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ORIGINAL ARTICLE

Evaluation of the clinical relevance of anti-annexin-A5 antibodies in Chinese patients with antiphospholipid syndrome

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Abstract A hallmark feature of antiphospholipid syndrome (APS) is the presence of antiphospholipid antibodies (aPLs). Few studies have addressed the clinical relevance of anti-annexin A5 antibodies (aANXA5) in Chinese patients with APS. In this study, we evaluated the clinical performance of aANXA5 in the diagnosis of APS. Sera from 313 subjects were tested, including 170 samples from patients with APS, 104 samples from patients with non-APS diseases as disease controls (DC), and 39 healthy controls (HC). Serum IgG and IgM aANXA5 were determined by ELISA. Overall, the levels of both IgG and IgM aANXA5 were significantly increased in patients with primary APS (PAPS) and APS associated to other diseases (APSAOD) compared with DC and HC. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for IgG and IgM aANXA5 in the diagnosis of APS were 33.5 and 15.3, 99.0 and 99.0, 98.3 and 96.3, and 47.7 and 41.7%, respectively. Significant associations between IgG aANXA5 and arterial thrombotic events (OR, 2.60; 95% CI, 1.44–4.71) and between IgG aANXA5 and venous thrombotic events (OR, 2.80; 95% CI, 1.55–5.06) were identified. No correlations were identified between IgG or IgM aANXA5 and obstetric complications. Our data suggest that aANXA5 could serve as a diagnosis biomarker for patients with APS. More importantly, our data highlighted a potential role of IgG aANXA5 in identifying APS patients with high risk of thrombosis.

Keywords Anti-annexin-A5 antibodies · Antiphospholipid antibodies · Antiphospholipid syndrome · IgG/IgM · Thrombosis

Introduction

Antiphospholipid syndrome (APS) is an autoimmune prothrombotic disorder characterized by recurrent vascular thrombosis and/or pregnancy morbidity [1]. The presence of the antiphospholipid antibodies (aPLs) represents a hallmark feature of APS. Detection of those aPLs has been considered as the first-line approach for the diagnosis of APS. The recent updated classification criteria for APS emphasize the persistent presence (for >12 weeks) of lupus anticoagulant (LAC), anticardiolipin (aCL), and anti-β2-glycoprotein 1 (aβ2GP1) antibodies [2].

The pathogenesis of APS remains largely unknown. Several potential mechanisms have been proposed. Increased resistance against the anticoagulant activity of annexin A5 as one of those mechanisms has gained increased attention [3]. Annexin A5 is a calcium-dependent phospholipid-binding protein with potent anti-coagulant properties [4]. The thromboregulatory role of annexin A5 comes from its ability to form a two-dimensional crystal anti-thrombotic shield over the phospholipid bilayers, thus preventing the coagulation factors from binding to phospholipid surfaces [5, 6].

It has been suggested that aPLs compete with annexin A5 for phospholipid binding, thereby conferring increased annexin A5 resistance and accelerating platelet procoagulant activity, leading to thrombosis and pregnancy loss [7]. Rand et al. showed that plasma from patients with aPLs and

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thromboembolism exhibited reduced annexin A5 anticoagulant activity compared with patients with aPLs but without thrombosis [8]. Data pooled from three studies indicated that annexin A5 resistance was present in 53.3% of patients with APS, whereas was present in 21.2% of disease controls, highlighting the role of annexin A5 resistance in APS [9].

Given the protective role of annexin A5 in anticoagulant effect, neutralization of annexin A5 by anti-annexin A5 antibodies (aANXA5), which exposes the phospholipids on cell membranes for the accessibility of phospholipid-dependent coagulation enzymes, represents a potential mechanism of annexin A5 resistance. Therefore, theoretically, aANXA5 may have predictive potential to serve as risk factors for thrombosis and pregnancy complications. Indeed, several studies reported elevated levels of aANXA5 in patients with APS compared with patients with other systemic autoimmune diseases [10–12]. Importantly, significantly higher frequency of thrombotic events was identified in patients with aANXA5 [10–12]. However, Ogawa et al. and Laat B et al. were not able to demonstrate significant association between aANXA5 and thrombosis in patients with APS [13, 14]. Similar conflicting conclusions have also been observed in patients with pregnancy morbidity. For instance, the NOHA study showed a strong association between aANXA5 and recurrent pregnancy loss [15], while other studies failed to demonstrate such association [16].

A possible explanation for the discrepancies mentioned above is the different genetic/environmental factors. To our knowledge, few, if any, studies have addressed the clinical relevance of aANXA5 in Chinese patients with APS. Given this information will enhance our appreciation of the clinical utility of aANXA5, it is of paramount importance to evaluate the clinical role of aANXA5 in APS, particularly in their prognostic value for thrombosis and pregnancy complications.

Patients and methods

Subjects and specimen collections

Sera from 313 subjects were collected and analyzed in this study (Table 1). The subjects included 73 patients with primary APS (PAPS), 97 patients with APS associated to other diseases (APSAOD) (including 93 patients with APS and systemic lupus erythematosus (SLE), 2 patients with APS and vasculitis, 1 patient with APS and Behcet's disease, 1 patient with APS and Sjögren's syndrome), 104 patients as disease controls (including 30 patients with non-APS thrombosis, 32 patients with non-APS pregnancy-related morbidity (PRM), 42 patients with SLE but without APS), and 39 healthy controls (HC). HC were defined as no signs of infection or inflammation or other significant illnesses. APS was diagnosed by the Sydney revised Sapporo guidelines [2], specifically, a combination of one positive clinical criterion and one positive laboratory criterion on two or more occasions, not less than 12 weeks apart (aCL and/or aβ2G1 antibodies determined by ELISA and/or LAC determined according to the guidelines of the International Society of Thrombosis and Hemostasis [2]). Clinical and laboratory features were collected from all subjects. The presence of arterial and venous thrombosis in patients with PAPS, APSAOD, non-APS thrombosis, non-APS PRM, and SLE was 45.2 and 45.2, 51.5 and 39.2, 16.7 and 86.7, 0 and 3.0, and 2.3 and 0%, respectively. The incidence of obstetric complications in patients with PAPS, APSAOD, non-APS thrombosis, non-APS PRM, and SLE was 52.8, 50.9, 0, 100, and 0%, respectively. Study protocols were reviewed and approved by the ethical committee of Peking Union Medical College Hospital (PUMCH), and informed consents were obtained from all participants. All sera were stored at −20 °C until analysis.

Serum aPL antibody determination

Serum IgG and IgM aCL antibodies, IgG and IgM aβ2GP1 antibodies, and IgG and IgM aANXA5 were determined by ELISA (Aesku Diagnostics, Wendelsheim, Germany) according to the manufacturer's instructions. IgG/IgM aANXA5 ELISA is a solid-phase enzyme immunoassay using native human annexin A5 for the quantitative and qualitative detection of IgG and IgM antibodies against annexin A5 in human serum. The cutoff values for positivity were set based on the recommendations by the manufacturer.

Statistical analysis

SPSS 20.0 statistical software package (SPSS Inc., Chicago, IL, USA) and Prism 5.02 (GraphPad Software, San Diego, CA, USA) were utilized for all statistical tests. One-way ANOVA was used to calculate the difference between groups. The χ^2 test or Fisher's exact test was utilized for comparison of categorical variables. p values of less than 0.05 were considered statistically significant.

Results

Levels of IgG and IgM aANXA5 were elevated in patients with APS

The values expressed as arbitrary units/ml of IgG/IgM aANXA5 from all subjects are presented in Fig. 1. Overall, the levels of both IgG and IgM aANXA5 were significantly increased in patients with PAPS and APSAOD compared with disease controls and HC ($p < 0.01$) (Fig. 1a, b). No significant differences in the levels of IgG or IgM aANXA5 were

Table 1 Demographic, clinical characteristic, and aPL profiles of patients with APS and controls

	Primary APS (n = 73)	APSOD (n = 97)	Non-APS thrombosis (n = 30)	Non-APS PRM (n = 32)	SLE controls (n = 42)	HC (n = 39)	p value[a]
Sex (female/male)	47/26	74/23	10/20	32/0	39/3	14/25	0.019
Median age at study (max, min)	34 (9, 76)	31 (5, 86)	53.5 (14, 85)	35 (24, 41)	30 (12, 68)	39 (25, 65)	0.03
Arterial thrombosis, n (%)	33 (45.2)	50 (51.5)	5 (16.7)	0 (0.0)	1 (2.3)	0 (0.0)	<0.0001
Venous thrombosis, n (%)	33 (45.2)	38 (39.2)	26 (86.7)	1 (3.0)	0 (0.0)	0 (0.0)	0.03
Obstetric complications, n (%)[b]	19/36 (52.8)	28/55 (50.9)	0/10 (0.0)	32/32 (100.0)	0/31 (0.0)	0/14 (0.0)	0.106
Annexin A5 IgG (>12), n (%)	30 (41.1)	35 (36.1)	1 (3.3)	0 (0.0)	3 (7.1)	0 (0.0)	<0.0001
Annexin A5 IgG (>18), n (%)	28 (38.4)	29 (29.9)	1 (3.3)	0 (0.0)	0 (0.0)	0 (0.0)	<0.0001
Annexin A5 IgM (>12), n (%)	13 (17.8)	24 (24.7)	0 (0.0)	1 (3.1)	0 (0.0)	0 (0.0)	<0.0001
Annexin A5 IgM (>18), n (%)	7 (9.6)	19 (19.6)	0 (0.0)	1 (3.1)	0 (0.0)	0 (0.0)	<0.0001

APS antiphospholipid syndrome, *APSOD* antiphospholipid syndrome associated with other diseases, *RPM* pregnancy-related morbidity, *SLE* systemic lupus erythematosus, *HC* health controls

[a] The difference between APS patients and controls

[b] Percentage among women with reproductive history

observed between patients with PAPS and APSAOD (Fig. 1a, b). Interestingly, significantly increased levels of IgG aANXA5 were observed in patients with SLE compared with HC (Fig. 1a). When the manufacturer's recommended cut off of >18 U/ml was used, the presence of IgG and IgM aANXA5 in patients with PAPS, APSAOD, non-APS thrombosis, non-APS PRM, and SLE was 38.4 and 9.6, 29.9 and 19.6, 3.3 and 0, 0 and 3.1, and 0 and 0%, respectively (Table 1).

The predictive power of IgG and IgM aANXA5 in the diagnosis of patients with APS

As shown in Table 2, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LR+), and negative likelihood ratio (LR−) for IgG (>18 U/ml) and IgM (>18 U/ml) of IgG and IgM aANXA5 in the diagnosis of APS were 33.5 and 15.3%, 99.0 and 99.0%, 98.3 and 96.3%, 47.7 and 41.7%, 34.87 and 15.91, and 0.67 and 0.86, respectively. Notably, when the gray zone value of 12 U/ml was applied to IgM aANXA5, the sensitivity was increased from 15.3 to 21.8% without loss of specificity (Table 2).

Association between IgG and IgM aANXA5 and thrombosis or obstetrical complications

As mentioned previously, aANXA5 has been shown to be associated with thrombosis and pregnancy morbidity

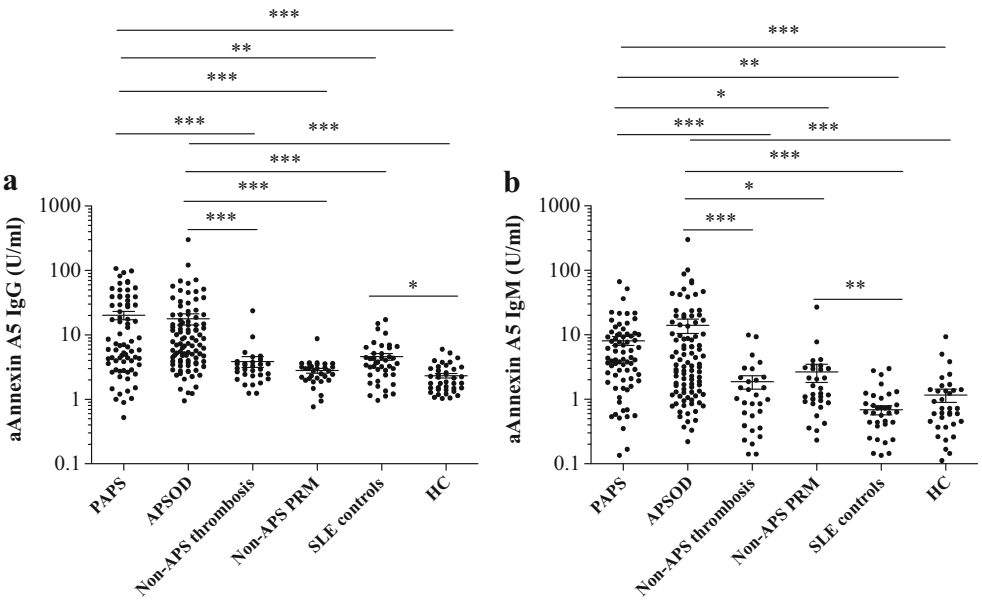

Fig. 1 Levels of IgG **a** and IgM **b** aANXA5 in patients with PAPS, APSAOD, non-APS-thrombosis, non-APS-RPM, SLE, and healthy controls. The values expressed as U/ml of IgG/IgM aANXA5. *U* arbitrary units, *aANXA5* anti-annexin A5 antibodies, *APS* antiphospholipid syndrome, *PAPS* primary APS, *APSAOD* APS associated to other diseases, *non-APS RPM* non-APS pregnancy-related morbidity, *SLE* systemic lupus erythematosus, *HC* healthy controls. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Table 2 The predictive power of IgG and IgM annexin A5 in the diagnosis of patients with APS

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	*LR+*	*LR−*
Annexin A5 IgG (>12)	38.2	96.2	94.2	48.8	9.94	0.64
Annexin A5 IgG (>18)	33.5	99.0	98.3	47.7	34.87	0.67
Annexin A5 IgM (>12)	21.8	99.0	97.4	43.6	22.64	0.79
Annexin A5 IgM (>18)	15.3	99.0	96.3	41.7	15.91	0.86

APS antiphospholipid syndrome, *PPV* positive predictive value, *NPV* negative predictive value, *LR+* positive likelihood ratio, *LR−* negative likelihood ratio

[10–12, 15, 16]. Thus, the levels of IgG and IgM aANXA5 were evaluated in patients with thrombosis or pregnancy morbidity. Importantly, significantly higher levels of IgG and IgM aANXA5 were observed in patients with arterial thrombosis as well as in patients with venous thrombosis, compared with patients without thrombosis (Fig. 2a, b). In contrast, no significant difference in the levels of both IgG and IgM aANXA5 were noted between patients with obstetrical complications and patients without obstetrical complications (Fig. 2c, d). Concordant with higher levels of IgG aANXA5 in patients with arterial thrombosis and in patients with venous thrombosis, significant associations between IgG aANXA5 and arterial thrombotic events (OR, 2.60; 95% CI, 1.44–4.71) and between IgG aANXA5 and venous thrombotic events were identified (OR, 2.80; 95% CI, 1.55–5.06) (Table 3). Interestingly, the associations were still observed when the gray zone value of 12 U/ml was used (Table 3). In contrast, no significant association was found between IgM aANXA5 and thrombosis (Table 3). In addition, no correlations were identified between IgG or IgM aANXA5 and obstetric complications (Table 3).

Levels of IgG and IgM aANXA5 in APS patients with different aPL profiles

As triple aPL positivity has been regarded as a risk factor for aPL-mediated clinical manifestations [17], the levels of IgG and IgM aANXA5 were evaluated in APS patients in terms of triple aPL positivity, double aPL positivity, and single aPL positivity. Importantly, significantly higher levels of both IgG and IgM aANXA5 were found in patients with triple aPL positivity compared with patients with double aPL positivity and patients with single aPL positivity (Fig. 3a, b). In addition, the levels of IgG aANXA5 were found significantly higher in patients with double aPL positivity than those in patients with single aPL positivity (Fig. 3a).

Discussion

Annexin A5 has been shown to play a central role in the pathophysiology of APS [7, 8, 10]. Reducing availability of annexin A5 by aPLs represents an important prothrombotic mechanism in APS [6, 18]. aANXA5 are suspected to play a

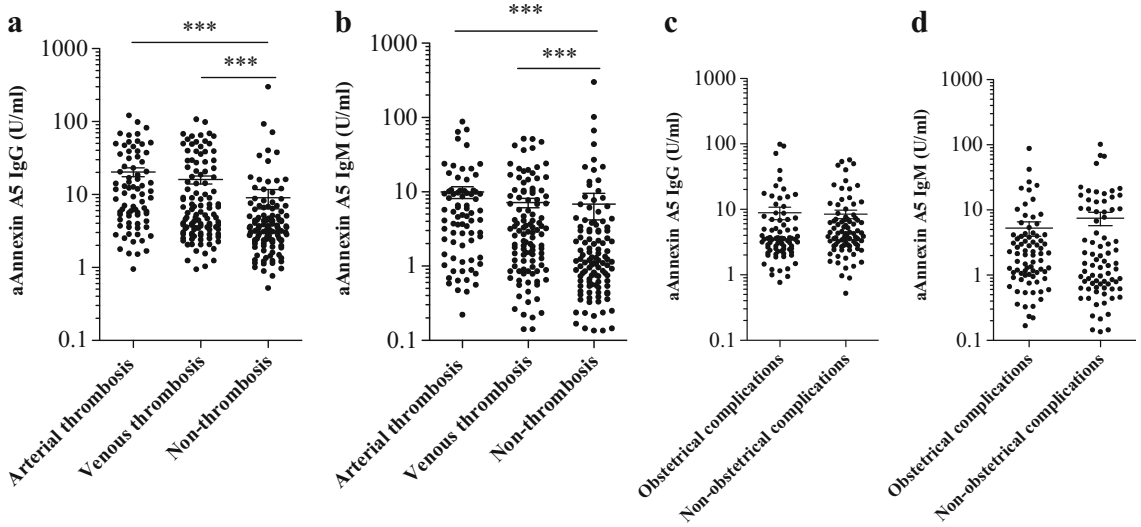

Fig. 2 Levels of IgG (**a** or **c**) and IgM (**b** or **d**) aANXA5 in patients with arterial thrombosis, venous thrombosis, and non-thrombosis and in patients with obstetrical complications and non-obstetrical complications. The values expressed as U/ml of IgG/IgM aANXA5. *U* arbitrary units, *aANXA5* anti-annexin A5 antibodies. $*p < 0.05$, $**p < 0.01$

Table 3 Correlations between anti-annexin A5 antibodies and thrombosis or obstetrical complications in clinical suspicious patients

	Odds ratio (95% confidence interval)			
	Thrombosis	Arterial thrombosis	Venous thrombosis	Obstetrical complications
Annexin A5 IgG (>12)	4.29 (2.25–8.20)	2.65 (1.51–4.66)	2.14 (1.23–3.74)	0.84 (0.37–1.94)
Annexin A5 IgG (>18)	5.07 (2.44–10.55)	2.60 (1.44–4.71)	2.80 (1.55–5.06)	1.00 (0.41–2.41)
Annexin A5 IgM (>12)	2.14 (1.02–4.52)	1.84 (0.92–3.70)	1.37 (0.68–2.74)	0.49 (0.20–1.23)
Annexin A5 IgM (>18)	2.00 (0.85–4.75)	1.77 (0.79–3.95)	1.06 (0.47–2.42)	0.70 (0.24–2.07)

pathogenic role by interfering with annexin V function. Unexpectedly, the correlation between aANXA5 and thrombotic events or pregnancy morbidity remains controversial [10–16, 19], indicating a clear need for characterizing the clinical relevance of aANXA5 in term of ethnic/geographic basis.

In the present study, we found that the levels of both IgG and IgM aANXA5 were significantly increased in patients with APS. Additionally, both IgG and IgM aANXA5 exhibited promising diagnosis potentials for APS with the sensitivity and specificity of 33.5 and 99.0% (IgG aANXA5) and 15.3 and 99.0% (IgM aANXA5), respectively. Importantly, IgG aANXA5 were significantly correlated with both arterial and venous thrombotic events. Our findings indicated that aANXA5, especially the IgG isotype, could serve as a promising biomarker to identify patients at risk of thrombosis in China.

In this study, IgG and IgM aANXA5 were detected in 38.4 and 9.6% of patients with PAPS and 29.9 and 19.6% of patients with APSAOD, which was similar to those reported by Satoh et al. [10]. In that study, Satoh et al. found that aANXA5 were present in 30.4% of patients with APS

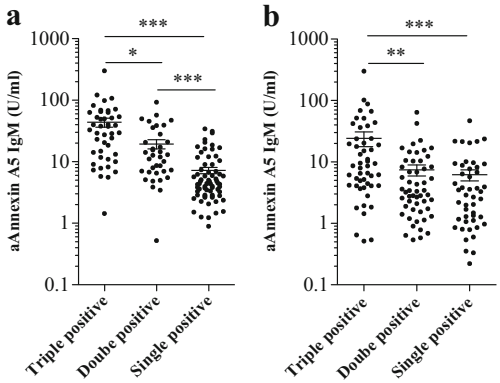

Fig. 3 **a** Levels of IgG aANXA5 in triple (LAC+, IgG aCL+, IgG aβ2GP1+), double (positive for any of the two IgG aPLs), and single aPLs positive (positive for any of the IgG aPLs) groups in patients with APS. **b** Levels of IgM aANXA5 in triple (LAC+, IgM aCL+, IgM aβ2GP1+), double (positive for any of the two IgM aPLs), and single aPLs positive (positive for any of the IgM aPLs) groups in patients with APS. The values expressed as U/ml of IgG/IgM aANXA5. *U* arbitrary units, *aCL* anticardiolipin antibodies, *aβ2GP1* anti-β2-glycoprotein I antibodies, *LA* lupus anticoagulant. $^{**}p < 0.01$

[10]. In another study conducted by Ogawa et al., the presence of IgG and IgM aANXA5 was 17 and 8%, respectively, which were lower than those in our study [13]. Interestingly, Singh et al. reported that the prevalence of IgG aANXA5 was 61.6% in patients with APS from India [12]. The striking difference in the presence of aANXA5 between their study and our study may be due to differences in ELISA kit (Zymutest kit, Hyphen Biomed, New Delhi, India vs Aesku Diagnostics ELISA, Wendelsheim, Germany) or differences in ethnic/geographic background.

Interestingly, aANXA5 were initially described in patients with SLE [20, 21]. Nevertheless, we did not detect the presence of aANXA5, in terms of both IgG and IgM isotypes, in Chinese patients with SLE. In a study by Satoh et al., the authors also reported low prevalence of aANXA5 in patients with SLE (3.8%) [10]. Remarkably, when the patients had both signs of SLE and APS, the presence of aANXA5 increased from 3.8 to 28.0% [10]. Another study from Hungary also showed that patients with APS had significantly higher frequency of aANXA5 compared with patients with other systemic autoimmune diseases [11]. Of note, in the initial publications describing the role of aANXA5 in SLE, a significant proportion of patients with SLE exhibited APS serological and clinical signs, indicating that those patients might belong to APSAOD [20, 21]. Taken together, our data suggest that aANXA5 could serve as a diagnosis biomarker for Chinese patients with APS.

A major controversy regarding the clinical relevance of aANXA5 in APS is whether a significant association exists between aANXA5 and APS clinical manifestations [10–15]. In this study, we did observe that aANXA5 were significantly correlated with thrombosis (both arterial thrombosis and venous thrombosis). Specifically, we found that IgG aANXA5 were significantly associated both arterial thrombosis and venous thrombosis. Interestingly, we did not find any association between IgM aANXA5 and thrombosis. Several studies demonstrated a significant association between IgG aANXA5 and thrombosis, which was similar to what we found [10–12]. Interestingly, Satoh et al. and Lakos et al. reported that aANXA5 correlated with both arterial and venous thrombosis, which were similar to our study [10, 11]. On the contrary, Ogawa et al. and de Laat et al. failed to show any association

between both IgG or IgM aANXA5 and arterial or venous thrombosis [13, 14].

Increasing evidence have highlighted that multiple positivities of aPLs are important parameters for risk assessment [17]. Analysis of the levels of aANXA5 in APS patients with different aPL profiles revealed a significantly higher level of IgG aANXA5 in patients with triple-positive aPL profile, further supporting the importance of IgG aANXA5 in evaluation of the APS clinical risks.

Although previous studies have suggested a link between aANXA5 and obstetric complications [14, 16, 22, 23], we did not observe such correlation, which is consistent with previous studies. However, further studies with more APS patients with obstetric complications are needed.

In summary, our data suggest that aANXA5 could serve as a diagnosis biomarker for patients with APS. More importantly, our data highlighted a potential role of IgG aANXA5 in identifying APS patients with high risk of thrombosis and thus could serve as a promising biomarker in clinical and therapeutic decision-making process.

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Compliance with ethical standards

Disclosures None.

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