THE STUDY OF ANTIPHOSPHOLIPID SYNDROME IN ALBANIAN POPULATION

Anila Mitre, Katerina Pikuli

Department of Biology, Tirana University, Tirana, Albania

Abstract

Antiphospholipid antibodies (aPL) are a family of autoantibodies with specificity for negatively charged phospholipids, and for their complex to phospholipid binding proteins. Their presence is associated with arterial or venous thrombosis and recurrent pregnancy loss. A combination of genetics with environmental factors are involved in development of APS syndrome. Antiphospholipid syndrome is diagnosed in young to middle-aged adults and is more common in females. There are two broad categories of assays that are used to diagnose APS: coagulation assays which study the effect of LAC on the clotting time and immunological assays, mostly ELISAs to detect IgG and IgM antibodies against cardiolipin, fosfatidilserine and β_2 glycoprotein I. For diagnosis of APS, at least one clinical criterion and one laboratory criteria (Sydney) should be present. From 2016-2019, 120 patients were evaluated with suspect diagnose: Antiphospholipid Syndrome and 94 cases are a control group which represent normal people who does not present health problems. The patients' age varies from 6-60 years. Based on the comparison of the parameters between the control group and the positive individuals, a very large variation of the main values of aPL antibodies was observed in persons with this syndrome. Risk for clinical manifestations appears to be associated with moderate to high levels of these autoantibodies. The most affected individuals belong to the age group 21-45 years and female gender. In this study we came to the conclusion that none of the markers studied is a perfect indicator of Antiphospholipid Syndrome. Lupus anticoagulant is the most sensitive marker in the detection of syndrome and after that β2GPI IgM.

Keywords: *antiphospholipid antibodies, recurrent pregnancy loss, venous and arterial thrombosis, Lupus anticoagulant*

1. INTRODUCTION

The antiphospholipid syndrome (APS) is an acquired autoimmune thrombophilic disorder that is characterized by thrombosis (venous, arterial and microvascular) and obstetric morbidity due to a diverse family of antibodies against phospholipid-binding proteins present in plasma. The term antiphospholipid antibody is actually a misnomer as the antibodies are not against the phospholipid per se, but target the plasma protein co-factors, which bind to anionic PLs [1]. aPL induced cellular activation, inhibition of natural anticoagulant and fibrinolytic systems, and complement activation, among others. Sign and symptoms depend on the place where the clot occur. A combination of environmental and genetics factors is involved in development of APS syndrome. Antiphospholipid syndrome is diagnosed in young to middle-aged adults and is more common in females. There is no cure for APS, but different treatments can help to prevent complications and blood clots [2]. Abortions of woman with APS can be mainly explained by the lack of blood supply to the fetus due to thrombosis or placental infarction.

It is known that placental spiral arteries present an abnormal development in APS. This may be due to an effect on endothelial function, however a PL binds specifically to placental antigens providing a possible mechanism for placental damage and fetal blood supply. Blood circulation blockages in placenta caused by thrombosis weaken fetal growth by not allowing blood exchange between mother and fetus and lead to loss of the fetus. APLs bind to endothelial cells, blood platelets or monocytes by promoting a pro-inflammatory and prothrombotic condition responsible for health conditions [3].

Although the scientific community has come a long way in understanding APS since its first description in 1983, correct and timely diagnosis of this entity still remains a challenge. In clinical practice, patients exist with typical clinical manifestations highly suggestive of antiphospholipid syndrome but remain

persistently negative for criteria aPLs. These patients have been termed seronegative APS (SNAPS) [4]. SNAPS patients, just as classic APS patients, manifest increased risk for thrombotic events and pregnancy morbidities.

There are three types of syndromes: *primary antiphospholipid syndrome* occurs in the absence of any other related disease, *secondary antiphospholipid syndrome* occurs with other autoimmune diseases and *catastrophic antiphospholipid syndrome (CAPS)* is defined as a "thrombosis storm" which multiple blood clots occur over a short period.

Two broad categories of assays are used to diagnose APS: coagulation assays which study the effect of LAC on the clotting time and immunological assays, mostly ELISAs to detect IgG and IgM antibodies against cardiolipin, phosphatidyl serine and/or β_2 glycoprotein I. For diagnosis of APS, at least one clinical criterion and one laboratory criteria (Sydney) should be present. The original APS "Classification Criteria" included only anti-cardiolipin (aCL) IgG/IgM and lupus anticoagulant (LA) as accepted laboratory criteria [5] . In 2006, the sensitivity of the classification criteria was improved by the inclusion of aβ2GPI IgG/IgM antibody. Still, in clinical practice, patients exist with typical clinical manifestations highly suggestive of antiphospholipid syndrome but remain persistently negative for criteria aPLs. Anti-phosphatidylserine/prothrombin antibodies (aPS/PT) are now acknowledged as a highly effective potential marker for APS [6]. In the current study, LAC and six promising antibodies namely aCL IgM/IgG, aβ2GPI IgM/IgG, aPS IgG/IgM were selected for evaluation.

2. THE STUDY AIM

To evaluate the diagnostic performance of Lupus anti-coagulant (LAC) and antibody profiles, anti-β_2 glykoprotein I (antiβ_2GPI) IgG/IgM, anti-cardiolipin (aCL) IgG/IgM and anti-phosphatidil serine (aPS) IgG/IgM antibodies in a group of Albanian patients classified with Antiphospholipid Syndrome.

3. MATERIAL AND METHODS

The first step of this study was the agreement with the clinics of rheumatology, cardiology and neurology in the University Hospital Center "Mother Teresa", with the University Hospital for Obstetrics and Gynecology "Queen Geraldine" and with Obstetric Gynecology Clinic "Gliozheni" in Tirana. The cases were collected from those centers and then analyzed in the medical laboratory "Intermedica" in Tirana. Diagnosis and classification of antiphospholipid syndrome were based on the 2006 International revised criteria. All patients included in the study had at least 1 clinical criterion for antiphospholipid syndrome positive.

Recommendations for the selection of groups were made by the respective clinician, and further participation in the study was voluntary. Cases with questionable diagnosis have excluded from the study. All the subjects provided written inform consent. Selection criteria for the sample in the study was: volunteering to complete all clinical tests. Before performing the tests, each patient filled in a form with data which were used in this study. Thrombotic events and pregnancy morbidities (PM) were recorded. Thrombosis was classified into arterial thrombosis and venous thrombosis, while pregnancy morbidities were divided into early and late pregnancy. Early pregnancy morbidity referred to at least one fetal loss before 10 weeks. The occurrence of stroke and deep vein thrombosis was also recorded.

None of the patients included in the study received anticoagulants. From 2016-2019, were evaluated 120 patients with suspect diagnose: Antiphospholipid Syndrome and 94 cases are a control group represent normal individuals who does not present health problems. The patient's age varies from 6-60 years. Patients have manifest various symptoms for a period of more than 12 weeks, or unexplained abortions in the absence of drug therapies. Patients are allowed to rest for 10 minutes in a room before taking blood samples. For each patient were taken three blood tubes, two for plasma extraction (2x2.5 ml Na Citrate 3.2%Tube) and one for serum. Blood is drawn into the vein with the help of specialized staff for this type of intervention. Plasma was prepared by two centrifugations. One Vial was centrifuged at 1500r.p.m and other at 3000 r.p.m for 10 min at room temperature and stored at −70°C in suitable

aliquots. The tube without anticoagulant is left to coagulate for 20 minutes at room temperature and then is subjected to centrifugation at 3200 rpm and stored at -20º and -80º C until use. Plasma will be tasted for lupus anticoagulant, while the serum will be used to test antibodies. Indirect ELISA with ELISA Euroimmun equipment and kite are used as techniques to detect specific antibodies. The tests that has been performed and the reference levels in the patients are: Lupus anti-coagulant Test (Clotting <43U/ml); Anti-Cardiolipin Antibody Test (ELISA Euroimmun aCL<12U/ml); Anti β_2-Glycoprotein I Test (ELISA Euroimmun β_2-GPI<20 RU/ml); Anti-Phosphatidylserine (aPS) Test (ELISA Euroimmun <12RU/ml).

4. RESULTS AND STATISTICAL ANALYSIS

After receiving the results for the patients and the control group, the data were arranged in specific tables. The IBM SPSS Statistic 20 program was used to analyze and present the results of this study. From the total of 120 individuals, 96 of them are females and 24 males. (Chart 1).

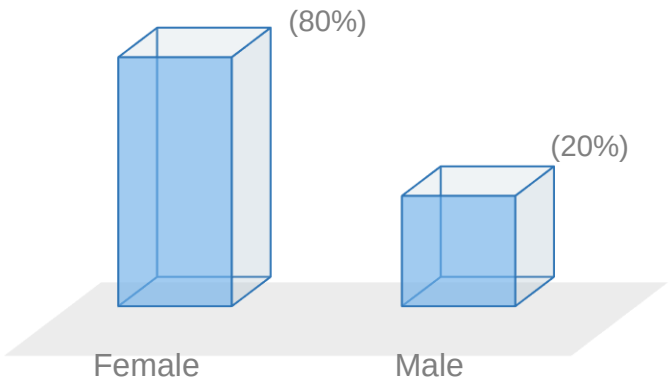


Chart 1. Gender repartition of the study population

The influence of age in the presence of aPL antibodies

At first, we test the normality of the distribution using a Kolmogorov-Smirnov test. From the results we find $p = 0.301, p > 0.05$ which leads us in the concluding that we have a normal distribution for the groups we are comparing. In order to find out whether the difference between variables is significant the parameter test ANOVA One Way was used. The values found from the test were the following:

$$p = 0.044, p < 0.05$$

Based on these values we it can be concluded that the group distribution is significantly different. *Hypothesis H_1 : Age influences the presence of aPL antibodies* Based on the literature and results obtained from data processing, it can be affirmed that the presence of the APS syndrome is influenced by age. Patients from the age group 21-45 years old were the ones who were the ones diagnosed the most frequently.

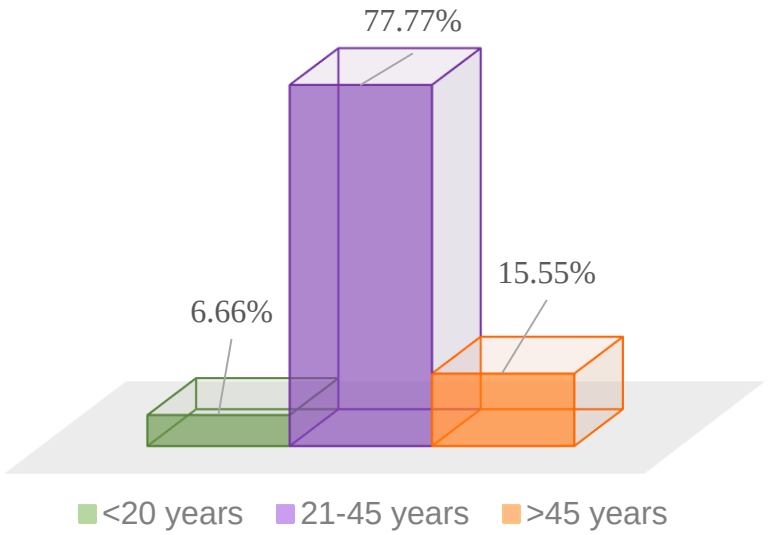


Chart 2. The influence of age on aPL antibody levels

The relationship between values of LAC in plasma and the age of patients' part of the study, From data processing the following results were obtained:

$$F(1;35) = 6.606\ (statistical\ value)$$

$$Equation\ Y = a + bX;\ Y = 31.30 + 11.15X$$

$$p = 0.024, p < 0.05$$

From these values we validate H_0:

Hypothesis H_0: The level of LAC is influenced by age, mainly in women between 21 to 45 years old

$$r^2 = 0.383$$

This goes on to show that age has an influence of 15% in the levels of LAC

$$Spearman\ coefficient\ \rho = 0.619$$
$$p = 0.016, p < 0.05$$

The value for this coefficient reinforces hypothesis H_1, which states that age and LAC levels are correlated.

As we have pointed out, APS is an autoimmune disorder characterized by thrombosis, fetal loss associated with thrombocytopenia (low platelet count) and increased number of aPL antibodies (LAC, β_2-GPI, aPS and aCL). Medical tests are a very effective way to diagnose APS Syndrome. Tests are important, but they are not 100% effective to diagnose the APS. There are a number of factors or other diseases that affect the level of antibodies causing false tests results.

In the Table 1 shows the difference in numerical values in our sample, the minimum, maximum and mean of antibody values. In individuals diagnosed with APS it is observed that the concentration of antibodies is much higher than in normal ones. Below is shown the difference in the distribution of antibody values between the patients and control group for the syndrome. The high level of these antibodies serves as an indication for diagnosis. The serum levels of different aPLs are significantly elevated in the APS patients compared with healthy controls.

Table 1. Summary of antibody values for patients vs. control group

Individuals	Patients			Control group		
(U/ml)	*Mean*	*Minimum*	*Maximum*	*Mean*	*Minimum*	*Maximum*
PTTLA	46.33	33.7	74.5	39.74	34.85	46.85
IgM Anti-$\boldsymbol{\beta_2}$GPI	26.26	8.9	83.1	11.88	3.5	34.6
IgG Anti-$\boldsymbol{\beta_2}$GPI	19.12	5.8	58.7	12.00	3	29.5
IgM Anti-Cardiolipin	14.41	3.7	80.74	7.23	2.3	24.5
IgG Anti-Cardiolipin	10.87	4.2	28.6	8.04	2.29	23.46
IgM Anti-Phosphatidyl Serine	13.98	5.3	41.1	8.15	3.01	23.32
IgG Anti-Phosphatidyl Serine	10.65	2.1	25.1	9.27	3.5	25.71

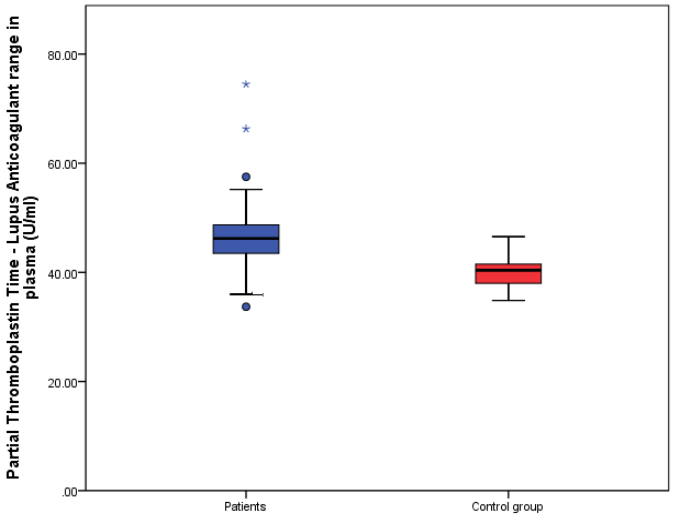


Chart 2. PTT-LA range in plasma for patients and control group individuals.

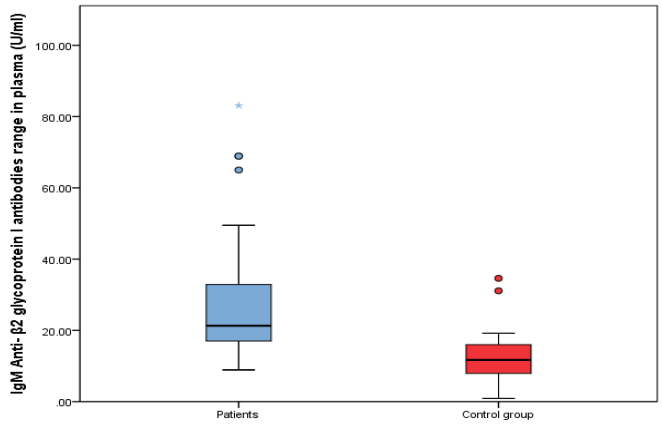


Chart 3. IgM Anti-β_2GPI range in plasma for patients and control group individuals.

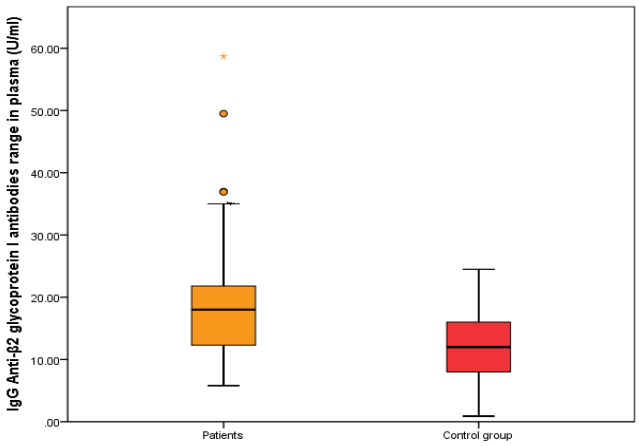


Chart 4. IgG Anti-β_2GPI antibodies range in plasma for patients and control group individuals.

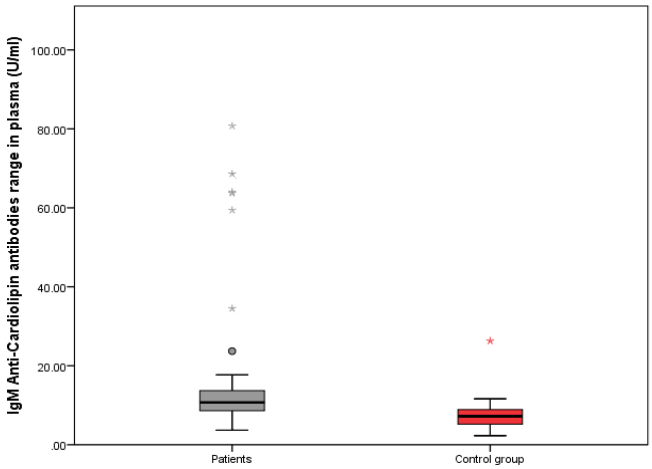


Chart 5. IgM Anti-Cardiolipin range in plasma for patients and control group individuals

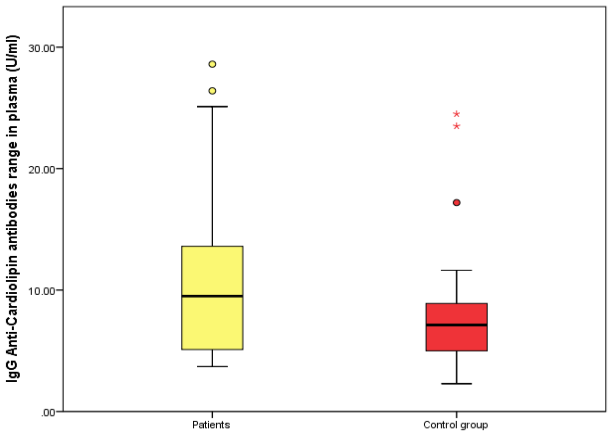


Chart 7. IgG Anti-Cardiolipin range in plasma for patients and control group individuals

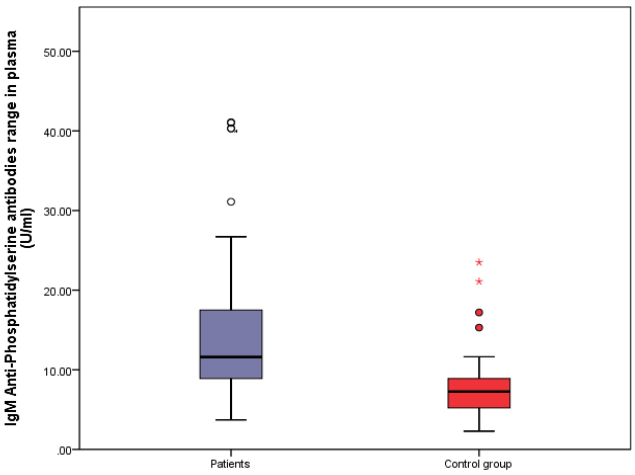


Chart 8. IgM Anti-Phosphatidylserine antibodies range in plasma for patients and control group individuals.

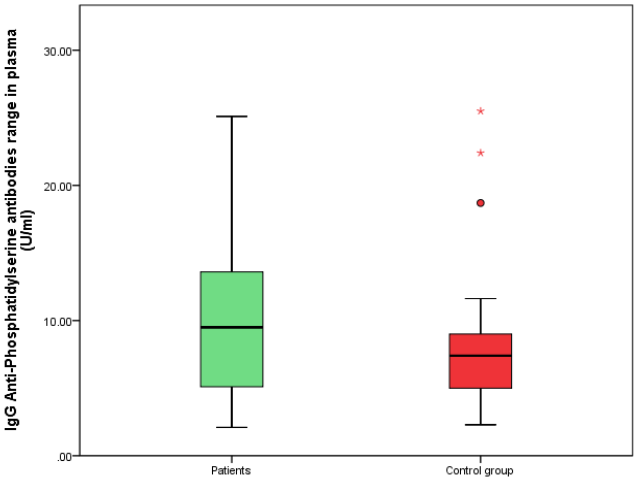


Chart 9. IgG Anti-Phosphatidylserine antibodies range in plasma for patients and control group individuals.

Antiphospholipid syndrome is a new syndrome discovered in recent years and presents difficulty in identification. We emphasize that the causes of formation are complex, ranging from genetic factors, others autoimmune disease, infections, medications, cholesterol, high blood pressure etc.

From the obtained data, false negative and positive tests were calculated and based on them it was possible to determine the sensitivity and specificity [7]. To make a comparison of tests which help to diagnose this syndrome we build ROC curves for each test based on the relevant data. These ROC curves quite efficiently assess the quality of diagnostic tests. Also, these graphs are made through sensitivity and specificity values.

One of the most important measurements, related to the ROC curve is the AUC coefficient, which evaluates the overall performance of a diagnostic test by combining sensitivity and specificity. The AUC coefficient value varies from 0-1. The closer to value 1, the better the test performance is. The data obtained through diagnostic tests were used to calculate the sensitivity and specificity which are statistical measurements of the quality of a solvent.

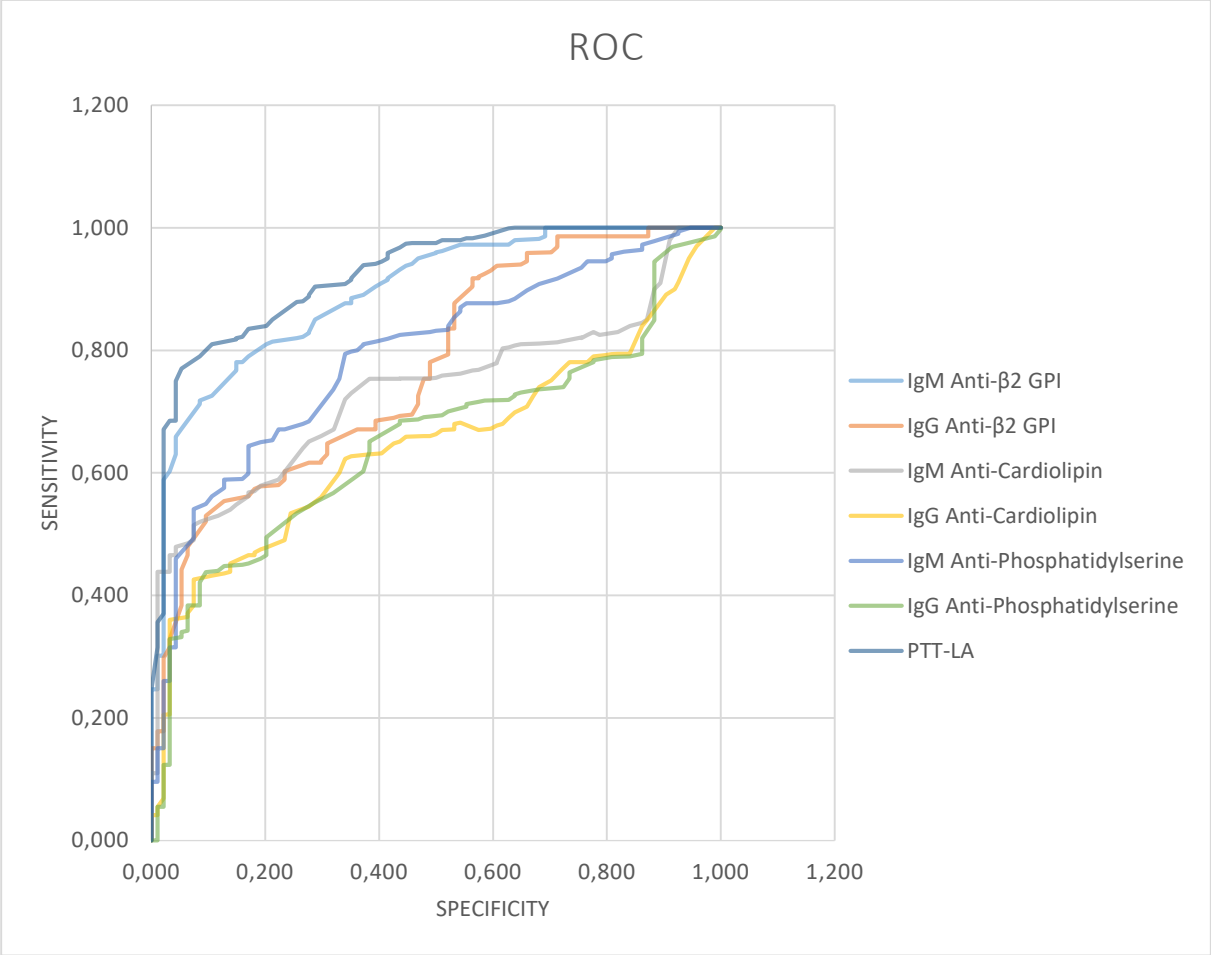


Chart 10. ROC Curves

Table 2. Results from data processing

	AUC	Sens	Spec
PTT-LA	0.896	0.781	0.989
IgM Anti-β_2GPI	0.854	0.589	0.978
IgG Anti-β_2GPI	0.773	0.315	0.968
IgM Anti-Cardiolipin	0.732	0.438	0.989
IgG Anti-Cardiolipin	0.661	0.356	0.968
IgM Anti-Phosphatidylserine	0.793	0.479	0.957
IgG Anti-Phosphatidylserine	0.660	0.328	0.968

Following data processing the Lupus anti-coagulant (LAC) showed the largest area under the curve (AUC, 0.896), followed by β_2-GPI IgM (AUC, 0.854). The value of specificity is very significant for all the tests used in the study. More precisely the tests with the highest sensitivity values are: PTT-LA 0.781; IgM Anti-β_2GPI 0.589; IgM Anti-Phosphatidylserine 0.793; IgG Anti-β_2GPI 0.773; IgM Anti-Cardiolipin 0.732; IgG Anti-Cardiolipin 0.661; IgG Anti-Phosphatidylserine 0.660.

5. DISCUSSION

The presence of phospholipid antibodies may be indicated by the detection of lupus anticoagulant (LAC). From our study the level of lupus anticoagulant is most specific and sensitive marker. LAC has been more consistently associated with thrombosis in both venous and arterial circulation, as compared to aCL and β2GPI-ELISA [8]. In the obstetric population also, LAC has been found to have a stronger association with recurrent pregnancy loss than other antibodies [9]. About 65% of individuals in our group have had recurrent pregnancy loss and so we can explain the high level of Lupus anticoagulants (LA). LA assays are not very analytically sensitive, i.e. a relatively high concentration of antibodies is required to prolong the clotting time in these tests. Thus, the presence of LA indicates a high titer of aPL and this, rather than the intrinsic functional characteristics of LA antibodies, may explain the high risk of clinical manifestations associated with LA [1]. The antigenic specificity of autoantibodies responsible for lupus anticoagulation includes prothrombin and β_2-glykoprotein I. β_2-glykoprotein I is important marker of antiphospholipid autoimmune antibodies. b2GPI is a PL-binding apolipoprotein which exists as open S type or closed loop conformation. The b2GPI structure is divided into five domains. In the closed type, cryptic epitope on domain 1 is hidden preventing autoantibodies (aAbs) from binding. When b2GPI is immobilized on negatively charged PL surfaces via domain V, it changes to open form-opening the cryptic domain1 to which the aAbs bind. It is the epitope 1 of b2GPI which is the target in APS patients [10]. Antibodies to β2GPI-D1, showed a close relationship with pregnancy morbidities, which is in accordance with a recently published retrospective study of obstetric patients [11].

In our study we found that IgM anti- β_2GPI is the second most important marker in signaling APS. Our data are consistent with those from the Italian study [12] in which it is stated that "approximately two-thirds of those with isolated IgM anti-B2GPI had obstetric manifestations of APS, perhaps indicating a possible association of IgM anti-B2GPI and this particular subtype of APS". By contrast, several other studies have reported a stronger association between IgG anti-B2GPI and clinical manifestations of APS compared with IgM anti-B2GPI, which has cast considerable doubt on the role of IgM anti-B2GPI in the first-line assessment of APS [13]. Another study looked at the ability of anti-B2GPI isotypes to stratify thrombotic risk in patients with lupus anticoagulant, and found that those with IgG anti-B2GPI had a higher incidence of thrombosis whereas there was no association for IgM anti-B2GPI [14]. aCL testing as a diagnostic criterion for APS is currently under debate. However, exclusion of aCL testing can lead to failure to diagnose APS in a small fraction of patients. It can be used as a screening test and the results should be read in conjunction with the other diagnostic tests**.** Anti-phosphatidylserine/prothrombin antibodies (aPS/PT) are now acknowledged as a highly effective potential marker for APS [14]. As a result of its remarkable diagnostic performance and high prevalence in the LA-positive patient group, as validated by multiple studies [15]. aPS/PT has gained much attention and was included in APS for patient thrombotic risk assessment [16] [17].

6. CONCLUSION

In the present study, we chose seven criteria for aPLs, namely the PTTLA, aCL IgG and IgM, aβ2GPI IgG and IgM, aPS/PT IgG and IgM. All seven of these markers can be detected in a considerable proportion of the APS patients and could help physicians to identify patients with an elevated risk of thrombosis and pregnancy morbidity. Patients female from the age group 21-45 years old were the ones who were diagnosed the most frequently. Lupus anticoagulant is the most sensitive and specific marker in the detection of syndrome. The second important marker is IgM anti- β_2GPI, indicating a possible association of IgM anti-B2GPI and obstetric manifestations of APS.

REFERENCES

1. Mallhi, J. S., Kushwaha, N., Chatterjee, T. & Philip, J., 2016. Antiphospholipid syndrome: A diagnostic challenge. *Med J Armed Forces India*, December, pp. 31-36.
2. Lockshin, M., Samaritano, L. & Schwartzman, S., 2000. Validation of the Sapporo criteria for antiphospholipid syndrome. *Arthritis Rheum*, Issue 43, pp. 440-443.
3. Peaceman, A. & Rehnberg, K., 1993. The effect of immunoglobulin G fractions from patients with lupus anticoagulant on placental prostacyclin and thromboxane production. *Am J Obstet Gynecol*, Volume 169, pp. 1403-1406.
4. Hughes, G. & Khamashta, M., 2003. Seronegative antiphospholipid syndrome. *Ann Rheum Dis*, December, 62(12), p. 1127.
5. Wilson, W. et al., 1999. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop. 42(7), pp. 1309-1311.
6. Sciascia, S. et al., 2014. Anti-prothrombin (aPT) and anti-phosphatidylserine/prothrombin (aPS/PT) antibodies and the risk of thrombosis in the antiphospholipid syndrome. A systematic review. Thromb Haemost, 111(2), pp. 354-364.
7. Mathai, A., Parikh, S., Sekhar, G. C. & Thomas, R., 2008. Understanding and using sensitivity, specificity and predictive values. Indian J Ophthalmol, Jan-Feb, 56(1), pp. 45-50.
8. de Groot, P. et al., 2005. Lupus anticoagulants and the risk of a first episode of deep venous thrombosis. J Thromb Haemost, Volume 3, pp. 1993-1997.
9. Opatrny, L., David, M., Kahn, S. & Shrier, I. R. E., 2006. Association between antiphospholipid antibodies and recurrent fetal loss in women without autoimmune disease: a metaanalysis. J Rheumatol, Volume 33, pp. 2214-2221.
10. Cucnik, S. et al., 2004. High avidity anti beta2-glycoprotein I antibodies in patients with antiphospholipid syndrome. Ann Rheum Dis, Volume 63, pp. 1478-1482.
11. Chighizola, C. et al., 2018. Beyond thrombosis: anti-beta2GPI domain 1 antibodies identify late pregnancy morbidity in anti-phospholipid syndrome. J Autoimmun, Volume 90, p. 76–83.
12. Tincani, A. et al., 2010. Antiphospholipid antibody profile: Implications for the evaluation and management of patients.. Lupus, Volume 19, pp. 432-435.
13. Lakos, G. et al., 1999. Isotype distribution and clinical relevance of anti-beta2-glycoprotein I (beta2-GPI) antibodies: Importance of IgA isotype.. Clinical and Experimental Immunology,, Volume 117, pp. 547-579.
14. Hoxha, A. et al., 2017. Antiphosphatidylserine/prothrombin antibodies as biomarkers to identify severe primary antiphospholipid syndrome. Clinical Chemistry and Laboratory Medicine, 55(6), pp. 890-898.
15. Shi, H. et al., 2018. Antiphosphatidylserine/prothrombin antibodies (aPS/PT) as potential diagnostic markers and risk predictors of venous thrombosis and obstetric complications in antiphospholipid syndrome. Clinical Chemistry and Laboratory Medicine (CCLM),, 56(4), pp. 614-624.
16. Sciascia, S. & Bertolaccini, M., 2014. Thrombotic risk assessment in APS: the Global APS Score (GAPSS). Lupus, 23(12), pp. 1286-1287.
17. Litvinova, E. et al., 2018. Prevalence and significance of non-conventional antiphospholipid antibodies in patients with clinical APS criteria. Front Immunol, Volume 9, p. 2971.