregnancy complications might be different from the aPL-mediated mechanism for thrombotic complications. The current results, in keeping with previous studies, indicate that this mechanism is not unique for pregnancy but is also applicable to both forms of APS.

The results of AnxA5 anticoagulant activity correlated with levels of anti-domain I IgG (Figure 3) and are consistent with our previous report in APS patient with previous thrombotic events (thrombotic APS). However, in contrast to that study, which was done with small, but well-defined, groups of patients, the correlation, although statistically significant, was more modest.

Based on the current results, we hypothesize that the patients with decreased AnxA5 anticoagulant ratios but without elevated antibodies against domain I of β2GPI represent individuals who have antiphospholipid antibodies that recognize other domains on the same protein or possibly against other phospholipid-binding cofactor proteins. Furthermore, the distribution of anti-domain I antibodies in both those with obstetric APS and those with thrombotic APS is not Gaussian, indicating that there may be different antibody populations of clinical interest, one of which has affinity for domain I. We plan to address these questions in future studies.

One of the major practical issues in managing patients with aPL is the lack of data available to advise asymptomatic individuals who have aPL antibodies of their future risks of complications because many may never develop either thrombotic or obstetric complications and others may develop them after several years. It will therefore be interesting to investigate prospectively whether assays for AnxA5 resistance and anti-β2GPI domain I antibodies may be risk predictors of obstetric and thrombotic complications in a cohort of asymptomatic patients with isolated aPL.

In summary, women with obstetric APS were more likely to have AnxA5 resistance and also anti-β2GPI domain I antibodies than the control group. These results are consistent with the hypothesis...
that AnxA5 resistance is a mechanism for pregnancy losses associated with aPL, and those antibodies with anti-domain I may mediate this effect along with antibodies of other, yet-to-be-described, epitope specificities. These new functional and epitope-specific immunosays may identify specific mechanisms for aPL-mediated complications in subsets of patients and may thereby open paths toward identifying targeted therapies for this disorder.

REFERENCES