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Title

Extra-criteria antiphospholipid antibodies in patients with small vessel brain lesions and clinical manifestations associated with antiphospholipid syndrome

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Key words

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Extra-criteria antiphospholipid antibodies. Small vessel brain lesions. Antiphospholipid Syndrome. IgA anti-β2 glycoprotein. IgG anti-phosphatidylserine/prothrombin.

Abstract

Objectives: Neurological manifestations compatible with small vessel brain lesions (SVBL), such as migraine, cognitive impairment, seizures, and transverse myelitis, may be related to antiphospholipid syndrome (APS) and patients could need APS therapies even though they do not fit into thrombosis or obstetric morbidity. Furthermore, extra-criteria antiphospholipid antibodies (aPL) provide an increase in sensitivity in patients with clinical manifestations related to APS but negative for IgG/IgM anticardiolipin (aCL), anti-β2 glycoprotein I (aβ2GPI), and lupus anticoagulant, which are the antibodies included in the classification criteria for APS.

Methods: We determined extra-criteria aPL in 65 SVBL patients with neurological traits and Magnetic Resonance Imaging suggestive of APS but negative for APS classification criteria, 47 of whom were prospectively followed and tested over three years. A group of 95 patients with autoimmune diseases (AD) but without clinical traits of APS was also studied.

Results: A persistent presence of extra-criteria aPL was detected in 27.7% of patients: 12.77 % IgM anti- prothrombin (PT), 6.38 % IgG anti-PT, 6.38% IgM anti-phosphatidylethanolamine (PE), 4.26% IgA aβ2GPI, 2.13% IgG anti-phosphatidylserine/prothrombin (PS/PT) and 2.13% IgM anti-PS/PT.

There was a tendency towards a higher prevalence of these aPL in SVBL patients than in AD – especially for IgA aβ2GPI – and a lack of IgG aPS/PT positivity in the AD group. We found no SVBL patient positive for IgA aCL, IgG anti-PE, annexin V, or aβ2GPI domain I.

Conclusions: Extra-criteria aPL can improve sensitivity for APS diagnosis in patients with SVBL, especially IgA aβ2GPI and IgG anti-PS/PT antibodies.

1. Introduction

Antiphospholipid syndrome (APS) – as included in the updated APS classification criteria – is characterized by circulating antibodies (Abs) directed against phospholipid-binding proteins or their complex with phospholipids (aPL) and clinical manifestations of thrombosis or gestational morbidity [1]. These criteria are very specific to ensure homogeneity for some purposes, such as clinical assays, yet they are not very sensitive for APS diagnosis [2]. Due to this lack of sensitivity, clinicians must be aware of some entities – distinct from thrombosis fetal losses, or eclampsia – that are not included in the classification criteria but may be related to APS [3,4].

Neuropsychiatric manifestations of APS are due to thrombotic events or direct interaction with surface neuronal molecules, among other mechanisms [5,6]. Some neuropsychiatric diseases associated with different levels of evidence of APS aside from thrombosis effects are migraine, cognitive impairment, seizures, movement disorders, and transverse myelitis. These disorders could need APS-related therapies even though they do not fit into the clinical criteria.

Besides clinical manifestations, at least one aPL laboratory result must be positive to fulfill APS classification criteria. These aPL are IgG/IgM anticardiolipin (aCL), anti-beta2glycoprotein I (aβ2GPI), or lupus anticoagulant (LA). Some extra-criteria or non-classical aPL have been postulated as related to APS [7,8,9]. The International Task Force on APS suggested the evaluation of additional aPL in order to increase sensitivity and/or specificity in APS diagnosis [10,11]. IgA aCL or aβ2GPI, Abs to annexin V, prothrombin (PT), phosphatidylserine/prothrombin (PS/PT), phospholipids other than cardiolipin such as phosphatidylethanolamine (PE), and domain I of β2GPI, among others, have been proposed as candidates to improve diagnostic accuracy in APS. In this way, a new entity has been defined as seronegative antiphospholipid syndrome (SNAPS) referring to patients with clinical profile suggestive of APS but


persistently negative for aPL included in the classification criteria [12].

In this work we determined extra-criteria aPL in 65 patients with small vessel brain lesions (SVBL), presenting Magnetic Resonance Imaging (MRI) and clinical findings compatible with neurological APS, but negative for clinical and laboratory APS classification criteria. Forty-seven of these patients were followed and tested over three years. In parallel, we studied a group of 95 patients with autoimmune diseases (AD) with no clinical or serological evidence of APS.

2. Patients and Methods

2.1 Patients

We recruited 65 patients: 42 from Hospital Universitari Son Espases (HUSE), 7 from Hospital de Alcorcón, and 16 from Hospital Miguel Servet. The inclusion criteria were the presence of SVBL compatible with APS by MRI with six or more supratentorial subcortical T2 lesions (Fig1), score 2 or above on the Fazekas scale [13], and over 17 years of age. The exclusion criteria were the presence of any disease responsible for SVBL or cardiovascular risk factors, positivity for aPL included in the classification criteria, over 70 years old, and on immunosuppressive treatment. Demographic and patients' main clinical data are depicted in Table 1. Informed consent was obtained and data and samples were anonymized following the ethical rules of the Balearic Islands Ethics Committee (CEIC): approval reference IB3288/16 PI.

Extra-criteria aPL (Table 2) were tested once a year over three years – except for anti-annexin II Abs, which was tested once a year over two years – in 47 patients. The remaining 18 patients were tested once for all extra-criteria aPL.

aPL can be produced as a result of an infection or by B lymphocyte polyclonal activation without pathogenic implications. For this

reason, we included 95 samples from patients with no clinical or serological evidence of APS, under follow-up for autoimmune diseases, most of which were organ-specific (Table 3). These patients were studied in order to evaluate the contribution of an autoimmune background to the presence of extra criteria aPL in the absence of APS clinical traits. Systemic lupus erythematosus patients were excluded due to their high prevalence of aPL and APS clinical manifestations.

Extra-criteria aPL were studied at the Immunology Service of HUSE. Other laboratory, clinical, and radiological tests were performed at each local Hospital.

2.2 Methods

Blood samples were collected without anticoagulant, centrifuged, and sera aliquots were frozen at -80ºC.

We determined IgA aCL and aβ2GPI and IgG/IgM anti-PS/PT by ELISA (INOVA diagnostics. San Diego. USA). The ELISA test was chosen for IgA aCL and aβ2GPI because it showed higher sensitivity in a pilot study (not shown), which is in accordance with previous studies that reported a better performance of ELISA assays *versus* chemiluminescence in IgA aPL detection [14,15].

We also tested IgG anti-annexin V, IgG/IgM anti-PE, and IgG/IgM anti-PT by ELISA (AESKU); IgG aβ2GPI domain I by chemiluminescence (INOVA diagnostics); and IgG+IgM to annexin II by competitive ELISA (Mybiosource, San Diego, CA, USA), (Table 2).

Cut-off values corresponded to those referred by manufacturers except for Abs to annexin II for which we calculated the 99th percentile of a cohort of healthy blood donors (n=50) in order to establish reference values.

2.3 Statistics

Results are expressed as absolute frequency and percentage of positive results. Comparisons were performed using Fisher's exact test. P values less than 0.05 were considered significant. Data were processed and analyzed using GraphPad Prism 5 software (San Diego, CA, USA).

3. Results

3.1 Patients and controls positive for extra-criteria aPL

SVBL (n=65) presented a slightly higher prevalence than AD (n=95) for extra-criteria aPL without statistical significance (n.s.), for IgM anti-PS/PT (15.4 % *vs* 12.4 %), IgG and IgM anti-PT (15.4 % *vs* 12.5 % and 13 % *vs* 8.3 %), and IgG anti-PE (6.2 % *vs* 4.5 %) (Figure 2).

No SVBL patients were positive for either anti-annexin V or IgG anti-domain I β2GPI *vs* 1.1 % and 6.8 % positive AD (n.s.). AD also showed a higher percentage of Abs to annexin II than patients (15.5 % *vs* 3.1 %, p<0.05).

Not one AD was positive for IgG anti-PS/PT *vs* 1.5% of patients; and while the frequency of IgA aβGPI in patients was low (3.1%), it was more than twice that found in AD: 1.5% (n.s.). The only AD positive for IgA aβ2GPI was a woman with M2 type of circulating antimitochondrial Abs (AMA), usually associated with primary biliary cholangitis (PBC), and Abs for anti-thyroid peroxidase. She had no clinical traits of PBC, but biochemical tests showed increased gamma-glutamyl transferase levels in serum. Besides that, she presented hyperthyroidism. She is at present under control by gastroenterologists for possible pre-PBC.

3.2 Patients persistently positive for extra-criteria aPL

The whole number of patients that were persistently positive for at least two years for extra-criteria aPL was 13 out of 47 (27.7 %): six of them presented IgM to PT (12.77 %), three IgM to PE (6.38 %), three IgG to PT (6.38 %), two IgA aβ2GPI (4.26 %), one IgG to PS/PT (2.13 %), and one IgM to PS/PT (2.13 %) (Figure 3).

Most of the persistently positive patients (8/13: 62 %) presented multiple reactivities (Figure 4). Four out of six patients with persistent IgM anti-PT Abs evolved to IgM anti-PS/PT positivity. IgA aβ2GPI and IgG anti-PS/PT were not associated with other Abs (Table 4).

3.3 Clinical traits of patients persistently positive for extra-criteria aPL

The main clinical manifestation in patients with persistent extra-criteria aPL was migraine: 8/13 (61.5 %). Migraine patients were in the age range of 28-60 (mean: 44.4) and mostly presented holocranial migraine (5/8). None of the patients developed aura. A majority of these migraine patients were women: 7/8, none of them under oral contraceptive therapy.

The remaining clinical traits were: memory loss: 6/13 (46.2 %); cognitive impairment: 4/13 (30.8 %); optical neuritis: 2/13 (15.4%); and seizures, anosmia, and hearing loss: 1/13 (7.7 %), respectively.

The small number of persistently positive patients did not allow for a statistical analysis of the association between extra-criteria aPL and clinical traits. There was no specific clinical profile for each Ab but it is remarkable that anosmia was only found in one IgA aβ2GPI-positive patient and hearing loss in one IgM anti-PE-positive one (Figure 5).

4. Discussion

Small vessel brain lesions constitute a neurological finding sometimes related to APS. In the present work, we detected a persistent presence – for at least two years – of extra-criteria aPL in 27.7% of patients with SVBL and MRI suggestive of APS, with no underlying disease or cardiovascular risk, but negative for classical aPL. Some of these Abs were also found in a cohort of patients with autoimmune diseases, mainly organ-specific, but free of APS symptoms and consensus aPL.

Prevalence of IgA aβ2GPI in SVBL patients was low but more than twice that in AD. Only one control with hyperthyroidism and AMA-positive was positive. IgA isotype Abs against β2GPI have already been detected in autoimmune liver diseases with a controversial correlation with thrombotic events [16, 17]. In contrast, isolated IgA aβ2GPI has been associated with an increased risk of thrombosis in systemic lupus erythematosus [19,20,21] and primary APS [22,23,24], but some reports have not shown an additional value of IgA aβ2GPI testing in SNAPS [25,26,27,28]. In favor of a pathogenic role of IgA aβ2GPI, a mouse model inoculated with this kind of purified Abs experienced thrombosis, revealing increased tissue factor activity in peritoneal macrophages [29]. These antibodies have also been reported in patients with central nervous system diseases related to APS but negative for criteria aPL. In particular, a longitudinal study conducted over 5 years in a cohort of asymptomatic individuals positive or negative for IgA aβ2GPI showed, during the follow-up, 15.6 % *versus* 3.2 %, respectively, of APS-related events, mainly arterial thrombosis [21]. The low sensitivity of chemiluminescence could explain the negative results for IgA aβ2GPI previously reported in APS and SAFSN patients [30,31,32,33]. A lack of standardization and bias in cohort composition to mainly women might also affect IgA aβ2GPI results, because these Abs are more prevalent in men [34]. In the present study we used a standardized ELISA (INOVA) with proven high sensitivity and specificity [35] but most patients were women (80%), which could influence towards a low frequency of IgA aβ2GPI. It is noteworthy that our positive patients for IgA aβ2GPI were women.

Both IgA aβ2GPI positive SVBL patients presented memory loss as well as three more positive patients for other extra-criteria aPL. The overall prevalence in our cohort of this neurological trait was 30.8 %. There is a high level of evidence in the association between aPL and memory loss or cognitive impairment [2]. The good correlation reported in most studies between isolated IgA aβ2GPI and clinical evidence of APS could substantiate the management of positive patients as APS [34,36].

We did not find any patient positive for IgA aCL. These Abs are, in general, redundant to criteria aPL and a correlation between their isolated presence and clinical manifestations of APS has not been demonstrated [23,34].

Prothrombin (PT) is a phospholipid-binding protein considered a cofactor for aPL. Abs to PT account for lupus anticoagulant activity and can be detected by two different ELISA assays [37], depending on how PT is exposed to patient serum. One kind of ELISA uses PT coated onto irradiated/activated plates (anti-PT) while the other uses PT in complex with PS (anti-PS/PT).

Anti-PS/PT Abs, mainly of IgG isotype, have been reported as very specific for APS, highly correlated to LA, and associated with thrombosis [37,38,39,40,41,42,43]. They can provide an additive value on aPL criteria to diagnose APS [28,44]. We found one patient positive for IgG anti-PS/PT who suffered from isolated migraine, which was the most prevalent clinical manifestation in extra-criteria aPL positive patients. Not a single AD control was positive for IgG anti-PS/PT. International consensus on APS criteria suggested that migraine could be an APS-related clinical trait [2] but the international Task Force on APS considered migraine as having a low level of evidence for being APS-related, therefore treatment with anticoagulants was not recommended if it was the only symptom [4]. Another patient was persistently positive for IgM anti-PS/PT and showed memory loss and cognitive impairment. As mentioned above, the level of evidence of this APS-related neurological manifestation is high [2].

We found a slightly higher frequency for IgM PS/PT and IgM/IgG anti-PT in SVBL than in AD. In some SVBL patients IgM a-PT preceded IgM a-PS/PT emergence. In fact, both aPL have been described as correlated to each other but belonging to different subpopulations of Abs [46,47,48]. Most studies comparing anti-PS/PT to anti-PT showed a higher specificity of the former Abs for APS diagnosis although anti-PT Abs of IgG isotype have been associated with thrombosis in SLE and APS [37,38,41] and found to be positive in some SNAPS patients [36].

Two of our three anti-PE persistently positive patients presented anti-PT Abs while the remaining one showed a very low level of anti-PE; thus, these antibodies did not have a relevant additive value to anti-PT. Anti-PE Abs have been correlated to thrombosis and other clinical traits of APS [48] and were the only aPL detected in a subset of SNAPS patients [33,49,50]. Some additional studies did not support a good clinical performance of anti-PE in APS [51,52].

We found no patient persistently positive for antibodies against annexin II, annexin V, or IgG anti-β2GPI domain I. Abs to annexin V were negative in all our patients and controls. Annexins II and V bind phospholipids with high affinity and regulate the coagulation cascade [53]. The clinical relevance of Abs to annexin V is controversial and the functional assay of annexin V resistance seems more useful [10,36]. Furthermore, pregnancy complications are the main APS clinical trait associated with anti-annexin V [53]. Annexin II mediates the binding of β2GPI to endothelial cells, playing a role in endothelium activation by aβ2GPI Abs [54]. Abs to annexin II have been reported to be significantly higher in patients with cerebral venous thrombosis [53]. We detected a subset of autoimmune controls positive for anti-annexin II antibodies, which has already been reported in autoimmune diseases [55,56,57]. Few studies show a significantly higher prevalence in APS or demonstrate positivity in SNAPS [54,57], in accordance with our results.

Antibodies to domain I of aβ2GPI were positive in some controls. Reactivity to domain I of β2GPI seems to be a trait of pathogenic IgG aβ2GPI Abs [58], which have been correlated to thrombosis [59] and whose positivity classifies patients at high risk of thromboembolic episodes [58,60,61]. In contrast, their value to increase sensitivity in APS diagnosis has not been confirmed [33,61]. Furthermore, they have previously been detected in autoimmune diseases without APS features [58], as in the present work.

~~A~~ limitation of our study could be the small size of the patient sample. Nevertheless, it is comparable to other published studies about extra-criteria aPL [50] and fulfills the recommendations of the Task Force to assess risk associated to aPL: it is a prospective study and performed over a long time to confirm Abs persistence [10,11]. AD controls were only tested once, as performed in previous studies [23].

We conclude that a subset of patients with SVBL and neurological diseases related to APS, but negative for criteria aPL, exhibit extra-criteria aPL. IgG and IgM anti-PS/PT, IgG and IgM anti-to PT, IgM to PE, IgA aβ2GPI, or IgG anti-PS/PT were consistently positive for at least two years in some SVBL patients. These aPL could constitute a set of new serological markers to provide more sensitivity in APS diagnosis, especially IgG anti-PS/PT, which was exclusively found in SVBL patients, and IgA aβ2GPI, which was detected at very low frequency in AD without APS clinical traits. Further research is needed to confirm the relevance of these antibodies.

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Data Availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no conflict of interest regarding the publication of this paper.

Author contributions

M.A.E., A.M., N.L., M.R.J-L., and M.R.J. performed the experimental assays; M.J.P. interpreted the radiological images; E.E., S.S., and L.P. recruited the patients and followed their clinical evolution; M.R.J. and M.A.E. analyzed the data and wrote the article. L.P. and M.R.J. coordinated the clinical and immunological parts of the project, respectively.

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