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ORIGINAL ARTICLE

Anti-vimentin/cardiolipin IgA in the anti-phospholipid syndrome: A new tool for 'seronegative' diagnosis

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Abstract

Anti-phospholipid syndrome (APS) is a systemic autoimmune disorder defined by the simultaneous presence of vascular clinical events, pregnancy morbidity and anti-phospholipid antibodies (aPL). In clinical practice, it is possible to find patients with APS who are persistently negative for the routine aPL tests (seronegative APS; SN-APS). Recently, the identification of aPL immunoglobulin (Ig)A and/or anti-β2-glycoprotein-I (β2-GPI) IgA was shown to represent a further test in SN-APS patients. In this study we analyzed the presence of anti-vimentin/cardiolipin (aVim/CL) IgA in a large cohort of patients with SN-APS, evaluating their possible association with clinical manifestations of the syndrome. This study includes 60 consecutive SN-APS patients, 30 patients with APS and 40 healthy donors. aVim/CL IgA were detected by enzyme-linked immunosorbent assay (ELISA). Results show that 12 of 30 APS patients (40%) and 16 of 60 SN-APS patients (26.7%) resulted positive for aVim/CL IgA. Interestingly, SN-APS patients who tested positive for aVim/CL IgA showed a higher prevalence of arterial thrombosis ($p = 0.017$, likelihood positive ratio = 5.7). This study demonstrates for the first time, to our knowledge, the presence of aVim/CL IgA in sera of patients with APS. In particular, they revealed a potential usefulness in identification of a significant proportion of SN-APS patients. Moreover, as patients tested positive for aVim/CL IgA reported a high likelihood ratio to have the clinical features of APS, this test may be considered a suitable approach in the clinical evaluation of SN-APS.

KEYWORDS

anti-phospholipid syndrome, aVim/CL antibodies, IgA isotype, seronegative APS

INTRODUCTION

Anti-phospholipid syndrome (APS) is a systemic autoimmune disorder defined by the simultaneous presence of vascular clinical events, pregnancy morbidity and anti-phospholipid antibodies (aPL). The aPL considered as diagnostics are anti-cardiolipin (aCL) antibodies, anti-β2 glycoprotein-I (aβ2-GPI) antibodies and lupus anti-coagulant (LA) [1–3].

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During the 11th International Conference on antiphospholipid antibodies held in 2004 in Sidney, Australia, only antibodies belonging to the immunoglobulin (Ig)M and IgG isotype were considered pathogenic. Only in recent studies have the pathogenetic importance and diagnostic value of aβ2-GPI of IgA isotype been demonstrated, including their role in the pathogenesis of thrombotic events and stroke [4–6].

Thus, from the 13th International Congress on Antiphospholipid Antibodies (2010; Galveston, Texas, USA), testing for aβ2-GPI IgA in patients negative for IgG and IgM aβ2-GPI with APS symptoms was recommended [7,8]. In the case of aβ2-GPI IgA, a higher incidence of APS events in carriers of these antibodies has been reported, although only in patients with particular situations: chronic renal disease treated with hemodialysis and those who received a kidney transplant [9,10].

In clinical practice, it is possible to find patients with APS who are persistently negative for the routine aPL tests. For these cases, the term 'seronegative APS' (SN-APS) has been proposed; this may depend upon the limitations of traditional technical approaches and/or the existence of antigenic targets other than those known. In recent years, new variants have emerged in aPL tests that support the non-criteria aPL concept [11,12].

In this regard, antibodies directed to vimentin/cardiolipin (Vim/CL) complex have been described in sera of SN-APS [13–15]. These data tried to explain the 'paradoxical' role of aPL in SN-APS by demonstrating that these unconventional antibodies, found in sera from SN-APS patients, are able to trigger a signal transduction pathway which may contribute to the pathogenesis of thrombosis and/or other clinical manifestations of the syndrome [16].

Finally, several studies have recently analyzed the role of antibodies of the IgA isotype, and the identification of aPL IgA and/or aβ2-GPI IgA was shown to represent a further test in SN-APS patients. In particular, recent studies have suggested that, while aPL IgG/IgM recognize an epitope in domain I β2-GPI, epitopes recognized by aPL IgA are localized within domains III, IV and V [17]. Thus, as reported by the 13th International Congress on Antiphospholipid Antibodies, aβ2-GPI IgA detection should be considered useful in patients negative for IgG and IgM isotypes with symptoms of APS and can be included as a 'non-criteria' test for detection of aPL in APS patients [8].

In this study we analyzed for the first time, to our knowledge, the presence of aVim/CL IgA in a large cohort of patients with SN-APS and evaluated their possible association with clinical manifestations of the syndrome.

MATERIALS AND METHODS

Patients

This study included 60 consecutive SN-APS patients with clinical features consistent with a diagnosis of APS [18], but persistently negative for 'conventional' aPL tests (aCL, aβ2-GPI and LA), 30 patients with APS classified according to 2006 criteria [18] attending the lupus clinic, Rheumatology Unit of Sapienza University of Rome. Finally, 40 sera from healthy controls (HC) matched for sex and age were also studied. Moreover, all the SN-APS patients were tested for common inherited thrombophilic defects, such as protein C and protein S deficiency, hyperhomocysteinemia, factor V Leiden, methylenetetrahydrofolate reductase (MTHFR) and prothrombin mutations to exclude other possible causes of thrombosis or obstetric morbidities. Sera were collected several times and stored at −20°C until use. This study was conducted in compliance with the Helsinki declaration, approved by the local ethic committee ('Sapienza' University of Rome; protocol 0215/2021), and participants gave written informed consent.

Detection of anti-nuclear and 'conventional' aPL antibodies

Anti-nuclear antibodies (ANA) were tested by an indirect immunofluorescence test on human epithelial HEp-2 cells (A. Menarini Diagnostics, Florence, Italy). To analyze 'conventional' aPL tests, aCL and aβ2-GPI antibodies (IgG, IgM) were detected by immune-enzymatic assay using the QUANTA Lite™ detection kit (Inova Diagnostic Inc., San Diego, California, USA), confirmed by chemiluminescence assay using a Zenit RA Immunoanalyzer (A. Menarini Diagnostics). In addition, LA was analyzed by two coagulation systems, a dilute sensitized activated partial thromboplastin time (aPTT) and a dilute Russell's viper venom time (dRVVT), also performing a confirmation test (Hemoliance Instrumentation Laboratory, Lexington, Massachusetts, USA).

Detection of anti-vimentin/cardiolipin antibodies by enzyme-linked immunosorbent assay (ELISA)

aVim/CL IgA and IgG were detected by ELISA, following the previously reported method [19]. Briefly, a 96-well polystyrene plate (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was coated with 100 µl/well of cardiolipin (50 µg/ml in methanol) (from bovine heart; Sigma-Aldrich, St Louis, Missouri, USA) and human recombinant vimentin (5 µg/ml in 0.05 mM $NaHCO_3$ buffer, pH 9.5) (R&D Systems, Minneapolis, Minnesota, USA). After coating, the plate was preserved overnight at 4°C and then washed three times with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T) before blocking with 100 µl 1% bovine serum albumin (BSA) in PBS (blocking buffer) for 2 h at

room temperature. After washing three times with PBS-T, the wells were incubated with 100 μl of patient sera (diluted 1:100 in the blocking buffer) for 1 h at room temperature. Goat polyclonal anti-vimentin (R&D Systems) was also added as a positive control. To verify antibody binding, the plates were first washed as above and then incubated with horseradish peroxidase (HRP)-conjugated antibodies with goat anti-human IgA, goat anti-human IgG or rabbit anti-goat IgG (Sigma-Aldrich), diluted in blocking buffer for 1 h at room temperature. The plates, washed three times with PBS-T, were incubated with 100 μl/well of *O*-phenylenediamine dihydrochloride development buffer to reveal the bound peroxidase. After observing the development reaction, the plate was stopped for 5 min with H_2SO_4 0.2 M (50 μl/well). Absorbance was measured at 492 nm in a microplate reader. Virtually no reactivity was detected in all the samples when the same ELISA assay was performed without vimentin/cardiolipin complex coating (data not shown).

Data were analyzed and cut-off values were calculated using the 99th percentile of 40 healthy donors. Each serum was analyzed in triplicate.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) or median ± interquartile range (IQR), according to the distribution of values. The χ^2-test or Fisher's exact test was utilized for comparison of categorical variables and the Mann–Whitney *U*-test to evaluate continuous variables. *p* values of less than 0.05 were considered statistically significant. Prism version 7 (GraphPad Software, San Diego, California, USA) was utilized for all statistical tests.

Results

Clinical characteristics of patients

We enrolled 60 SN-APS patients (52 female and eight male) with a mean age of 40.8 years (SD = 11.02) who were tested negative for conventional aPL tests, aCL and aβ2-GPI (IgG, IgM) and LA, 30 patients affected by APS (26 female and four male) with a mean age of 44.7 years (SD = 14.16) and 40 healthy donors (34 female and six male) with a mean age of 39.2 (SD = 12.20). Clinical and demographic characteristics of patients are reported in Table 1. Fifteen of the 60 (25%) SN-APS patients were ANA-positive, 13 met the criteria for systemic lupus erythematosus (SLE) and two patients were diagnosed with undifferentiated connective tissue disease (UCTD).

Occurrence of IgA antibodies in APS and SN-APS patients

We preliminarily analyzed the occurrence of IgA autoantibodies in patients with APS. As expected, our results showed the presence of aCL IgA in 11 of 30 (36.6%) and the presence of aβ2-GPI IgA in eight of 30 (26.6%) APS patients.

TABLE 1 Clinical and demographic characteristics of patients studied

Features	APS *n* = 30 (%)	SN-APS *n* = 60 (%)	
Male/female	April-26	August-52	*P* = 0.74
Mean age in years (SD)	44.7 (14.16)	40.8 (11.02)	*P* = 0.26
Thrombosis	26 (86.7)	29 (48.3)	*P* = 0.001
Arterial thrombosis	11 (36.7)	13 (21.7)	*P* = 0.2
Venous thrombosis	19 (63.3)	19 (31.7)	*P* = 0.008
Recurrent thrombosis	16 (53.3)	13 (21.7)	*P* = 0.005
Pregnancy morbidity	8/26 (30.8)	37/52 (71)	*P* = 0.1
Spontaneous abortions	5 (19.2)	26 (50)	*P* = 0.02
Normal fetus deaths	2 (7.7)	15 (28.9)	*P* = 0.07
Premature births	1 (3.8)	2 (3.8)	*P* = 0.5
Thrombosis + pregnancy morbidity	4 (13.3)	6 (10)	*P* = 0.9
Non-criteria APS features	21 (70)	25 (41.7)	*P* = 0.02
Livedo reticularis	7 (23.3)	11 (18.3)	*P* = 0.8
Thrombocytopenia	6 (20)	6 (10)	*P* = 0.3
Migraine	6 (20)	12 (20)	*P* = 0.8
Seizures	3 (10)	2 (3.3)	*P* = 0.4

APS = anti-phospholipid syndrome; SN-APS = seronegative APS; SD = standard deviation.

Interestingly, 12 of the 30 (40%) APS patients resulted positive for aVim/CL IgA (Table 2, Figure 1).

Among the SN-APS patient group, three of 60 (5%) and two of 60 (3.3%) resulted positive for aCL and aβ2-GPI isotype IgA, respectively. Moreover, 16 of 60 (26.7%) of the SN-APS patients resulted positive for aVim/CL IgA (Figure 2), one at a dilution of 1:800, one at 1:400, three at 1:200 and 11 at 1:100 (Supporting information, Table S1). In addition, 21 of these patients (36.7%) were positive for aVim/CL IgG (Table 2), but the isotype IgA allowed to detect the positivity in eight patients who tested negative for aVim/CL IgG.

Thus, 17 of 60 (28.3%) SN-APS patients resulted positive for at least one IgA isotype of aPL. Indeed, aVim/CL IgA reached almost all positives; in fact, among the positive patients only one was negative for aVim/CL IgA and positive for both aCL and aβ2GPI IgA. Figure 3 reports the distribution of the positivity among the tests.

None of the 40 healthy controls resulted positive for aCL IgA, aβ2-GPI IgA or aVim/CL IgA. The receiver operating characteristic (ROC) analysis for aVim/CL IgA test in SN-APS is shown in Figure 4.

TABLE 2 Prevalence of antibodies specific for vimentin/cardiolipin complex.

Autoantibodies to vimentin/cardiolipin complex	APS (60) *n* %	SN-APS (30) *n* %	HC (40) *n* %
Anti-vimentin/cardiolipin IgA	12/30 (40)	16/60 (26.7)	0/40 (0)
Anti-vimentin/cardiolipin IgG	24/30 (80)	22/60 (36.7)	0/40 (0)

APS = anti-phospholipid syndrome; SN-APS = seronegative APS; HC = healthy control; Ig = immunoglobulin.

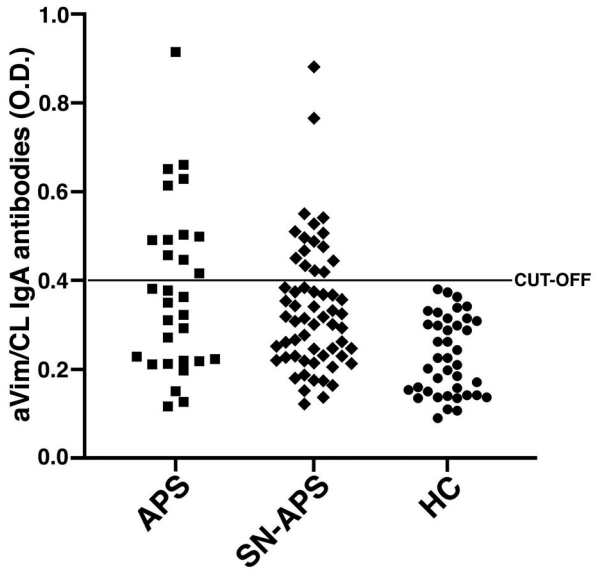

FIGURE 1 Levels of anti-vimentin/cardiolipin (aVim/CL) immunoglobulin (Ig)A in patients [anti-phospholipid syndrome (APS), seronegative (SN)-APS] and in healthy controls (HC). For detection of aVim/CL IgA all the sera were analyzed by enzyme-linked immunosorbent assay (ELISA). The cut-off level has been calculated as the 99th percentile of 40 HC sera

Correlation of IgA aVim/CL with clinical features of SN-APS patients

SN-APS patients who tested positive for aVim/CL IgA showed a higher prevalence of arterial thrombosis ($p = 0.017$, likelihood positive ratio = 5.7); aVim/CL IgG resulted in a likelihood positive ratio of 4.07 to have livedo reticularis ($p = 0.044$) and thrombocytopenia ($p = 0.015$, likelihood positive ratio = 5.86). APS patients tested positive for aVim/CL IgG showed a higher prevalence of pregnancy morbidity ($p = 0.039$, likelihood positive ratio = 4.24) and thrombocytopenia ($p = 0.046$, likelihood positive ratio = 3.97).

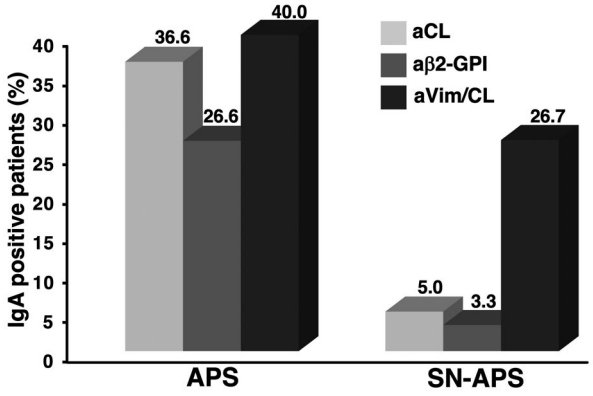

FIGURE 2 Percentage of patients [anti-phospholipid syndrome (APS), seronegative (SN)-APS] positive for at least one immunoglobulin (Ig)A assay. aβ2-GPI = anti-β2-glycoprotein I

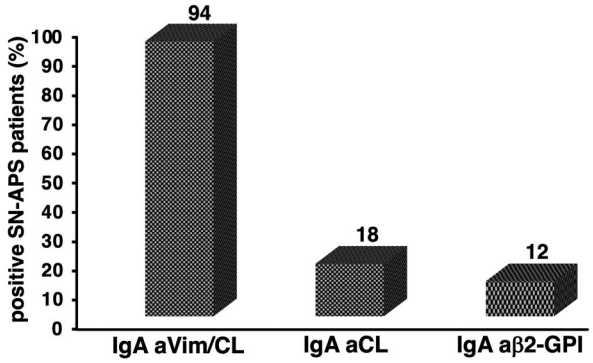

FIGURE 3 Distribution of positive seronegative-anti-phospholipid syndrome (SN-APS) patients among those tested positive for at least one immunoglobulin (Ig)A assay: anti-cardiolipin (aCL), anti-β2-glycoprotein I (aβ2-GPI); anti-vimentin/cardiolipin (aVim/CL)

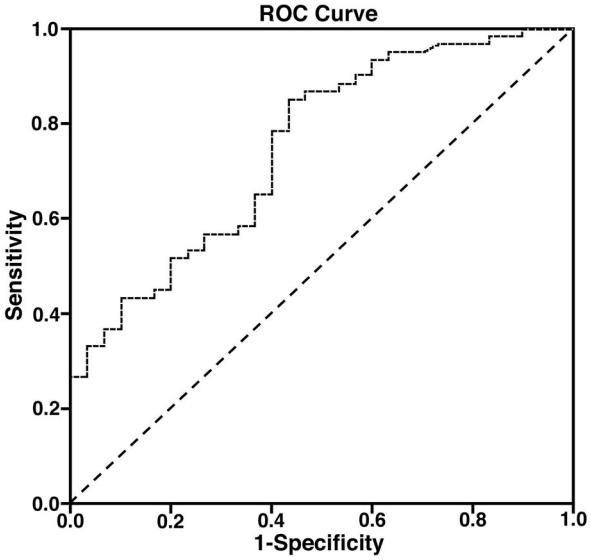

FIGURE 4 Receiver operating characteristic (ROC) analysis of seronegative-anti-phospholipid syndrome (SN-APS). A Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) variation presented an area under the curve (AUC) of 0.697 [95% confidence interval (CI) = 0.628–0.766, $p < 0.0005$]. The variation in anti-vimentin/cardiolipin (aVim/CL) immunoglobulin (Ig)A levels presented an area under the curve (AUC) of 0.75 (95% CI = 0.645–0.856, $p < 0.0001$, Youden's J = 0.213) for the development of thrombotic or pregnancy events, as indicated in the APS classification criteria

DISCUSSION

This study demonstrates for the first time the presence of aVim/CL IgA in sera of APS, as well as in a large cohort of patients with SN-APS.

The IgA isotype of aCL and aβ2-GPI is not included in the latest classification criteria for APS [18], although in 2012 the task force from the 13th International Congress on APS recommended testing the IgA isotype in the so-called SN-APS [7]. Thus, some studies reported a variable prevalence of aCL and aβ2-GPI IgA in SN-APS patients, ranging from 0 to 12% and 0 to 10%, respectively [15,20–24]. In 2018, aβ2-GPI IgA were defined as 'Cinderella' for their unjust marginalization in the pathogenesis of APS [25], while some papers have begun to show their potential pathogenic role in the disease [9,17,26]. Indeed, the presence of immune complexes has been detected in patients with aβ2-GPI IgA. In these patients, the aβ2-GPI IgA alone has a positive predictive value for APS events. The presence of aβ2-GPI IgA immune complexes shows a very strong correlation with APS events in carriers of aβ2-GPI IgA [27]. In addition, the pathogenicity of aβ2-GPI IgA was conclusively demonstrated in animal models. Mice inoculated with purified aβ2-GPI IgA developed a significantly higher thrombotic area than mice inoculated with control IgA. Additionally, affinity-purified aβ-GPI IgA induced thrombus in the femoral vein of these animals [28].

Hu and colleagues described the results of a large cross-sectional study on 7293 Chinese subjects, 212 with a diagnosis of APS. The overall prevalence of both aCL and aβ2-GPI IgA was quite low (2.48 and 2.13%, respectively). Furthermore, the positivity rate of these aPL was similar in APS patients and healthy controls, resulting in the absence of a diagnostic role of aPL IgA in the Chinese population [29].

A recent study reported the results of non-criteria aPL in 90 SN-APS patients; all the patients positive for aCL and aβ2-GPI IgA (12 and 10%, respectively) were also positive for other non-criteria aPL [24]. These data are confirmed and extended by the present study, where the IgA isotype of aCL and aβ2-GPI seems to appear together with other antibodies and has a quite low prevalence in SN-APS patients. Moreover, in this study, we also tested aVim/CL IgA, as we had previously identified by the proteomic approach with vimentin as a potential co-factor protein and the Vim/CL complex as an immunoreactive antigen in SN-APS sera [30]. Our results showed a significantly higher prevalence of aVim/CL IgA compared to aCL and aβ2-GPI IgA. Our findings suggest potential usefulness for these antibodies both in diagnosis and clinical associations. aVim/CL IgA also ensured the achievement of positivity in eight patients who tested negative for all non-criteria aPL tests performed in the SN-APS patients, including aVim/CL IgG, which resulted in the most prevalent non-criteria aPL in SN-APS patients [15,21]. Regarding clinical associations, patients who tested positive for aVim/CL IgA showed a higher prevalence of arterial thrombosis with a likelihood positive ratio of 5.7. This finding is not surprising, as patients who tested positive for aVim/CL IgG were shown to have at an least fourfold increase in the odds of having clinical features included in the classification criteria (arterial thrombosis, pregnancy morbidity) and extra-criteria manifestations (livedo reticularis, thrombocytopenia). Thus, aVim/CL IgA and IgG not only have a high prevalence in SN-APS patients, but patients who tested positive for these antibodies more likely to have APS-related clinical characteristics. The main limitation of this study was its one-center design. Further studies are in progress in a larger and multi-centric cohort of patients to validate the role of aVim/CL IgA in the diagnosis of SN-APS patients, clinical associations and risk stratification.

These findings demonstrate that in a wide range of SN-APS patients it could be possible to detect aPL and to make the diagnosis of APS using new autoantigens, such as aVim/CL IgA. Patients who tested positive for IgA as well as IgG aVim/CL reported a high likelihood ratio to have the clinical features of the APS. Therefore, these tests may be considered a suitable approach in the evaluation of SN-APS patients.

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CONFLICTS OF INTEREST

The authors state no conflicts of interest.

AUTHOR CONTRIBUTIONS

Maurizio Sorice, Fabrizio Conti and Simona Truglia designed the study; Antonella Capozzi, Gloria Riitano and Serena Recalchi performed the experiments; Silvia Mancuso and Simona Truglia enrolled patients; Silvia Mancuso and Valeria Manganelli performed statistical analysis; Antonella Capozzi, Roberta Misasi, Fabrizio Conti, Simona Truglia and Maurizio Sorice wrote the paper; Tina Garofalo, Cristiano Alessandri and Agostina Longo read and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

Data from this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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