Clinical and laboratory evaluation of patients with primary antiphospholipid syndrome according to the frequency of antinuclear antibodies (ANA Hep-2)

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ABSTRACT

Objective: To evaluate the frequency of clinical and laboratory manifestations in patients with primary antiphospholipid syndrome (PAPS) with positive antinuclear antibodies (ANA Hep-2+) compared to those in whom this antibody is negative (ANA Hep-2-). **Patients and Methods:** This is a transversal study with 58 patients (82.8% females) with PAPS. Demographic and clinical data, comorbidities, medications, and antiphospholipid antibodies were evaluated. **Results:** Twenty (34.5%) out of 58 patients were positive for ANA Hep-2. Comparing the group of patients ANA Hep-2+ with those that were ANA Hep-2-, it was observed that both groups of patients with APS did not show statistically significant differences regarding demographic data, as well as the duration of the disease. As for clinical and laboratorial manifestations, the ANA Hep-2+ group showed higher frequency of deep venous thrombosis (85 *versus* 52.6%, P = 0.04), a statistically higher frequency of anticardiolipin IgG (85 *versus* 52.6%, P = 0.02), and a tendency for anticardiolipin IgM (80% *versus* 52.6%, P = 0.05), as well as greater medians of those antibodies [33 (0-128) *versus* 20 (0-120) GPL, P = 0.008] and [33 (0-120) *versus* 18,5 (0-120) MPL, P = 0.009]. Such difference was not observed regarding other manifestations of APS, presence of comorbidities, lifestyle, and medications used. **Conclusions:** Patients with PAPS with ANA Hep-2+ have a higher frequency of deep venous thrombosis and anticardiolipin IgG and IgM.

Keywords: antiphospholipid syndrome, primary antiphospholipid syndrome, cellular antibodies in Hep-2 cells, autoantibodies, anticardiolipin.

INTRODUCTION

Antiphospholipid syndrome (APS) is an acquired autoimmune disease characterized by vascular thrombosis (arterial and/or venous) that can evolve with obstetric events in the presence of persistent antiphospholipid antibodies (anticardiolipin, anti-beta-2-glycoprotein I, or lupus anticoagulant).[1]

Autoantibodies against antigens present in Hep-2 cells are practically universal in systemic lupus erythematosus (SLE). The finding of those antibodies supports the confirmation of this disorder, considering that they are present in 95% of the patients. However, although the remaining lupus patients included in the other 5% are negative for these antibodies on indirect immunofluorescence (IIF), they can be positive for other antibodies, such as SS-A/Ro of 60 kDa, using other techniques.[2-6]

The study of the IIF technique helps the detection of autoantibodies that are highly specific for SLE, such as anti-native DNA, anti-ribosomal P and anti-Sm.[7-9] Additionally, some of those antibodies (anti-native DNA and anti-ribosomal P) can be markers of disease activity, alternating the titers in moments of exacerbation and clinical remission.[7,9] A prior

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study compared lupus patients with and without antinuclear antibodies in Hep-2 cells on IIF.[10] The authors, evaluating 25 lupus patients without ANA Hep-2 and 91 patients with those antibodies, demonstrated that the cutaneous involvement was uncommon in the group negative for ANA Hep-2. Moreover, the presence of thrombocytopenia, arterial and venous thrombosis, and stroke was more frequent in the group negative for ANA Hep-2. This study demonstrated the existence of a distinct clinical and laboratorial aspect in lupus patients without positive ANA in Hep-2 and also detected a higher frequency of manifestations that are fairly common in APS.

A recent study demonstrated that patients with primary APS (PAPS) and the presence of antinucleosome antibodies could develop lupus.[11] These data led us to undertake the present study whose objective was to evaluate whether the presence of antigens against Hep-2 cells in a population of PAPS patients is capable of identifying a subgroup of patients with clinical or laboratory manifestations that are distinct from those without these antibodies.

PATIENTS AND METHODS

Fifty-eight patients of both genders, aged 18 to 55 years, with a diagnosis of PAPS according to Sapporo criteria, were included in this study. They were treated at the APS outpatient clinic of the Rheumatology Department of Hospital das Clínicas of the Medical School of Universidade de São Paulo, from February to May 2008. The study was approved by the Ethics Commission of the same hospital under protocol #1109/08.

A clinical evaluation followed with data from medical records was undertaken. Arterial and venous events were all confirmed by imaging exams, such as Doppler ultrasound, ventilation-perfusion pulmonary scintigraphy, computed tomography, MRI, arteriography, and angiotomography and angio-MRI. Thrombocytopenia was defined as platelet levels bellow 100.000/mm^3 in at least two consecutive occasions. The presence of comorbidities, such as hypertension (BP ≥ 140 x 90 mmHg or the use of anti-hypertensive medication), dyslipidemia, and drugs used were also evaluated. Blood for laboratory assessment was not drawn, as these tests had been previously carried out as part of the routine follow-up.

Patients with other diseases that lead to secondary APS, such as SLE, rheumatoid arthritis, systemic sclerosis, and Sjögren syndrome, as well as those with silicone implants and/or who were taking drugs that can induce the formation of autoantibodies, such as hydralazine, phenytoin, and others, were excluded from the study. Age was limited to 55 years, as it is known that the prevalence of autoantibodies is higher in elderly individuals.[13]

Detection of autoantibodies

Indirect immunofluorescence (IIF) was used to detect anti-cellular antigens using Hep-2 cells as the substrate.[14,15] Results greater than 1:80 were considered positive. All serum samples were tested by a standardized methodology at the laboratory where this study was undertaken and the results were also confirmed by a commercial kit (Euroimmun, Lubeck, Germany).

The presence of antinative DNA antibodies was also detected by IIF using *Crithidia luciliae*[16] as the substrate, and results ≥ 1:10 were considered positive. Antibodies against extractable nuclear antigens (anti-ENA), specifically anti-U1RNP and anti-Sm, were detected by immunoagglutination using rabbit thymus extract as the substrate (Sigma Chem Co., St. Louis, USA). Anti-Ro/SS-A and anti-La/SS-B were detected by counter-electrophoresis in 1.0% agarose gel with barbital buffer, pH 8.2, using dog spleen extract as antigen.[17,18] Positive results did not show agglutination of erythrocytes visible to the naked eye for anti-ENA, as well as precipitation line for anti-Ro/SS-A and anti/La-SS-B. To characterize precipitating antibodies, reference sera with known antigenic specificities were used.

Detection of antiphospholipid antibody

Anticardiolipin antibodies (aCL) were detected by ELISA.[19] In brief, wells on polystyrene plates were sensitized with cardiolipin (Sigma Chem. Co., USA) at a concentration of 50 µg/mL in cold ethanol (50 µl/well) for 16 hours at 4° C. After washing, the plate was blocked for two hours with inactive fetal bovine serum (56° C for 30 minutes) at 30% in PBS. The wells were sequentially incubated, in duplicate, with diluted sera at 1:50 and goat IgG against peroxidase marked human anti-IgG and anti-IgM (Sigma Chem. Co., USA). Results were expressed in GPL and MPL units determined by a curve plotted from the values of optical density obtained from internationally-defined reference standards. To diagnose the syndrome, values above 20 U were considered positive according to Sapporo criteria.[12]

The presence of lupus anticoagulant (LAC) was detected by functional hematologic tests, initially by screening activated with partial thromboplastin time (aPTT), followed by the dilute Russel's viper venom test (dRVVT) when the aPTT was

negative. When the ratio between the patient's aPTT and the normal control was > 1.2, the test of mixing 50% normal plasma with 50% test-plasma was carried out. If a correction of the aPTT or dRVVT was observed, a deficiency of the coagulation factor was diagnosed. If a correction was not observed, but this was obtained with a mixture with phospholipid-rich plasma (platelets), LAC was diagnosed. This test was performed after verifying whether the INR of the prothrombin time was lower than 3.5; if it was greater, a pre-mixture with normal plasma was done before proceeding with the aPTT.

Statistical analysis

Results were presented as means and standard deviations. The software GraphPad InStat, version 2.0, was used for the statistical analysis. The Student's *t* test was used to compare the means, the Mann-Whitney test to compare medians and the Fisher exact test was used for frequencies. Results were considered significant when $P < 0.05$.

RESULTS

The mean age of all 58 patients with APS at the time of the investigation was 39.0 ± 10.0 years; 48 (82.8%) were females, and 79.3% were Caucasian. The mean duration of the disease was 71.8 ± 58.5 months. As for vascular events, 53.4% of the patients had arterial thrombosis, 51.7%, venous thrombosis, and 31%, obstetric events.

Twenty (34.5%) of 58 patients had a positive ANA Hep-2. The predominant pattern was nuclear homogenous (n = 9), followed by speckled nuclear (n = 7) and the mixed pattern (n = 4). Most patients had titers ≥ 1/320 (n = 10), followed by 1/160 (n = 7) and equal to 1:80 (n = 3) (Table 1).

Characterization of pattern specificity showed that six (30%) of the 20 patients with positive ANA Hep-2 had specific antibodies. One of them was positive for antinative DNA, but with a low titer (1:20) and the analysis of other serum samples of the same patient did not detect this antibody. Two patients were positive for anti-ENA antibodies – one of them was anti-Sm positive and the other anti-U1RNP. Two patients were positive for anti-Ro/SS-A. The diagnostic work-up of dry eyes and dry mouth in these patients was negative. One last patient had low titers of rheumatoid factor in latex fixation test; however, this antibody was negative in the Waaler-Rose test. Anti-P and anti-La/SS-B antibodies were not found (Table 1). Currently, approximately two years after the data collection, all antibody-positive patients are asymptomatic and without evidence of SLE or other connective tissue disease. When we compared patients positive for ANA Hep-2 with those that were negative, significant differences in age (37.1 ± 9.5 *versus* 40.0 ± 10.9 years, P = 0.30), in female gender (95 *versus* 78.9%, P = 0.14), Caucasian ethnicity (85 *versus* 78.9%, P = 0.73), mean weight (60.3 ± 16.8 *versus* 77.0 ± 20.5 kg, P = 0.10), height (158.7 ± 8.8 *versus* 162.3 ± 7.6 cm, P = 0.11), and body mass index (26.8 ± 5.7 *versus* 29.2 ± 7.3 kg/m^2, P = 0.19), were not observed. The duration of the disease (77.7 ± 52.5 *versus* 68.4 ± 61.3 months, P = 0.28) was similar in both groups (Table 2).

Table 1
Twenty ANA Hep-2 positive patients with primary antiphospholipid syndrome, titers of these antibodies, and corresponding specific autoantibodies

Patient	Pattern of positive ANA Hep-2	Titer of positive ANA Hep-2	Specific autoantibodies
1	Nuclear homogeneous	1/160	-
2	Nuclear homogeneous	1/160	Anti-Ro/SS-A
3	Mixed nuclear homogeneous + nuclear speckled	> 1/320	-
4	Nuclear homogeneous	> 1/320	-
5	Nuclear homogeneous	> 1/320	-
6	Nuclear homogeneous	> 1/320	Anti-U1RNP
7	Mixed nuclear homogeneous + nuclear speckled	> 1/320	-
8	Nuclear homogeneous	> 1/320	-
9	Nuclear homogeneous	1/160	-
10	Mixed nuclear homogeneous + nuclear speckled	1/160	Anti-Sm
11	Nuclear speckled	1/80	-
12	Nuclear speckled	1/160	-
13	Nuclear speckled	1/160	-
14	Nuclear speckled	1/80	Positive rheumatoid factor (latex), with Waaler-Rose negative
15	Nuclear homogeneous	> 1/320	-
16	Nuclear speckled	≥ 1/320	-
17	Nuclear speckled	1/80	-
18	Nuclear homogenous	> 1/320	Antinative DNA 1/20
19	Mixed nuclear homogeneous + nuclear speckled	> 1/320	Anti-Ro/SS-A
20	Nuclear speckled	1/160	-

Regarding clinical manifestations, the group positive for ANA Hep-2 had a higher frequency of deep venous thrombosis (85 *versus* 57.9%, P = 0.04). Both groups of patients were similar regarding other clinical manifestations and comorbidities related to the disease, such as arterial, venous, and obstetric events, pulmonary thromboembolism, thrombocytopenia, stroke, Sneddon syndrome, ischemia of the extremities, acute myocardial infarction, angina, hypertension, dyslipidemia, and osteonecrosis. Similarly, the lifestyle, the frequency of physical activity, and past and current smoking status also were similar in both groups (Table 3).

Both groups were also similar regarding the use of the following drugs: corticosteroids (current and past use), chloroquine, warfarin, statins, and acetylsalicylic acid. The use of corticosteroids in patients with PAPS was due to thrombocytopenia, and in two of them for transverse myelitis (Table 4).

The medians of aCL IgG antibodies [33 (0-128) *versus* 20 (0-120) GPL, P = 0.008] and IgM [33 (0-120) *versus* 18.5 (0-120) MPL, P = 0.009] were significantly higher in ANA Hep-2 positive patients than in negative individuals. The levels of aCL did not show a Gaussian distribution and, therefore, the Mann-Whitney test was used. Thus, a higher frequency of aCL IgG (85 *versus* 52.6%, P = 0.02) and a tendency for anticardiolipin IgM (80% *versus* 68.4%, P = 0.05) were observed in ANA Hep-2 positive patients. The presence of lupus anticoagulant was comparable in both groups (Table 5).

Table 2
Comparison of the demographic and anthropometric data and disease duration in ANA Hep-2 positive and ANA Hep-2 negative patients with primary antiphospholipid syndrome (PAPS)

Data	PAPS ANA Hep-2 + n = 20	PAPS ANA Hep-2 - n = 38	P
Age (years)	37.1 ± 9.5	40.0 ± 10.9	0.30
Female, n(%)	19 (95)	29 (76.3)	0.14
Caucasian, n(%)	17 (85)	29 (76.3)	0.73
Weight (kg)	68.3 ± 16.8	77.0 ± 20.5	0.10
Height (cm)	158.7 ± 8.,8	162.3 ± 7.6	0.11
Body mass index (kg/m^2)	26.8 ± 5.7	29.2 ± 7.3	0.19
Disease duration (months)	77.7 ± 52.5	68.4 ± 61.3	0.28

Data are presented as means ± standard deviations or percentages.

Table 3
Comparison of clinical data, cardiovascular events, comorbidities and lifestyle in ANA Hep-2 positive and ANA Hep-2 negative patients with primary antiphospholipid syndrome (PAPS)

Data	PAPS ANA Hep-2 + n = 20	PAPS ANA Hep-2 - n = 38	P
Arterial event, n(%)	9 (45)	23 (60.5)	0.28
Venous event, n(%)	11 (55)	19 (50)	0.79
Obstetric event, n(%)	4 (20)	14 (36.8)	0.24
Stroke, n(%)	6 (30)	17 (44.7)	0.40
Sneddon syndrome, n(%)	3 (15)	4 (10.5)	0.68
Ischemia of extremities, n(%)	2 (10)	6 (15.8)	0.70
Acute myocardial infarction, n(%)	0	3 (7.9)	0.54
Angina, n(%)	0	3 (7.9)	0.54
Deep venous thrombosis, n(%)	17 (85)	22 (57.9)	0.04
Pulmonary thromboembolism, n(%)	5 (25)	9 (23.7)	1.00
Thrombocytopenia, n(%)	4 (20)	3 (7.9)	0.22
Osteonecrosis, n(%)	1 (5)	2 (5.3)	1.00
Hypertension, n(%)	6 (30)	17 (44.7)	0.40
Dyslipidemia, n(%)	4 (20)	11 (28.9)	0.54
Physical activity, n(%)	4 (20)	12 (31.6)	0.54
Current smoking, n(%)	4 (20)	2 (5.3)	0.17
Past smoking, n(%)	6 (30)	16 ((42.1)	0.41

Table 4
Comparison of the frequencies of the drugs used by ANA Hep-2 positive and ANA Hep-2 negative patients with primary antiphospholipid syndrome (PAPS)

Data	PAPS ANA Hep-2 + n = 20	PAPS ANA Hep-2 - n = 38	P
Current use of corticosteroids, n(%)	3 (15)	2 (5.3)	0.73
Past use of corticosteroids, n(%)	9 (23.7)	14 (36.8)	0.58
Warfarin, n(%)	16 (42.1)	30 (78.9)	1.00
Chloroquine, n(%)	6 (30)	6 (15.8)	0.31
Statins, n(%)	5 (13.2)	10 (26.3)	1.00
Current use of acetylsalicylic acid, n(%)	4 (10.5)	13 (34.2)	0.37

Table 5
Comparison of the frequency of antiphospholipid antibodies in ANA HE-p2 positive and ANA Hep-2 negative patients with primary antiphospholipid syndrome (PAPS)

Data	PAPS ANA Hep-2 + n = 20	PAPS ANA Hep-2 - n = 38	P
Lupus anticoagulant, n(%)	19 (95)	31 (81.2)	0.24
aCL IgG, GPL*	33 (0-128)	20 (0-120)	0.008
aCL IgM, MPL*	33 (0-120)	18.5 (0-120)	0.009
Positivity of aCL IgG, n(%)	17 (85)	20 (52.6)	0.02
Positivity of aCL IgM, n(%)	16 (80)	20 (52.6)	0.05
Positivity of aCL IgG or IgM	17 (85)	26 (68.4)	0.22

Data are presented as means ± standard deviation, median*, or percentage. aCL = anticardiolipin.

DISCUSSION

The present study demonstrated a distinct pattern of clinical and laboratory manifestations in patients with PAPS positive for ANA Hep-2 when compared to those without these autoantibodies.

Several clinical conditions can show the presence of ANA Hep-2, being represented, mainly, by diffuse connective tissue diseases and individuals with silicone implants. Thus, those individuals were excluded from the study to guarantee the clinical relevance of the results.[20-14] Similarly, studies in the literature have demonstrated the association of the presence of antinuclear antibodies with older age, including in healthy individuals.[13] Thus, the present study excluded patients older than 55 years.

Studies with a design similar to that of the present study cannot be found in the literature. Studies, such as that of Blanco *et al*.,[25] have demonstrated that the presence of ANA in PAPS can be associated to the subsequent development of SLE. Likewise, Carbone *et al*. demonstrated that the presence of ANA in 33 women with PAPS represented a greater risk of evolution of PAPS into SLE in six patients.[26]

The association found in the present study between ANA Hep-2 positivity and a higher frequency of aCL antibodies cold suggest a polyclonal activation of lymphocytes in APS. This activation has been demonstrated by the presence of at least 30 different types of autoantibodies related to APS.[27]

The higher frequency of deep venous thrombosis in ANA Hep-2 positive individuals had been demonstrated in patients with SLE;[28,29] however, studies demonstrating these findings in patients with PAPS cannot be found in the literature. It is known that SLE patients are at higher risk to develop thromboembolic phenomena and that this risk is higher when antiphospholipid antibodies are present.[28-30] In addition, there seems to be clusters of autoantibodies, such as a combination of aCL, antinative DNA, and LAC, that discriminate a population of SLE patients with a higher risk of thromboembolic events.[28-30]

The present study demonstrated that approximately 1/3 of PAPS patients could be ANA Hep-2 positive and most of these individuals had titers > 1/320. The finding of six patients with specific antibodies, except for rheumatoid factor, at low titers and not confirmed by a more specific test, raises the possibility that these individuals might develop other connective tissue diseases. The study by Arbuckle *et al*. demonstrated the presence of specific antibodies in sera of normal individuals who, after approximately five years, developed SLE.[31]

Thus, the present study showed, for the first time, that ANA Hep-2 positive patients with PAPS had a higher frequency of deep venous thrombosis and aCL antibodies, as well as higher titers of these antibodies.

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