# PRIMARY ANTIPHOSPHOLIPID SYNDROME

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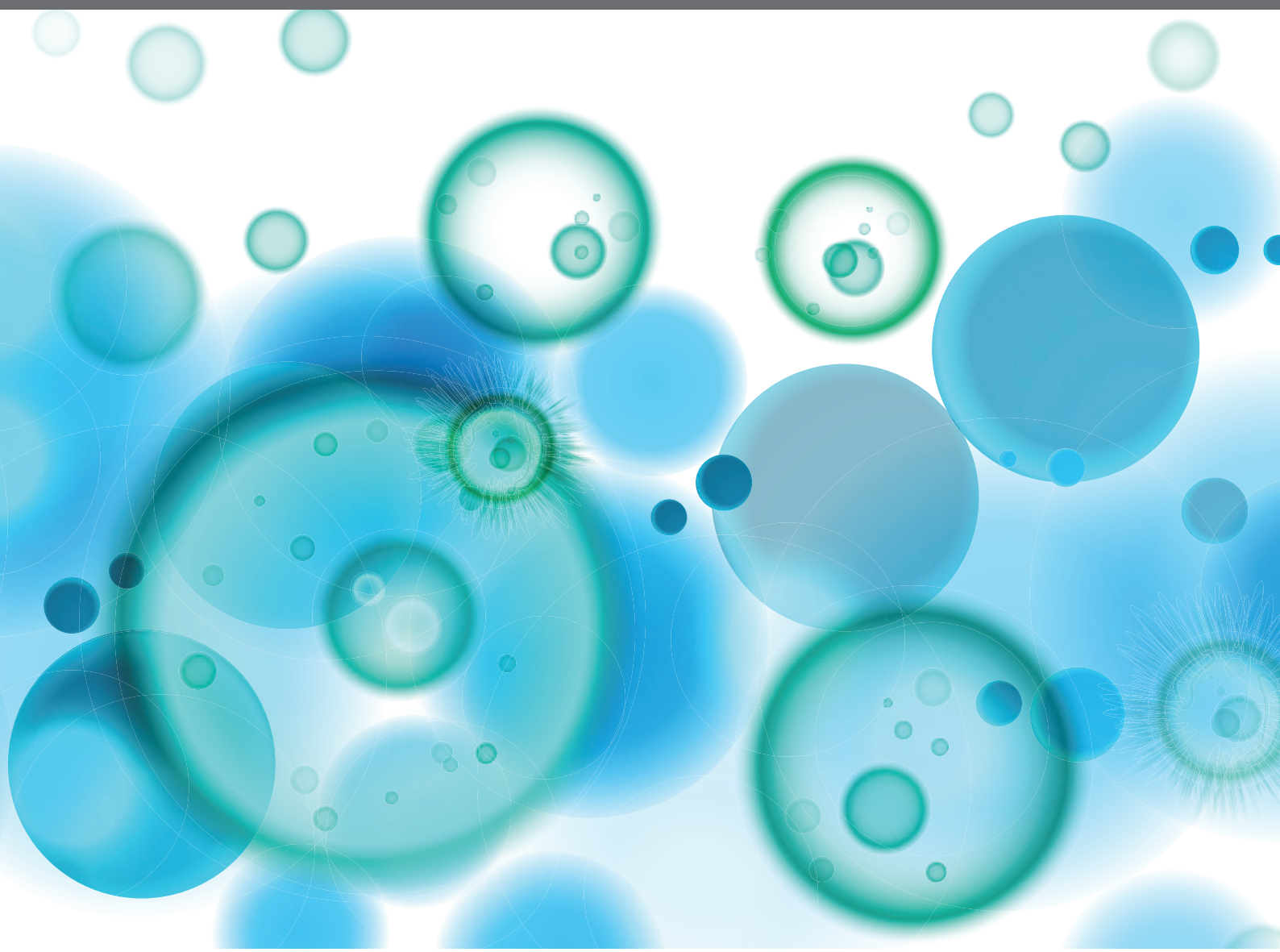

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# PRIMARY ANTIPHOSPHOLIPID SYNDROME

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EDITORIAL
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# Editorial: Primary Antiphospholipid Syndrome

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**Keywords: PAPS, APS, thrombosis, B2GP1, PS/PT, cardiolipin**

**Editorial on the Research Topic**

**Primary Antiphospholipid Syndrome**

The antiphospholipid syndrome (APS) was described in 1983 as a systemic autoimmune disease characterized by the presence of thrombotic events or gestational morbidity in people carrying antiphospholipid antibodies (aPL). Although the disease was mainly detected in patients who already suffered from other autoimmune diseases, such as systemic lupus erythematosus (SLE), it was soon perceived that people with APS who did not suffer from other autoimmune diseases constituted a clearly differentiated clinical entity -the so called primary APS (PAPS) (1–3).

In recent years the interest of the scientific community on the APS has markedly grown. This e-book brings together basic, translational, and clinical research studies as well as reviews on the current situation of this disease.

The articles can be grouped into three blocks: New APS-associated biomarkers, pathogenesis and clinical associations, and reviews about specific aspects of the APS.

## NEW BIOMARKERS

In addition to patients with well-diagnosed APS, there are also asymptomatic people carrying aPL who never had an APS event (4). Conversely, there are patients with clear clinical APS criteria who are negative for the aPL included in the classification criteria, but may be positive for other aPL (5). It is very important to identify people at real risk to suffer APS events; therefore, the discovery of new biomarkers for diagnosis and monitoring the disease is an important aspect in this e-book.

López-Pedrera et al. address genomic/epigenetic changes related to the clinical profile of patients with APS and its modulation due to the effect of specific therapies.

Non-consensus anti-phosphatidylserine/prothrombin (aPS/PT), anti-domain 1 and anti-β2-glycoprotein I (aβ2GPI) antibodies of IgA isotypes have been discussed in several articles. McDonnell, Artim-Esen et al. determine the IgG subclass distribution for anti-domain1 and aβ2GPI antibodies. Litvinova et al. describe the potential value of aPS/PT antibodies as a strong marker of APS and propose that anti-PS/PT antibodies could be a surrogate APS biological marker of the lupus anticoagulant (LA) in patients whom LA detection cannot be achieved.

Sciascia et al. describe up to 45% of overall discrepant results for LA—and even higher in patients on vitamin K antagonists—and propose that the introduction of aPS/PT testing might represent a further diagnostic tool, especially when LA is not available or their results are uncertain.



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Serrano, Martinez-Flores et al. describe that antibodies of patients with thrombotic APS and IgA anti-β2GPI isolated positivity do not bind to domain 1 of β2GPI but bind other sites on domains 3 and 4 of β2GPI previously described as thrombosis-related epitopes.

Other non-aPL serum markers have been also studied. Manganelli et al. describe that serum levels of HMGB1 and sRAGE in APS patients are significantly increased when comparing to healthy subjects, highlighting that patients with APS and recurrent abortion showed significantly higher levels of sRAGE.

Serrano, Morán et al. studied patients with transplant-associated APS and show that pretransplant presence of circulating immune complexes predicts which aPL positive patients are at risk of thrombosis or early mortality after heart transplantation.

## PATHOGENESIS AND CLINICAL ASSOCIATIONS

In two articles a differential phenotype for the cells of the immune system that circulate in the blood is described in patients with APS. Lonati et al. describe that the percentage of blood cells with C4d in the membrane was significantly higher in patients with APS than in aPL negative controls, suggesting an important role of complement activation in APS. Álvarez-Rodríguez et al. found a less inflammatory profile in patients with PAPS that in SLE with higher levels of FoxP3 mRNA expression and reduced presence of circulating Th17 cells.

Palli et al. describe variations in type I interferon (INF) signature in APS patients. Type I IFN score is increased in PAPS and correlated positively with anti-β2GPI antibodies and negatively with hydroxychloroquine use.

Pérez et al. describe that the presence in the blood of APS patient of immune complexes of IgG/IgM bound to β2GPI is strongly associated with thrombocytopenia, leukopenia and complement consumption. In addition, these patients present non-criteria APS clinical manifestation, such as livedo reticularis and dry eyes.

McDonnell, Willis et al. propose a new therapeutic alternative using a PEGylated domain I of β2GPI pegylated. In laboratory assays, pegylated is capable of neutralizing IgG aβ2GPI of APS patients blocking its coagulopathic and thrombogenic properties.

## REVIEWS

Two revisions are also included in this topic. Kolitz et al. review APS cardiac damage, including treatment recommendations for each cardiac complication and Chaturvedi et al. review the role of complement in the pathophysiology of the APS and the use of its modulation in the treatment of catastrophic APS and thrombotic microangiopathy.

We hope that this collection of articles will help readers to better understand the characteristics of the APS. The progressive improvement of our knowledge about the origin, pathogenesis and mechanisms involved in the damage generated by the disease, together with the incorporation of new markers that help us to identify risks and assess the disease's staging, will help us to improve our therapeutic management and, ultimately, to help our patients in a more effective way.

## AUTHOR CONTRIBUTIONS

AS, RC, and JG had a substantial contribution to the conception of the work, drafted the work, revised it critically and approved it for publication. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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# Antiphospholipid Antibodies to Domain I of Beta-2-Glycoprotein I Show Different Subclass Predominance in Comparison to Antibodies to Whole Beta-2-glycoprotein I

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Antiphospholipid antibodies (aPL), the serological hallmark of antiphospholipid syndrome (APS), are a heterogeneous group of autoantibodies raised against circulating blood proteins. Of these proteins, the phospholipid-binding b2-glycoprotein I (β2GPI) is considered to be the main autoantigen in APS. Indeed, IgG antibodies targeting b2GPI (ab2GPI) directly cause both thrombosis and pregnancy morbidity in several mouse models. While antibodies raised against all five domains of b2GPI have been reported, a subgroup of IgG ab2GPI raised against the first domain (DI) of b2GPI (aDI), strongly correlate with thrombotic APS, and drive thrombosis and pregnancy loss *in vivo*. Few studies have focused on determining the type of IgG subclass(es) for aPL. The subclass of an antibody is important as this dictates the potential activity of an antibody; for example, IgG1 and IgG3 can fix complement better and are able to cross the placenta compared to IgG2 and IgG4. It is unknown what subclass IgG aDI are, and whether they are the same as ab2GPI. To determine IgG subclass distribution for ab2GPI and aDI, we purified total IgG from the serum of 19 APS patients with known ab2GPI and aDI activity. Using subclass-specific conjugated antibodies, we modified our established in-house ab2GPI and aDI ELISAs to individually measure IgG1, IgG2, IgG3, and IgG4. We found that while IgG1, IgG2, and IgG3 ab2GPI levels were similar, a marked difference was seen in IgG subclass aDI levels. Specifically, significantly higher levels of IgG3 aDI were detected compared to IgG1, IgG2, or IgG4 ($p < 0.05$ for all comparisons). Correlation analysis of subclass-specific ab2GPI vs. aDI demonstrated that IgG3 showed the weakest correlation ($r = 0.45$, $p = 0.0023$) compared to IgG1 ($r = 0.61$, $p = 0.0001$) and IgG2 ($r = 0.81$, $p = 0.0001$). Importantly, total subclass levels in IgG purified from APS and healthy serum ($n = 10$ HC $n = 12$ APS) did not differ, suggesting that the increased IgG3 aDI signal seen in APS-derived IgG is antigen-specific. To conclude, our data suggests that aDI show a different

IgG subclass distribution to ab2GPI. Our results highlight the importance of aDI testing for patient stratification and may point toward differential underlying aPL-driven pathogenic processes that may be subclass restricted.

**Keywords: antiphosholipid antibodies, IgG 3, Antiphospholid syndrome, domain I, Beta 2 glycoprotein**

## INTRODUCTION

Antiphospholipid Syndrome is an autoimmune rheumatic disorder with an estimated population prevalence of between 0.3 and 1% of the population (1), with a female predominance. Often presenting in association with systemic lupus erythematosus (SLE, secondary APS), APS is the commonest cause of acquired hypercoagulability, accounting for one in six strokes in patients under 50 years old (2), one in nine heart attacks (3), and associated with an almost 3-fold greater risk of atherosclerosis, even in the absence of SLE (primary APS), compared to matched controls (4).

Antibodies play a key role in the pathogenesis of APS. The accepted international classification criteria for the disease (5) require positive tests for antiphospholipid antibodies (aPL) on at least two occasions, at least 12 weeks apart. There are three different tests routinely used to detect aPL in clinical practice. Two of these are enzyme-linked immunosorbent assays (ELISAs) for IgG and IgM antibodies to cardiolipin (aCL ELISA) or β2 glycoprotein I (aβ2GPI ELISA), while the third is a functional test that detects the effect of aPL on clotting time in the presence or absence of excess phospholipid (the lupus anticoagulant or LA test). aCL and aβ2GPI antibodies are found in various isotypes. Though only IgG and IgM measurements are defined in the classification criteria (5), there is increasing interest in the role played by IgA aPL. Despite this, the IgG isotype best correlates with clinical events in patients (6–8) and is the most often studied *in vitro* and *in vivo* (9–14).

There is extensive evidence from clinical studies and mouse models of both thrombosis and pregnancy loss (15–19) that IgG aPL play a direct pathogenic role in APS. However, not all aPL are equally pathogenic. McNeil et al (20) showed that non-pathogenic aPL found in healthy people could bind both neutral and anionic PL without the need for a co-factor, whereas aPL from patients with APS showed preferential binding to anionic PL but required the presence of the serum co-factor β2GPI. Subsequently, it was shown that these pathogenic aPL could bind β2GPI in the absence of PL. β2GPI has five domains, and multiple studies have shown that antibodies to the N-terminal domain (Domain I or DI, anti-DI antibodies or aDI) are most closely linked to development of thrombosis (12, 13, 21–24). Our group has shown that when IgG from patients with APS is fractionated into aDI-rich and aDI-poor fractions by affinity purification on a DI column and the fractions used to stimulate thrombosis in a mouse model of APS, the thrombogenic potential is concentrated in the aDI fraction (25). In the same mouse model, we also showed that recombinant DI can block induction of thrombosis by IgG from patients with APS IgG antibodies are produced in four subclasses: IgG1, IgG2, IgG3, and IgG4 (26). Each of these subclasses has different avidities, affinities and abilities, for example IgG1 and IgG3 can cross the placenta whilst IgG2 and IgG4 cannot. They also have different structures, with IgG3 displaying a far longer hinge region than the other subclasses. Their ability to interact with Fc receptors and their affinity for those receptors also differs, whilst their alternative structures lend themselves to different kinetics in serum, with IgG3 displaying a range of half-lives (27). In SLE, there is literature showing that IgG antibodies to key autoantigens are concentrated in certain subclasses, with confounding results in some cases. For example, Zahir et al. (28) studied 120 SLE patients and showed that IgG3 anti-nucleosome antibodies were present at high levels in active but not inactive SLE, rose during flares of disease activity and showed a particularly close association with nephritis. In contrast, Ravirajan et al. (29) showed that in 31 patients with SLE, anti-nucleosome were predominantly IgG2, anti-dsDNA antibodies were IgG1 and IgG3, and anti-heparan sulfate were IgG2 and IgG3. There is also a precedent for investigating IgG subclass distribution in APS with studies examining the subclass distribution of IgG aCL (30, 31), which seemingly appear to be of all four subclasses but with a potentially pathogenic predominance for IgG2 and IgG4. Previous research had suggested that IgG1 or IgG2 predominate for aβ2GPI (32, 33), however, no-one has investigated the subclass distribution of patient antibodies for IgG aDI subclasses.

This led us to test whether the more pathogenic aDI subgroup of aβ2GPI has a different subclass distribution compared to aβ2GPI as a whole.

## METHODOLOGY

### Patients and Controls

Serum was obtained from 19 patients with APS and 5 healthy controls (HC). Serum samples were collected by informed consent following local institutional ethical approval from 19 patients from 2 centers: University College London [London, UK], University of Istanbul [Istanbul, Turkey]. All 19 patients fulfilled the revised classification criteria for APS (5).

### IgG Purification

Polyclonal IgG was purified from serum of patients and healthy controls as follows. Serum was diluted with physiological phosphate buffer (pH 7.4) and run through a protein G sepharose column (Pierce) before elution with 0.1 M Glycine (pH 2.7) and neutralization with 1 M Tris buffer. Eluted IgG was desalted and dialysed in physiological phosphate buffer (pH 7.4) using a centrifugal concentrator. Samples were quantified by BCA (Pierce).

## Establishing an ELISA Method for Direct Comparison of IgG Subclass Levels Using Optical Density (OD) Units

Determination of the appropriate conditions for detecting all four IgG subclasses was carried out using our in-house whole IgG ELISA. Briefly, anti- human IgG (Sigma, I8885) is coated at 400 ng/ml on half a maxisorp plate, the other half coated with PBS alone. Samples are diluted in PBS, plates are washed 3 times before application of sample with PBST (0.1%). Secondary antibody (anti-human IgG, HRP Conjugated) was applied at 1:1000 and incubated for 1 h at room temperature. Substrate was warmed to room temperature and applied to the plate post washing (100 μl) for 30 min. Plates were stopped with acid and read at 450 nM. Specificity for subclass was achieved using subclass specific HRP conjugated secondary antibodies (Sigma). Secondary antibody concentrations were determined using a serum standard of known subclass composition. All secondary antibodies were diluted to give OD values matching the expected outcome from the known serum.

Goat anti-human IgG against the Fc portion (I8885, Sigma) was used to coat maxisorp plates (400 ng/ml) overnight at 4°C. Plates were blocked with 2% BSA/PBS for 1 h at 37°C, and the commercial serum sample loaded at a dilution of 1:100000 in 1% BSA/PBS for 1 h at room temperature (RT). Detection was carried out using specific mouse anti-human secondary antibodies at a range of values from 1:1000 to 1:80000 for 1 h at RT. Optimal concentrations were as follows: anti-IgG1 at 1:1000, anti-IgG2 at 1:2000, anti-IgG3 at 1:10000 and anti-IgG4 at 1:80000. Plates were washed and substrate added and developed in the dark for 10 min. The reaction was stopped with 1% HCl and plates were read at 450nm.

## Measurement of Total IgG1, IgG2, IgG3, and IgG4 in Purified IgG Samples

In order to be sure that differences between samples in the amount of each IgG subclass binding to β2GPI or DI were indeed antigen-specific findings and due to biasing of an individual subclass, we carried out an ELISA to measure total IgG1, IgG2, IgG3, or IgG4. In essence, this assay was the reverse of our whole IgG ELISA, as described above. Four separate lanes of a maxisorp plate were coated with mouse anti-human IgG1, IgG2, IgG3, or IgG4 respectively. After blocking, purified IgG samples from patients and controls were diluted to 500 μg/ml in 1% BSA/PBS and added to the plates, followed by incubation for 1 h at 37°C. Anti-human IgG secondary antibody conjugated to HRP (A6029, Sigma) was added for 1 h at 37 °C. Substrate was added for 10 min, the reaction was stopped with 1% HCL and OD was read at 450nm. We compared OD values obtained from 10 HC and 12 APS patients.

## aβ2GPI Subclasses ELISA

To measure IgG subclass specific aβ2GPI, we employed our in-house aβ2GPI ELISA (35). Plates were prepared by coating overnight with 4 μg/ml of commercial purified human β2GPI at 4°C (Enzyme Research Laboratories). Plates were then blocked for 1 h at 37°C with 2% BSA/PBS. Purified IgG samples from patients and controls were prepared in 1% BSA/PBS at a protein concentration of 500 μg/ml. Samples were applied to the plate for 1 h before washing with PBS Tween (0.01%) and subclass specific secondary antibodies (as established above) applied. Secondary antibodies were diluted to the levels determined against the calibrant material. Incubation with secondary antibody was for 1 h before washing and application of substrate for 15 min, followed by addition of 1% HCl to stop the reaction. Samples were read at 450 nm. Binding for the individual subclasses was compared. For each patient, all four secondary antibodies against the four IgG subclasses were tested on the same plate.

## aDI Subclasses ELISA

Similarly to the aβ2GPI assay, we utilized our in-house aDI ELISA to measure IgG subclass specific aDI (34). The assay was performed in exactly the same manner as per our aβ2GPI ELISA, with the exception of the DI plates being coated with 10 μg/ml of folded, conformationally correct DI for 2 h at 37°C. For each patient, all four secondary antibodies against the four IgG subclasses were tested on the same plate.

## Statistical Analysis

Statistical analysis was carried out using Prism V5.0. We performed a 1 way ANOVA with a Kruskal-Wallis post test to determine significant differences between IgG subclass distributions. Linear regression analysis was carried out for correlating subclasses between the two antigens, β2GPI and DI.

# RESULTS

Samples from 19 different patients with APS were tested. Their clinical and serological characteristics are shown in **Table 1**. Twelve patients with APS had a history of vascular thrombosis (VT) only, four had experienced pregnancy morbidity (PM) only, one had VT and PM and two had catastrophic antiphospholipid syndrome (CAPS).

## The Pattern of IgG Subclass Detection Is Different for aβ2GPI Compared to aDI

All 19 APS samples tested positive for aβ2GPI, 18 of which were positive for aDI. The mean activity for all samples is shown in **Table 1**.

**TABLE 1 |** This table shows the characteristics of the patients and healthy controls included in the study.

| | APS | HC |
|---|---|---|
| Age | 36.8 (11.8) | 33.3 (8.5) |
| Sex | 12F, 6M | 3F 1M |
| aβ2GPI | 77.7 (30.4) | – |
| aDI | 45.19 (35.9) | – |
| Other ARD | 11 | – |
| LA | 13 | – |

*Mean age, aβ2GPI and aDI levels are shown, with standard deviations in parentheses. aβ2GPI positivity cut off: 8 GBU, aDI positivity cut off: 10 GDIU.*

**Figures 1A,B** show the relative percentages of subclass for each antigen (1A, aβ2GPI and 1B, aDI). This was calculated by expressing the OD for each subclass as a percentage of the cumulative OD generated from all four subclasses, for each individual patient. The mean OD, as well as the average percentage of cumulative binding for all four IgG subclasses in both aβ2GPI and aDI assays can be seen in **Table 2**.

IgG4 was the lowest detected subclass in both aβ2GPI and aDI ($p < 0.05$ across all comparisons). In aβ2GPI assays, the IgG1 subclass was the most predominant, followed by IgG2 and IgG3, with no statistically significant difference between these three subclasses (**Table 2**, **Figure 1A**, $p > 0.05$ across all comparisons). In aDI assays however, the percentage of IgG3 was significantly higher than both IgG1 and IgG2 (**Table 2**, **Figure 1B**), demonstrating that in our patients, aβ2GPI and aDI can be of a different subclass. The average percentage of cumulative binding for all patients for each IgG subclass is summarized as a pie distribution chart in **Figure 2** (**2A**, aβ2GPI and **2B**, aDI). Raw OD values can be seen in the **Figure S1**.

Analysing the patients individually, 12/18 patients positive for aDI had the majority of their antibody binding specific to the IgG3 subtype. In contrast, only 4/18 had the highest for IgG1. In the aDI assay, over 50% of the cumulative total OD was seen in the IgG3 subtype for 9/18 patients, and 4 of these patients showed over 75% of their cumulative antibody binding to be of the IgG3 subclass. Comparatively, only 1/18 showed >50% of the cumulative binding for IgG1 subclass (51%) and no patients showed >50% of cumulative antibody binding for either IgG2 or IgG4. In contrast, when analyzing the aβ2GPI subclass pattern, only 3/19 patients had >50% antibody binding for IgG3 whilst 7/19 has >50% cumulative antibody binding for IgG1, and one patient showed >50% cumulative antibody binding in IgG2.

Correlation analysis of each IgG subclass for the two antigens revealed strong correlations between aβ2GPI and aDI of the same subclass for: IgG1 ($r = 0.61$, **Figure 3A**), IgG2 ($r = 0.81$, **Figure 3B**) and IgG4 ($r = 0.85$, data not shown) The difference is highlighted in IgG3 where the correlation is much lower ($r = 0.45$, **Figure 3C**), reflecting the increased level of IgG3 aDI antibodies compared to the IgG3 aβ2GPI antibodies.

When stratified by clinical phenotype (thrombosis vs. pregnancy morbidity), the results were very similar across all IgG subclasses, with no clear association between a single subclass and clinical history (data not shown). However, this may be confounded by low patient numbers.

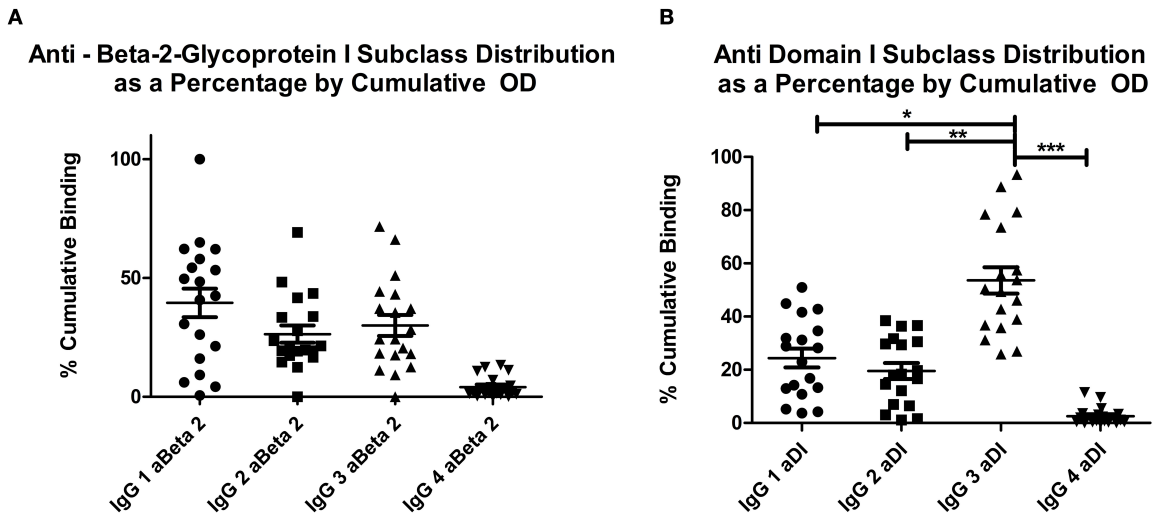


**FIGURE 1 | (A)** shows the distribution of OD as a percentage of cumulative OD for aβ2GPI antibodies, **(B)** shows the same measure for aDI antibodies. The increase in IgG3 percentage can be seen in **(B)**, this was significantly higher than any other subclass. Both panels show IgG4 significantly lower than any other subclass for both antigens. *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. IgG4 vs. IgG1, IgG2 or IgG3 in both **(A)** and **(B)** = $p < 0.05$.

**TABLE 2 |** This table contains the raw OD and the average percentage of the cumulative OD for each subclass against both antigens.

| | aβ2GPI | | aDI | |
|---|---|---|---|---|
| | **Mean OD (±*SD*)** | **Average % of cumulative binding (±*SD*)** | **Mean OD (±*SD*)** | **Average % of cumulative binding (±*SD*)** |
| IgG 1 | 0.6 (±0.7) | 39.5 (±26.0) | 0.3 (±0.6) | 24.3 (±14.9) |
| IgG 2 | 0.31(±0.27) | 26.4 (±15.7) | 0.2 (±0.3) | 19.5 (±12.7) |
| IgG 3 | 0.37 (±0.35) | 29.9 (±19.1) | 0.45 (±0.4) | 53.5 (±21.0) |
| IgG 4 | 0.07 (±0.15) | 4.1 (±4.5) | 0.02 (±0.04) | 2.5 (±3.3) |

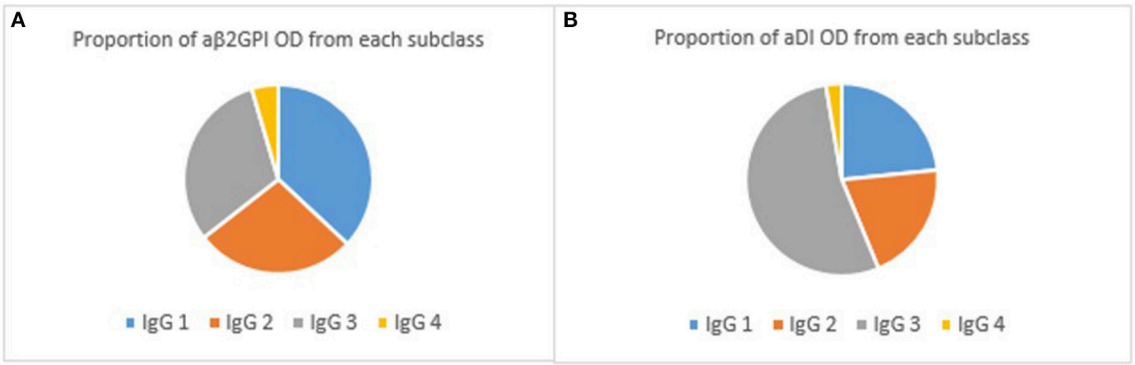


**FIGURE 2 | (A)** shows the proportion of aβ2GPI OD associated with each subclass. As can be seen, IgG1 predominates in the aβ2GPI assay whilst IgG2 and IgG3 are similar. **(B)** shows the same proportions for aDI subclasses. It is clear here the predominant subclass is IgG3.

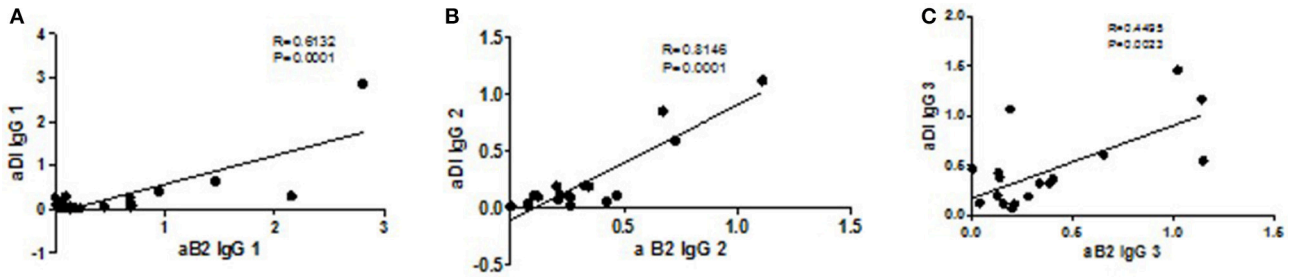


**FIGURE 3 |** Correlations of subclasses between antigens. The strongest correlations are seen for IgG1 (**A**, $p = 0.0001$, $r = 0.61$), IgG2 (**B**, $p = 0.0001$, $r = 0.81$) and IgG4 ($p = 0.0001$, $r = 0.85$; data not shown). The weakest correlation is seen with the IgG3 subclass (**C**, $p = 0.02$, $r = 0.45$) reflecting the difference between the antigens in the IgG3 distribution.

## Total Levels of Each IgG Subclass Were Similar in Patients With APS Than Controls

To exclude the possibility that the high IgG3 aDI levels in patients with APS were simply a reflection of over-production of total IgG3, we measured total levels of each subclass in 10 healthy controls and 12 patients with APS. As can be seen in **Figure 4**, there are no significant differences between the two groups for total IgG 2, IgG 3 and IgG 4, thus underlining that the high IgG3 aDI levels we detected in patients are indeed antigen-specific. IgG 1 was significantly different between APS and HC, with lower levels in HC ($p < 0.045$) however, removal of the outlier in the HC group (1.53) loses the significance.

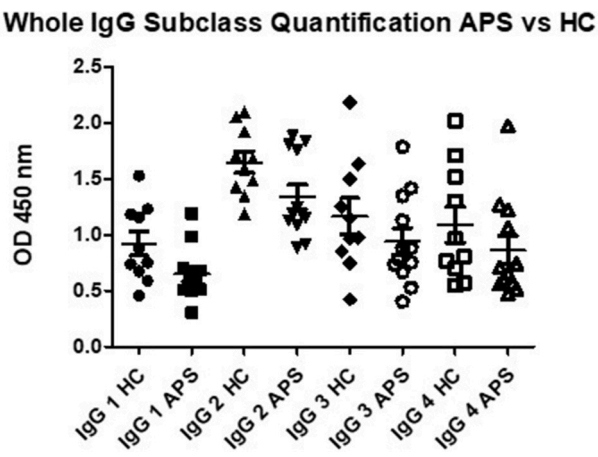


**FIGURE 4 |** Total capture IgG ELISA results demonstrate that there are no significant differences in OD for IgG 2, IgG 3 and IgG 4 subclass in comparison of APS patients to healthy controls (HC). Subclass IgG 1 shows significantly lower levels in APS, however, removal of the outlier in the HC group shows no significant difference between APS and HC.

## CONCLUSIONS

Our data suggests that antibodies to DI are of a different subclass to antibodies against whole β2GPI. In our cohort, antibodies to DI are predominantly IgG3, whilst antibodies to β2GPI are more prominently IgG1.

Our results conflict with previous data, which suggested an IgG2 class restriction for aβ2GPI antibodies. These studies however, were 20 years ago and our ability to characterize subclasses of IgG has improved, as has the methodology for detecting antibodies to both DI and β2GPI. The production of subclass specific monoclonal antibodies has improved and aβ2GPI antibody assays have been developed and refined in that

time, although no consensus on the source of antigen exists. Another possibility for the discrepancy between our results and others', is that by selecting for patients who are both aβ2GPI and aDI positive, we have isolated a patient population with less overall IgG2 aβ2GPI and an overall different serological profile. We knowingly selected patients with positivity to both β2GPI and DI, allowing us to directly compare the subclasses detected in the two assays. The dominance of IgG1 and IgG3 subclasses is interesting as these are heavily involved in complement activation, particularly IgG3, and may offer an insight to the mechanisms of pathogenesis in APS. Interestingly, a difference in subclass between these antibodies may imply several mechanisms are active in APS patients. The IgG subclasses differ in their affinity to receptors, their half lives and their potential activity *in vivo*.

It was expected that the aβ2GPI and aDI subclasses would be similar, as β2GPI contains the aDI epitope, however, the results are quite disparate. This would suggest that the aβ2GPI assay does not necessarily detect all circulating aDI antibodies, and indicates a clinical utility for the aDI assay in patients. Indeed, recommendations for development, standardization and application of aDI assays in APS have existed for some years now, first highlighted at the 14th International Congress for Antiphospholipid Antibodies (35). Of note, we reported good qualitative and quantitative agreement between our IgG aDI ELISA and the USDA approved chemiluminescent assay for aDI, developed by Inova Diagnostics (36). Despite this, it is possible that the structure or presentation of the protein on the plate may have influenced the results presented. Ideally this would be repeated in the fluid phase to confirm binding to subclasses.

To guard against bias from abnormally high or low IgG subclass concentrations, patient antibodies were tested along with healthy controls the results of which showed similar proportions of IgG subclass antibodies between patients and controls. It could be argued that the use of another irrelevant antigen would aid in proving that the effect seen is not just a skewing of antibody production, however, we felt that (a) given the aDI and aβ2GPI subclasses differed, and (b) similar quantification was seen in the total IgG subclass ELISA, that this was not required.

To conclude, our findings suggest that IgG aDI are of a different subclass to aβ2GPI. While unexpected, our results may demonstrate IgG subclass switching of aβ2GPI to aDI, or vice versa, and thus epitope restriction or spreading respectively. Importantly, our study highlights the potential for aDI testing to stratify patients. Further studies are required to validate these findings in larger patient cohorts. The ability of IgG1 aβ2GPI vs. IgG3 aDI to exert pathogenic biological effects also warrants investigation, as autoantibody skewness to specific IgG subclasses may indicate an association of these antigen-specific antibodies with clinical outcomes.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of London Hampstead Research Ethics Committee Ref No 12/LO/0373 with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the London Hampstead Research Ethics Committee Ref No 12/LO/0373.

## AUTHOR CONTRIBUTIONS

The project was conceived by TM and CP. BA-E supplied patients, CW, DI, CP, IG, AR, and VR gave intellectual advice. CW also provided data for the review process.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02244/full#supplementary-material

**Figure S1 |** Panel **(A)** shows aβ2GPI levels as raw OD for each of the subclass specific secondary antibodies. **(B)** mirrors the aβ2GPI assay in an aDI assay.

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**Conflict of Interest Statement:** TM, CP, IG, RA are inventors on a patent for Domain I in the US.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# PEGylated Domain I of Beta-2-Glycoprotein I Inhibits the Binding, Coagulopathic, and Thrombogenic Properties of IgG From Patients With the Antiphospholipid Syndrome

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APS is an autoimmune disease in which antiphospholipid antibodies (aPL) cause vascular thrombosis and pregnancy morbidity. In patients with APS, aPL exert pathogenic actions by binding serum beta-2-glycoprotein I (β2GPI) via its N-terminal domain I (DI). We previously showed that bacterially-expressed recombinant DI inhibits biological actions of IgG derived from serum of patients with APS (APS-IgG). DI is too small (7 kDa) to be a viable therapeutic agent. Addition of polyethylene glycol (PEGylation) to small molecules enhances the serum half-life, reduces proteolytic targeting and can decrease immunogenicity. It is a common method of tailoring pharmacokinetic parameters and has been used in the production of many therapies in the clinic. However, PEGylation of molecules may reduce their biological activity, and the size of the PEG group can alter the balance between activity and half-life extension. Here we achieve production of site-specific PEGylation of recombinant DI (PEG-DI) and describe the activities *in vitro* and *in vivo* of three variants with different size PEG groups. All variants were able to inhibit APS-IgG from: binding to whole β2GPI in ELISA, altering the clotting properties of human plasma and promoting thrombosis and tissue factor expression in mice. These findings provide an important step on the path to developing DI into a first-in-class therapeutic in APS.

**Keywords: antiphospholipid syndrome, PEGylation, domain I, therapeutics, biologics**

## INTRODUCTION

Antiphospholipid syndrome (APS) is an autoimmune disease in which antiphospholipid antibodies (aPL) interact with phospholipid/protein complexes in the body to cause vascular thrombosis and/or pregnancy morbidity (1). The exact population prevalence of APS is unknown but it has been estimated that aPL may contribute to 6.1% of cases of pregnancy morbidity, 13.5% of

strokes, 11.5% of myocardial infarctions, and 9.5% of deep vein thromboses (DVT) (2). The only evidence-based treatment to prevent recurrent thrombosis in patients with APS is long-term anti-coagulation, generally with warfarin (3) though more recently new oral anticoagulants such as rivaroxaban have been used (4). Utility of these new oral anticoagulants however, is not yet fully established in treatment of thrombotic APS. In APS pregnancies, the standard of care remains daily treatment with oral aspirin plus subcutaneous heparin (5). Unfortunately recent data from the TRAPS trial has been disappointing with thrombosis rates in patients on Rivaroxaban being significantly higher (19 vs. 3%) (6). There is currently no therapeutic agent that targets the biologic actions of aPL specifically.

The key autoantigen targeted by pathogenic aPL is beta-2-glycoprotein I (β2GPI) (7), which consists of five domains [Domain I to Domain V (DI to DV)]. The N-terminal DI carries the key epitope recognized by aPL (8, 9). It is believed that the aPL-β2GPI complex interacts with the surface of target cells by means of binding between DV and negatively charged phospholipids on the cell membrane (10). This interaction leads to activation of cell surface receptors and intracellular signaling cascades causing changes in cellular behavior that promote thrombosis and/or pregnancy morbidity (10). Activation of target endothelial cells (ECs), monocytes and platelets by aPL induces the upregulation of several proinflammatory cytokines, key among them being tissue factor (TF) (11, 12). In our mouse thrombosis model utilizing knockout mice, we have previously shown that aPL-mediated increases in inflammatory cytokines, particularly TF levels, correlate with increased thrombus formation *in vivo* (13).

Our hypothesis is that an agent containing DI alone could inhibit binding of the pathogenic aPL to whole β2GPI (14). We have developed a method of expressing recombinant human DI in bacteria and have optimized this method to obtain high yield and purity of DI (15). We and others previously showed that recombinant DI inhibits binding of polyclonal IgG from patients with APS (APS-IgG) to whole human β2GPI (8, 9). We have also shown that recombinant DI inhibits thrombosis induced by human APS-IgG in a murine model (16). In these experiments, we tested wild-type DI as well as two variants created by site-directed mutation. One variant, with mutations of aspartic acid to serine at position 8 and glycine at position 9 (D8S,D9G) retained the ability to block both binding and thrombogenic properties of APS-IgG (16). We therefore decided to pursue the development of both wild-type DI and DI (D8S,D9G) as potential therapeutic agents for APS.

The small size (7 kDa) of DI would lead to a short half-life *in-vivo*, making it unsuitable as a therapeutic agent. There are a number of different ways to modify small biological molecules to improve half-life. These include addition of an immunoglobulin Fc region, as with the TNF blocking drug etanercept, and the chemical addition of polyethylene glycol groups (PEGylation). PEGylation has been used to develop a number of drugs used in rheumatic diseases notably pegloticase for gout and certolizumab pegol for rheumatoid arthritis [reviewed in (17)]. The advantages of PEGylation include improved half-life and reduced immunogenicity but potential disadvantages include loss of biological efficacy due to the large PEG group(s) blocking interaction of the protein with its physiological ligand (18–21). Larger PEG groups enhance half-life but reduce activity (19, 20, 22, 23) thus it is important first to assess activity of different PEGylated variants of a protein before going on to select a specific PEG size as successful development of a PEGylated therapeutic relies on efficiently balancing these two opposing conditions.

Site-directed PEGylation on disulfide bonds enables control of both the number and location of the PEG groups added to a protein, so that the properties of the PEGylated product are predictable and reproducible (24). DI has two disulfide bonds making it suitable for this method. The ideal product is monoPEGylated DI because two separate PEG groups attached to the same DI molecule would probably interfere with the ability of aPL to bind to their epitope (25–27).

In this paper we describe the PEGylation of both recombinant wild-type DI (WT-PEG-DI) and the D8S,D9G variant [PEG-DI (D8S,D9G)], and the characterization of their chemical and biological properties. We include a comparison of variants carrying PEG molecules of different sizes to facilitate future selection of a lead product to take forward to pharmacokinetic testing.

## METHODS

A full description of the methods, including extended details of pH, media, buffers, and mouse experiments is included in **Supplementary Material**.

### Expression of Recombinant DI and DI(D8S,D9G) in Bacteria

Production was carried out as described previously (15). *E. coli* BL21* cells were transfected with the recombinant DI plasmid and expression of DI was achieved by adding 1 mM IPTG followed by incubation with shaking overnight at 20°C. The bacteria were dissolved in lysis buffer, sonicated, and centrifuged to collect inclusion bodies containing the protein of interest. Inclusion bodies were dissolved and ground using a pestle and mortar into a chaotropic buffer before sonication (50% intensity, 50% cycles, 8 min) to increase solubilization. The expression plasmids are designed such that a nickel-binding hexahistidine tag is present at the N-terminal end of expressed DI, separated from it by a site for the protease Factor Xa (FXa) (15). The expressed protein from the solubilized inclusion bodies was therefore purified on a nickel column, re-folded in 0.6 M arginine buffer with a cysteine redox buffer (pH 8.5) and dialysed against 20 mM Tris, 0.1 M NaCl, pH 8. Protein was again purified post-folding using a nickel column and dialysed against phosphate buffered saline (PBS).

### PEGylation

Protein was reduced at a concentration of 0.4 mg/ml in 2 M arginine, 20 mM sodium phosphate (NaPO4, 0.1 M NaCl), 40 mM EDTA at pH 8.0 with 0.1 M DTT for 1 h at 20°C. This process was followed by removal of the reductant and buffer exchange on a PD-10 column to an identical buffer with 25 mM arginine rather than 2 M. PEGylation reagent was added (1:0.8

molar ratio) and incubated for 4 h at 4°C. This solution was then buffer exchanged to 20 mM sodium acetate with 0.05% Tween at pH 6.0 for cation exchange purification on a 5 ml SP-HP column (GE Healthcare) with a linear gradient from 20% buffer containing 1 M NaCl to 100% of the same buffer at 2 ml/min for 1 h. Fractions containing protein of the expected size of PEG-DI were identified by peaks on a chromatogram at 280 nm and then pooled. The hexahistidine tag was cleaved using FXa as in McDonnell et al. (15). Quantification from this point onwards was by BCA for both PEGylated and non-PEGylated form, thus 20 μg of WT-DI contains the same amount of DI as 20 μg WT-PEG-DI.

## Quantification

All concentrations of constructs were quantified using the BCA method. This method measures only the protein component of the construct (i.e., DI) and is unaffected by PEG. The concentrations expressed are based on protein concentration of the PEGylated constructs. This method was chosen as the form of quantification as previous work showed UV quantification was biased by the presence of a ring group in the PEGylation reagent. Thus, when we refer to 20 μg PEG-DI, we mean 20 μg of DI within the PEG-DI construct. Therefore, the PEGylated and non-PEGylated constructs contain the same amount of antigenic sites.

## Chemical Characterization

Proteins were characterized for purity by reverse phase high performance liquid chromatography (RP-HPLC) using a C8 column with a linear gradient between 2% Acetonitrile (AcN), 0.05% trifluoroacetic acid (TFA), and 100% AcN 0.065% TFA. Proteins were also characterized by SDS PAGE for size.

## Production of APS-IgG Samples From Serum of Patients

The clinical and serological data for the patients are shown in **Table 1**. IgG was purified from serum of these patients by passing these samples down a protein G column (Pierce). Eluted IgG was neutralized with 500 μl of 1 M Tris solution. Samples were then dialysed against PBS and total IgG content was quantified using a BCA assay.

## Competitive Inhibition Anti-β2GPI ELISA

This ELISA was carried out as described previously (15). In brief, serum was diluted 1:50 in PBS/1% bovine serum albumin (BSA) and tested for binding in an anti-β2GPI ELISA. For each serum sample, the dilution level giving 50% of maximum binding in this assay was selected for use in the inhibition assay. Samples at this dilution were incubated with varying concentrations of a DI construct for 2 h at room temperature then tested again in the anti-β2GPI ELISA. The results were plotted as "% Binding remaining" on the y-axis against concentration of inhibitor on the x-axis where "% Binding remaining" = (Binding in presence of inhibitor)/(Binding in absence of inhibitor) × 100.

**TABLE 1 |** Clinical and serological data for patients with APS whose samples were used in these experiments.

| | APS patients |
|---|---|
| Number | 18 |
| Age | 49.5 (±12) |
| Female | 13 |
| Male | 5 |
| Caucasian | 17 |
| Asian | 1 |
| SLE/APS | 8 |
| VT | 11 |
| VT PM | 6 |
| CAPS | 1 |
| LA | 17 |
| Mean aCL (GPLU) | 64 (*SD* 36) |
| Mean aB2GPI (GBU) | 54 (*SD* 44) |
| Mean aDI (GDIU) | 45 (*SD* 41) |
| aDI positive | 10 |
| aβ2GPI positive | 12 |
| aCL positive | 13 |

*Patient data for those used to test for activity for D8S,D9G and WT protein. Note that cut-off for positivity in the aCL assay is >17GPLU, in the β2GPI assay it is >8GBU, and for DI positivity it is >10 GDIU. All cut off values were derived from 200 healthy patients.*

## Modified Direct Russell Viper Venom Test (dRVVT)

Although APS is characterized by increased clotting caused by aPL, one of the tests used in clinical practice to detect aPL in serum is called the lupus anticoagulant (LA) test. The rationale of this test is that when clotting is triggered *in-vitro* using a reagent containing dilute Russell Viper Venom, the effect of adding aPL from a patient with APS is to inhibit clotting, prolonging the clotting time. This effect is reversed by adding an excess of phospholipids. The result is therefore expressed as the ratio of dRVVT-stimulated clotting time of the patient's plasma in the absence of phospholipid (LA-sensitive reagent or LS) to the clotting time in the presence of phospholipid (LA-resistant reagent or LR). A ratio above 1.1 suggests the presence of a circulating inhibitor of coagulation. This ratio was devised based on healthy control data, previously published studies and is suggested in the manufacturer kit insert.

We modified the dRVVT to test whether our PEG-DI products inhibits prolongation of the dRVVT clotting time caused by APS-IgG. APS-IgG samples were added to commercially available healthy human plasma at a concentration of 500 μg/ml for 15 min at 37°C before testing in the modified dRVVT assay. Those APS-IgG that gave LS/LR ratios >1.1 were used for inhibition assays. APS-IgG was incubated with inhibitor (DI or PEG-DI) at a 1:1 molar ratio with 50 μl of plasma for 15 min at 37°C. This mixture was then added to 350 μl of plasma and re-incubated for 15 min before testing for an LA-like effect. The outcome measure was ratio of clotting times seen in the presence of LS and LR reagents (LS/LR ratio). Reduction in this LS/LR ratio in the presence of DI or PEG-DI signified an

inhibitory effect on the prolongation of the modified dRVVT caused by addition of APS-IgG. In control experiments, we used octreotide (a kind gift from Dr. Kozakowska of PolyTherics) or albumin (Sigma Aldrich) instead of DI or PEG-DI to exclude a non-specific effect of adding extraneous proteins to this assay.

## Passive Transfer Mouse Model

The method was as described in previous papers (16). Male CD-1 mice ($n = 5$ per group) (Charles River Laboratories, Wilmington, MA) between 6 and 8 weeks in age were injected intraperitoneally (IP) with 500 μg in 1 ml APS-IgG in (all injections from a single preparation of IgG) and then 30–60 min later with either DI, PEG-DI conjugate or PBS control. IgG was isolated from a Caucasian 37 year old female APS/SLE patient with a history of pregnancy morbidity and venous thrombosis, serum levels of aCL, aβ2GPI, and aDI were 114 GPLU, 361 GBIU, and GDIU, respectively. IgG aβ2GPI levels were measured in the purified IgG as 132 SGU by INOVA Quantalite ELISA assay, aDI levels previously measured were 60 GDIU (at 100μg/ml). Negative control mice were injected IP with 500 μg in 1 ml normal healthy serum (NHS)-IgG. All materials had endotoxin levels below 1.5 EU/ml. These injections were repeated after 48 h and the thrombogenicity of aPL was assessed at 72 h after the first injection. At this time, mice were anesthetized and one of the femoral veins was exposed for observation with an approximate 0.5 mm segment trans-illuminated using a microscope equipped with a closed-circuit video system. The isolated vein segment was pinched to introduce a standardized injury and thrombus formation and dissolution was visualized and recorded. The treatment groups were as follows: APS-IgG + PBS control, NHS-IgG Alone, APS-IgG + 40 μg non-PEGylated WT-DI, APS-IgG + 40 μg non-PEGylated DI (D8S,D9G), APS-IgG + 40 μg 20 kDa PEGylated DI, APS-IgG + 40 μg 20 kDa PEGylated DI (D8S,D9G), APS-IgG + 20 μg 20 kDa PEGylated DI, PEG alone, and APS-IgG + PEG.

Three outcome measures were assessed, as fully described in previous papers and in Supplemental Data (16). These were:

a) Induced thrombus size: A total of three thrombi were generated in each mouse and the largest cross-sectional area of each thrombus during the formation-dissolution cycle was measured five times and a mean value calculated (in $\mu m^2$).
b) Activity of tissue factor (TF) in peritoneal macrophages by a chromogenic assay. Results were standardized with reference to the protein concentration of lysates and expressed in pM/mg/ml.
c) Tissue Factor expression in mouse carotid homogenates [measured as described in (b) above].

All animals were housed in the viral antibody-free barrier facility at the University of Texas Medical Branch (UTMB). Animal use and care were in accordance with the UTMB Institutional Animal Care and Use Committee (IACUC) guidelines.

## Statistical Analysis

Results were expressed as means plus or minus standard deviation as appropriate. A one way analysis of variance by ANOVA followed by the Tukey-multiple comparison test was used to compare differences among mouse groups. These analyses were performed using the xlStat. Statistics were also carried out in prism using ANOVA and *T*-Tests. Statistical analysis primarily compared PEGylated DI variants with their non-modified equivalent.

# RESULTS

## WT-PEG-DI Was Produced at Over 95% Purity and monoPEGylated Variants Containing 20, 30, and 40 kDa PEG Were Obtained

As can be seen in **Figures 1A,B**, 20 kDa WT-PEG-DI, 30 kDa WT-PEG-DI, and 40 kDa WT-PEG-DI were generated and the purity of this product exceeded 95%. Since DI contains two disulfide bonds, the potential products of the reaction were diPEGylated DI, monoPEGylated DI, and residual non-PEGylated DI. The chromatogram in **Figure 1C** shows that monoPEGylated DI was the dominant product and could be easily separated in different fractions from the other two products. The fractions shown in the chromatogram were run on an SDS-PAGE gel (**Figure 1D**), which shows that monoPEGylated, diPEGylated, and non-PEGylated DI were all obtained separately.

## WT-PEG-DI Retains the Ability to Inhibit Binding of Serum From Patients With APS to Whole β2GPI

**Figures 2A,B** demonstrate the results obtained in the inhibition ELISA with serum samples from six patients with APS. Binding to β2GPI on the plate was inhibited by increasing concentrations of the following products; non-PEGylated wild-type DI (WT-DI), WT-PEG-DI, non-PEGylated DI (D8S,D9G), and PEG-DI (D8S,D9G). Greater inhibition is shown by lower curves, signifying that less of the original binding capacity was maintained in the presence of the inhibitor.

For most of the samples there is little difference between the DI and DI(D8S,D9) curves, whether PEGylated (compare Green with Purple), or non-PEGylated (compare Red with Blue) showing that introduction of the D8S,D9G mutations had little effect on inhibitory capacity of DI in this assay.

The effect of PEGylation, this can be seen by comparing the WT-DI (Red) to WT-PEG-DI (Green) and the DI(D8S,D9G) (Blue) to PEGylated DI(D8S,D9G) (Purple). The samples can be divided into two types. One type, exemplified by panel A was characterized by strong inhibition (80% or more) by the two non-PEGylated products whereas their PEGylated equivalents were still able to inhibit binding but not by as much (typically a maximum of 40–50%). The other type of patient, exemplified by panel B showed less inhibition of binding but this level of inhibition (typically a maximum of 30–50%) was similar for the non-PEGylated and PEGylated DI molecules. Of note, in direct anti-DI ELISA the type A samples had higher binding to DI (mean 87.5GDIU) than the type B samples (mean 44.4GDIU).

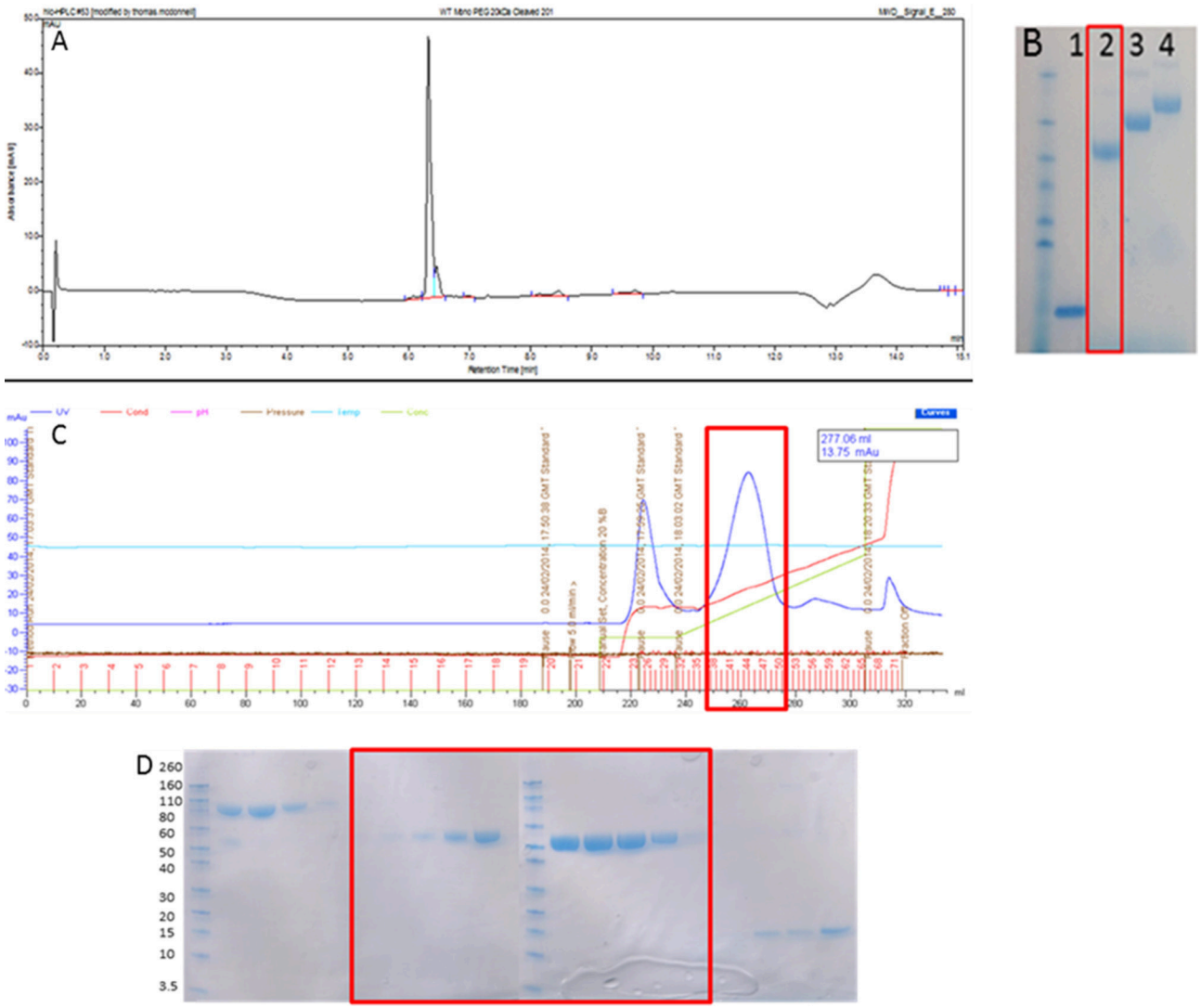


**FIGURE 1 |** Production of monoPEGylated DI. **(A)** A chromatogram demonstrating the production of >95% pure conjugated WT-PEG-DI shown as a single peak by Reverse Phase HPLC using a C18 column. **(B)** SDS PAGE gel showing unmodified WT-DI (lane 1), WT-DI conjugated to 20 kDa PEG (lane 2), WT-DI conjugated to 30 kDa PEG (lane 3), and WT-DI conjugated to 40 kDa PEG (lane 4). The red box around lane 2 indicates the sample shown in chromatogram **(A)**. **(C)** Chromatogram showing the result of cation exchange purification to separate non-conjugated WT-DI from PEG-WT-DI. The peak at 225 ml is diPEGylated WT-DI. The large peak at 260 ml, highlighted by the red box, is monoPEGylated WT-DI. The small peak at 320 ml is residual non-PEGylated WT-DI. **(D)** SDS PAGE gel showing the different forms of WT-PEG-DI obtained from the cation exchange purification **(C)**. The Bands in the three lanes at the far left are diPEGylated WT-DI. Bands in the center, highlighted by the red box, are monoPEGylated WT-DI. The faint bands in the lanes on the far right are residual non-PEGylated WT-DI.

We then compiled all the patient data into a single graph displaying average inhibition within groups (**Figure 2C**). We utilized the 100 mcg dose as this concentration was before the plateau of inhibition; these data are shown in **Figure 2C**. It demonstrates that 20 kDa WT-PEG-DI ($n = 10$), 30 kDa WT-PEG-DI ($n = 6$), and 40-kDa WT-PEG-DI ($n = 9$) all inhibit binding to a similar level. When combining the results from testing multiple serum samples, there was no significant loss of inhibitory capacity with the PEGylated compared to the non-PEGylated variants.

## PEGylated DI Retains the Ability to Inhibit the Effect of APS-IgG in the Modified dRVVT Assay

The outcome measure in this assay is the ratio of clotting time in the presence of the lupus anticoagulant-sensitive (LS) reagent to clotting time in the presence of the lupus anticoagulant-resistant (LR) reagent—referred to in the figure as LS:LR ratio or ratio of reagents. As described in the methods section, a reduction in this ratio in the presence of DI or PEG-DI indicates inhibition of the prolongation of the dRVVT.

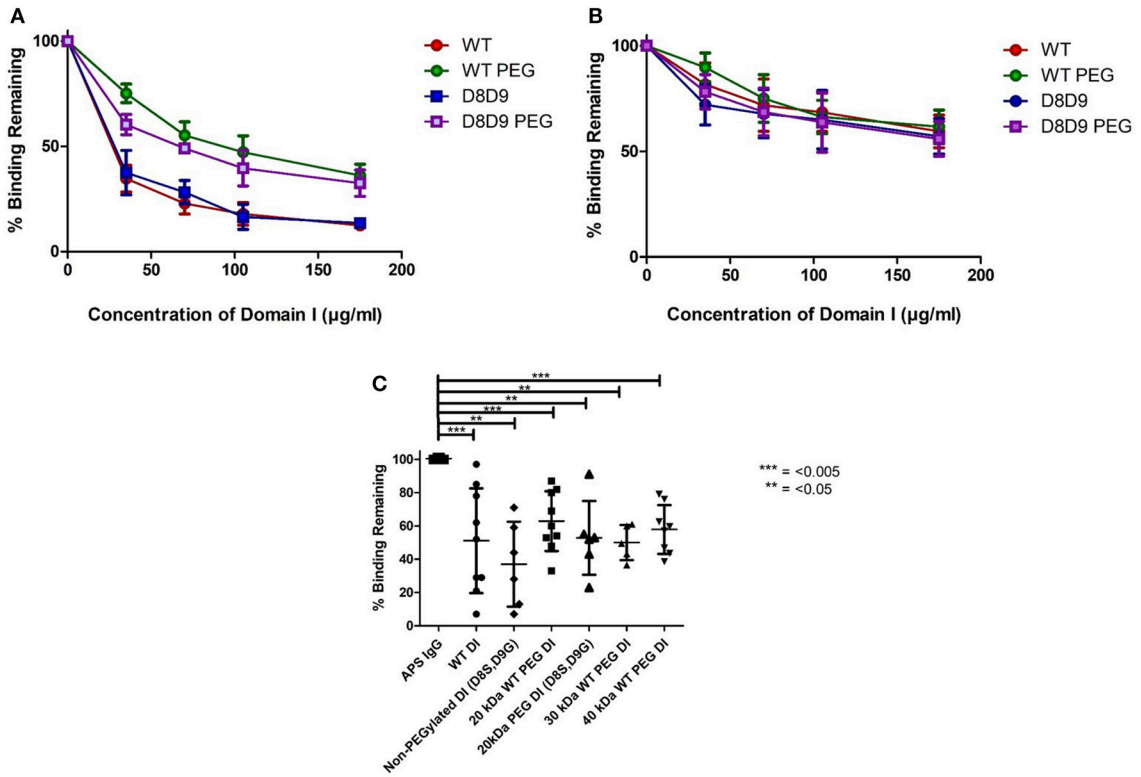


**FIGURE 2 |** PEGylated DI blocks binding of IgG in APS serum samples to whole β2GPI. **(A)** This graph shows the combined results from inhibition ELISAs of serum samples from three patients whose binding to whole β2GPI was inhibited more strongly by non-PEGylated than by PEGylated DI constructs. **(A)** Contains patients with a high level of serum aDI antibodies (average >80 GDIU). **(B)** This graph shows the combined results from inhibition ELISAs of serum samples from three patients whose binding to whole β2GPI was inhibited equally by non-PEGylated and PEGylated DI constructs. These patients have a lower level of serum aDI (average <45 GDIU). **(C)** The dot bot shows the combined results from testing samples from 9 patients [including all six from **(A,B)**]. The first column on the left shows binding to β2GPI in the absence of any inhibitor and the other columns show the binding in the presence of different inhibitors at a concentration of 100 μg/ml. For both WT-DI and DI(D8S,D9G), addition of 20 kDa PEG does not alter the inhibitory capacity of DI in this assay and the same is true for 30 and 40 kDa PEG in the case of WT-DI (these larger PEG sizes were not tested for DI(D8S,D9G). Significant differences were seen between the results obtained with APS IgG alone and those obtained with all inhibitors PEGylated or non-PEGylated (***$p < 0.005$, **$p < 0.05$) but no significant differences were seen between any of the inhibitors.

Results from 9 samples can be seen in **Figure 3A**. There was significant inhibition ($P < 0.05$) with non-PEGylated WT-DI and both 20 kDa WT-PEG-DI and 40 kDa WT-PEG-DI. PEGylation did not reduce the ability of WT-DI to inhibit the action of APS-IgG in this assay. Furthermore, **Figure 3B** shows the results for each sample separately. For two samples, the PEGylated variants reduced LS:LR ratio more than non-PEG-DI. WT-PEG-DI showed no effect on the clotting of normal plasma in the absence of APS-IgG. PEG alone showed no inhibitory effects (data not shown).

**Figure 3C** shows the results of control experiments designed to show that only proteins containing DI inhibited APS-IgG in this assay. We added octreotide or two different preparations of albumin to either APS-IgG or control IgG preparations [from a patient with systemic lupus erythematosus (SLE) or a healthy subject]. The figure shows that neither albumin nor octreotide had any effect on the LS:LR ratio obtained with any of these samples.

**Figure 3D** shows the results of samples from three patients for which inhibitory effects of both WT-DI and DI(D8S,D9G) were tested. Both PEGylated and non-PEGylated forms of both these agents inhibited the LA-like effect of APS-IgG in these experiments.

## PEGylated DI Retains the Ability to Inhibit the Effect of APS-IgG on Development of Thrombosis in a Mouse Model of APS

In each panel of **Figure 4**, the two columns on the far left show the difference in outcome obtained after administering APS-IgG and NHS-IgG in the absence of any inhibitor. In **Figure 4A** the following two columns show that PEG alone does not cause enhanced thrombosis (compared to NHS-IgG) nor does it inhibit the thrombotic effect of APS-IgG. The next two columns compare the WT-DI and DI(D8S,D9G) non-PEGylated proteins for their ability to inhibit thrombosis; both significantly reduce thrombus size. The following two columns show that 20 KDa WT-PEG-DI also inhibits thrombosis, but not as well as non-PEGylated WT-DI, with little difference between the 20 and 40 μg doses. However, the two columns on the far right

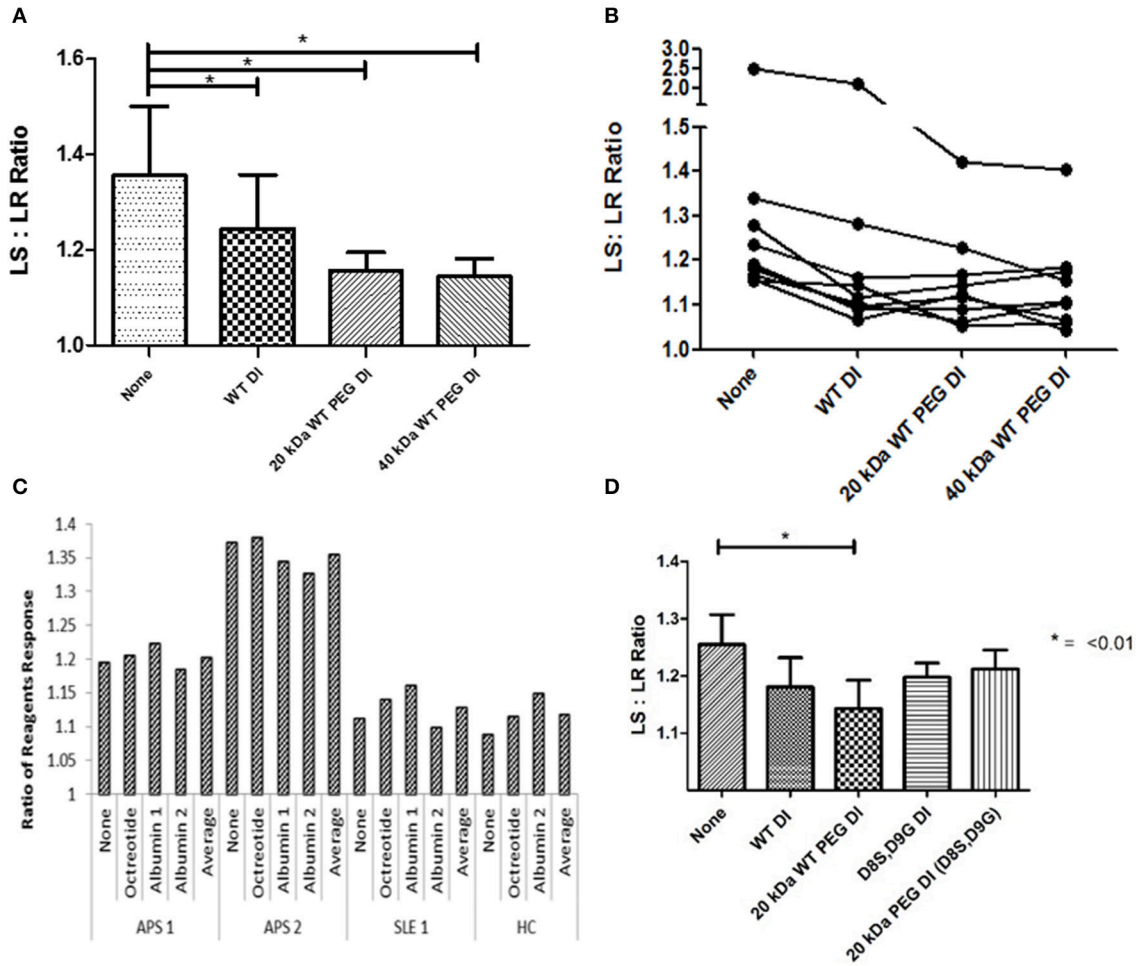


**FIGURE 3 |** PEGylated DI inhibits the LA effect of APS-IgG samples. In each figure, the y-axis shows the ratio of clotting times obtained in the presence of LS and LR reagents. LS:LR value >1.1 suggests an LA effect. **(A)** Demonstration of the effect of inhibitors on a modified dRVVT assay, this graph shows combined results from testing 9 samples. The mean LS:LR ratio was significantly lower in the presence of non-PEG-DI, 20 or 40 kDa WT-PEG-DI than in the absence of any of these agents. Significance was seen with all inhibitors compared to no inhibition (*$p < 0.05$) but there were no significant differences between the results obtained with non-PEG-DI, 20 and 40-kDa-PEG-DI. **(B)** Each separate line shows results from IgG of an individual patient inhibited with PEGylated and non-PEGylated Domain I in the modified dRVVT assay. The LS:LR ratios obtained with PEG-DI are either similar to or lower than those obtained with non-PEG-DI for all samples. **(C)** Control experiment showing that addition of non-DI proteins (albumin and octreotide) has no effect on LS:LR ratios in this coagulation assay. Each group of five columns shows the results from blood of a single individual (two with APS, one with SLE but not APS, and one HC) and in each group there are no significant differences between the columns. **(D)** Comparison of WT-DI and DI(D8S,D9G) in tests on three samples. Here PEG-DI(D8S,D9G) and non-PEG-DI(D8S,D9G) have similar effects to each other but less than WT-PEG-DI.

show a marked loss of inhibitory activity of DI(D8S,D9G) after PEGylation, especially at the 20 μg dose.

**Figure 4B** shows TF expression in peritoneal macrophages of mice exposed to APS-IgG in the presence of various inhibitors. The columns on the left (3–5) show inhibition by both PEGylated and non-PEGylated WT-DI whereas the columns on the right (8–10) show that the inhibitory effect of DI(D8S,D9G) in this assay is dramatically reduced after PEGylation. In fact, **Figures 4A,B** show that a dose of 20 μg PEG-DI(D8S,D9G) has no inhibitory effect at all. PEG in the absence of APS-IgG does not have any effect on TF expression in peritoneal macrophages.

As can be seen in **Figure 4C** both non-PEGylated WT-DI and WT-PEG-DI inhibit the expression of TF in mouse carotid exposed to APS-IgG by similar amounts. Conversely DI (D8S,D9G) does not significantly reduce carotid TF expression, whether or not it is PEGylated.

Since the mice in this model are subjected to anesthesia and a surgical procedure, some premature deaths were expected. All but one of these deaths occurred while mice were still under anesthesia. Thus, in the control groups that were not exposed to DI, 1/5 mice died in each of the following groups; APS-IgG alone, NHS IgG alone, and PEG alone and no mice died in the group of 7 mice given APS IgG and PEG. The results were similar in the mice given APS-IgG plus non-PEGylated WT-DI (1/5 died) and APS-IgG plus non-PEGylated DI (D8S,D9G) (0/5 died). Regarding the PEGylated constructs, two different doses

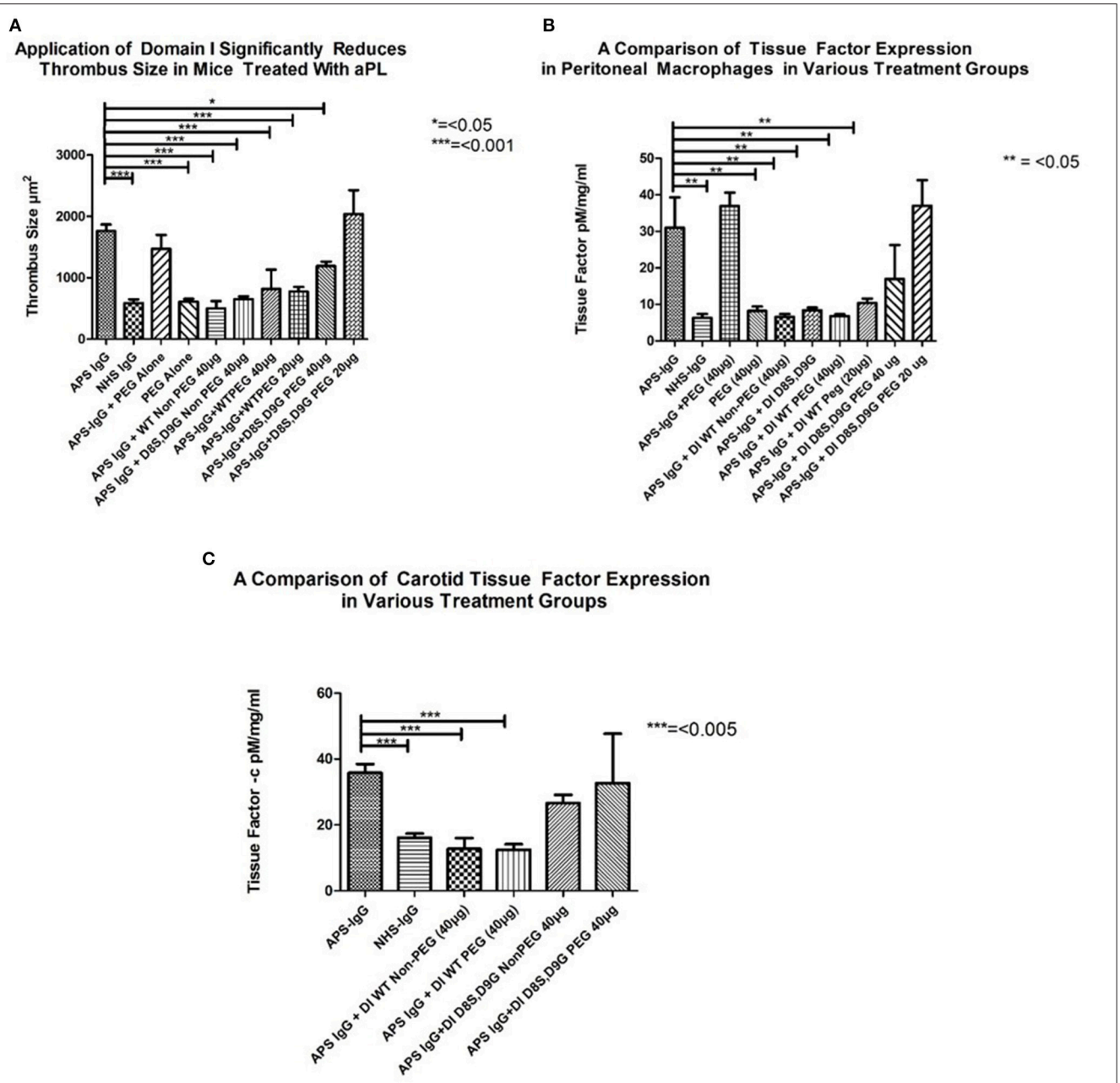


**FIGURE 4 |** Inhibitory effects of non-PEG-DI and PEG-DI in a passive transfer mouse model of APS. Mice received either NHS-IgG (500 µg), APS-IgG (500 µg), or 40 µg PEG alone with no IgG. APS-IgG, but not NHS-IgG or PEG alone, stimulated increased thrombosis **(A)**, increased peritoneal macrophage TF expression **(B),** and increased carotid TF expression **(C)**. The effects of the various PEGylated and non-PEGylated DI constructs are shown in the columns on the right of the graphs and explained fully in the results section.

of PEG-DI were tested. At high dose (40 µg) 2/5 mice died in both the APS-IgG plus PEG-WT-DI and the APS-IgG plus PEG-DI(D8S/D9G) group. However, at low dose (20 µg) 0/5 mice died in the APS-IgG plus PEG-WT-DI and 2/5 died in the APS-IgG plus PEG-DI(D8S/D9G) group.

We carried out analyses to investigate possible causes of toxicity in these mice. Mice treated with high dose of PEG-WT-DI and either dose of PEG-DI(D8S/D9G) displayed some signs of inflammatory damage localized to the peritoneal cavity manifesting as erythema, exudation, and in the case of some of the animals treated with the PEG-DI(D8S,D9G) agent, petechial hemorrhage. However, none of the mice exhibited any of the typical signs of a systemic inflammatory response, namely coat ruffling, impaired consciousness, activity, eye movements, respiration, or response to stimuli. Importantly, the mice exposed to low dose PEG-WT-DI had neither

localized nor systemic inflammation and none of them died prematurely.

We measured the levels of the acute phase reactants interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in mice treated with the 40 μg dose of PEG-DI(D8S,D9G) or PEG-WT-DI and found that they were similar to those seen in mice treated with a low dose of endotoxin (0.001 mg), which does not cause systemic inflammation. These levels were ∼10 to 60-fold less than those seen in mice treated with a typical endotoxin dose to induce sepsis (0.5 mg).

In summary, these mortality data showed that administration of the non-PEG-DI and low dose PEG-20kDaWT-DI did not demonstrate any excess deaths compared to control groups, whereas the PEG-20kDa DI(D8S/D9G) group had a slight excess (2/5 deaths compared to 1/5 in control groups) at either dose. Thus, the PEGylated mutant version of DI showed unfavorable properties in this *in-vivo* model compared to PEGylated WT-DI both in terms of activity (see above) and toxicity.

## DISCUSSION

We previously showed that recombinant DI expressed in bacteria blocked the binding and thrombogenic actions of APS-IgG (16). Since DI alone is not a viable therapeutic agent due to its small size, we have pursued development of a PEGylated form of DI. The use of PEGylation is common in the production of therapeutics due to its positive pharmacokinetic effects, however, this is weighed against a loss of activity for the protein. For this reason it is essential to characterize protein activity to determine which size of PEG would be optimal in terms of balancing increased half-life with maximal activity, prior to testing pharmacokinetics. Equally, before proceeding to trials of efficacy, it was important to optimize the method of production of pure monoPEGylated DI. The optimization of expression of DI in *E. coli* has been reported in a previous paper (15) and the optimization of PEGylation and purification is reported here.

Total inhibition of binding of APS-IgG to β2GPI was not seen even at high concentrations of PEG-DI because the serum anti-β2GPI response in patients with APS is polyclonal and comprises antibodies to different domains of β2GPI. The anti-DI component of this polyclonal response is believed to play the predominant role in pathogenesis of the APS. Both a Dutch group de Laat et al. (28) and our group have shown that anti-DI levels are more closely associated with risk of thrombosis than anti-β2GPI or anti-cardiolipin antibody levels (29). Andreoli et al. tested serum from a variety of groups for antibodies to DI and to DIV/V (30). Whereas anti-DI and anti-DIV/V were equally likely to be found in subjects with no autoimmune disease, anti-DI was found four times more commonly than anti-DIV/V in autoimmune patients (30). In a mouse model, we showed that when APS-IgG was separated into anti-DI-rich and anti-DI-poor fractions by affinity-purification on a column carrying recombinant DI, the ability to cause vascular thrombosis was concentrated in the anti-DI rich fraction (31). Thus, we believe that 50% inhibition of binding of APS-IgG to β2GPI that we have demonstrated with PEG-DI is of potential clinical benefit, because the subpopulation of anti-β2GPI being inhibited is also the group with the major thrombogenic effect.

Our description of a modified dRVVT assay to test the ability of potential therapeutic agents to block the ability of APS-IgG to prolong the modified dRVVT is itself novel and could be used to test other potential therapeutic agents in APS, however, further testing is required before the modified dRVVT could be considered a reliable marker. Testing the LA-like effect is relevant as several studies have shown that LA may be a stronger predictor of clinical outcomes than the anti-cardiolipin or anti-β2GPI tests in patients with SLE and APS (32), particularly in pregnancy (33). WT-PEG-DI was at least as effective an inhibitor in this assay as non-PEG WT-DI. However, unlike the binding assay [where we obtained similar results with WT-DI and DI (D8S,D9G)] in the modified dRVVT assay PEGylation seems to reduce the efficacy of DI (D8S,D9G). Replacing two larger negatively charged amino acids with smaller neutral ones could alter exposure of the disulfide bond to which the PEG binds and might therefore alter the orientation of the PEG group itself, thus influencing factors such as steric hindrance.

In the mouse model experiments, it was also clear that WT–PEG-DI had advantages over PEG-DI (D8S,D9G). Whereas, both 20 μg WT-PEG-DI and 40 μg WT-PEG-DI inhibited the biological effects of APS-IgG on thrombosis and TF expression, the PEGylated forms of DI(D8S,D9G) had reduced efficacy as inhibitors. Furthermore, significant toxicity was seen with the PEG-DI(D8S,D9G) agent but not the WT-PEG-DI agent. While the 20 and 40 μg doses of the PEG-DI(D8S,D9G) agent produced inflammatory damage, resulting in premature deaths of 40% of the mice in each group, the WT-PEG-DI agent did not have this effect when given at a dose of 20 μg.

It was not possible to use the mouse model to establish half-lives of the DI or DI-PEG conjugates for several reasons. In this acute model, mice are sacrificed after 72 h, as such little data would be generated regarding the half-lives of protein from such a small exposure. Equally, DI or PEG-DI is bound by circulating antibody, this would alter the values detected if we attempted to use this model to characterize half-lives. The aim of the mouse model was to characterize the activity of the conjugates before establishing a lead candidate for therapeutic development, it is only through initially testing the activity of the protein conjugates that the correct PEG size can be tailored to the protein.

Although the ultimate purpose in PEGylating DI was to improve its serum half-life, we have not yet carried out pharmacokinetic experiments to prove that this has been achieved. Clearly these experiments must be done before clinical application of PEG-DI can be considered, furthermore, we also plan to test ability of PEG-DI to inhibit thrombus formation in a chronic mouse model of APS, in which mice are immunized with human β2GPI and thus develop their own anti-β2GPI antibodies (34) (as opposed to the passive transfer of human APS-IgG reported in this paper).

We are unsure of the exact mechanism of toxicity attributed to the PEGylated DI(D8S,D9G) but it seems clear that it is

localized to the site of inoculation and given the changes in size and charge to the DI molecule imparted by the PEG conjugate, impaired absorption of the agent across the peritoneum may be a contributing factor. Importantly, we ruled out the formation of immune complexes (CIC) in the peritoneal cavity as a possible cause by measuring levels in collected peritoneal exudates (data not shown). Future toxicity studies will focus on determining specific effects of PEGylated agents when introduced in the intraperitoneal vs. intravascular compartments.

We hypothesize that the difference in activity *in-vitro* and toxicity *in-vivo* between the two conjugates may be due to a difference in the binding site of the PEG. The PEGylation technology used requires access to surface exposed disulphide bonds. The dual mutation in the DI(D8S,D9G) protein allows access to the disulphide from two sides whilst the WT-DI protein restricts access to a single site. The mutations may therefore allow a different PEGylation which in turn may lead to masking the active site. This difference in site could also explain the increased toxicity seen with the PEG-DI(D8S,D9G) agent as the difference in PEGylation site may also effect diffusion constants and solubility. This theory is, however, speculative and would require significant modeling work to fully confirm.

Overall, the results of our programme of experiments clearly identify WT-PEG-DI as a strong potential lead for APS and a better candidate than PEG-DI(D8S,D9G), though the optimal size of PEG cannot be identified until pharmacokinetic experiments have been carried out.

Limitations of the work include the fact that we have tested samples from a relatively small number of patients with APS. Particularly for the *in-vivo* experiments there are practical limitations to the number of different samples that can be tested, since large numbers of mice are required for tests on each APS-IgG sample. For the modified dRVVT assay we can only use samples that have LS:LR $>1.1$ in the absence of inhibitor. We have mitigated the problem of limited sample numbers by using three complementary assays to test efficacy of PEG-DI as an inhibitor of APS-IgG. We should stress however, PEG-DI may not be effective in all patients; potentially it may show greatest effect in patients with high anti-DI levels. Measuring anti-DI levels could identify the sub-population of patients with APS likely to respond to PEG-DI. A study by Pericleous et al. showed ∼40% of patients with APS had aDI positivity (29), a finding mirrored by a recent review (35).

Possible limitations of PEG-DI include potential toxicity or immunogenicity of the PEG molecule. PEG is harmless to animals at concentrations as high as 16 g per kg [1,000-fold higher than the normal therapeutic dose of protein-PEG conjugates in humans (26)] but anti-PEG antibodies are found in between 8 and 25% of the healthy population (36, 37). This phenomenon is possibly due to use of PEG in everyday products such as hand creams. It is not clear whether those anti-PEG antibodies would affect efficacy of PEGylated drugs. Although anti-PEG and anti-drug antibodies adversely affected efficacy of PEGylated uricase used in gout, the same was not true in large clinical trials of certolizumab pegol, a PEGylated anti-tumor necrosis factor Fab used in rheumatoid arthritis. Though antibodies to this drug were seen in 5–6% of patients in the RAPID 1 (38) and RAPID 2 (39) clinical trials, there was no evidence that presence of these antibodies affected clinical outcomes.

In conclusion, there is a pressing need for targeted therapies in APS given that none have yet been developed for this disease. We have developed PEG-DI, an agent designed to block binding of pathogenic aPL to β2GPI, and have shown that it is effective at blocking the effects of APS-IgG in three different assays, two *in-vitro* and one *in-vivo*. These data support pharmacokinetic and toxicology experiments which are now underway to develop PEG-DI as a first-in-class therapeutic for APS.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of London Hampstead Research Ethics Committee Ref No 12/LO/0373 with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the London Hampstead Research Ethics Committee Ref No 12/LO/0373. All animal research was conducted in a responsible, humane manner under the guidance of the University of Texas Medical Branch Institutional Animal Care and Use Committee (UTMB IACUC). UTMB IACUC is a Category I institution accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and is in full compliance with the US Federal Animal Welfare Act (1966) and Texas animal care laws.

## AUTHOR CONTRIBUTIONS

TM carried out laboratory work, developed methodology, and co-wrote the paper. RW carried out mouse work in Texas. CP and YI developed methodology and gave intellectual guidance. VR and IG gave intellectual guidance. DI, AB, and EG gave direction and support. EP, ZR-P, and MJ carried out practical work in Texas regarding mouse models. AR gave intellectual input, co-wrote the paper and supervised the project.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02413/full#supplementary-material

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**Conflict of Interest Statement:** TM, CP, YI, IG, and AR are named inventors on a Patent filed in the US for PEG-DI.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Presence of Immune Complexes of IgG/IgM Bound to B2-glycoprotein I Is Associated With Non-criteria Clinical Manifestations in Patients With Antiphospholipid Syndrome

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**Background:** Antiphospholipid syndrome (APS) is an acquired autoimmune disorder defined by the presence of both clinical (thromboembolic events or pregnancy morbidity) and laboratory (antiphospholipid antibodies, aPL) manifestations. Despite their importance, several clinical manifestations strongly associated with APS such as livedo reticularis (LR), thrombocytopenia, sicca-ophthalmic(sicca), heart, or neurological manifestations are not included in the APS clinical classification criteria. Circulating immune complexes (CIC) formed by Beta-2-glycoprotein I (B2GPI) and aPL (B2-CIC) have been described and their presence has been related with thrombotic events.

**Methods:** Cross-sectional and observational cohort study in APS patients with thrombotic symptomatology.

**Setting and Participants:** Fifty-seven patients from the University Hospital Center Bezanijska Kosa (Belgrade, Serbia) who met the APS classification criteria (35 with primary APS and 22 with APS associated to systemic lupus erythematosus). This study aimed to determine the prevalence of B2-CIC in APS patients and to evaluate their association with clinical manifestations of APS not included in the classification criteria.

**Results:** B2-CIC prevalence in APS patients was 19.3%. The presence of thrombocytopenia (OR:5.7), livedo reticularis (OR:5.6), sicca (OR:12.6), and leukopenia (OR:5.6) was significantly higher in patients with B2-CIC than in the rest of APS patients. C3 and C4 complement factor levels were significantly lower in B2-CIC positive patients, which suggests a greater consumption of complement. Patients with quadruple aPL positivity (triple aPL-positivity plus the presence of B2-CIC) showed a higher prevalence of thrombocytopenia, leucopenia and LR than those with single/double aPL-positivity. No significant differences were found in the frequencies observed in patients with triple-only vs. single/double aPL-positivity. There were no significant differences between patients with primary APS and lupus-associated APS regarding the prevalence of B2-CIC and outcomes.

**Conclusions:** Presence of B2-CIC is strongly associated with several non-criteria clinical manifestations related to APS and to higher complement consumption. More studies are required to better understand the clinical significance of B2-CIC.

**Keywords: antiphospholipid, livedo reticularis, sicca, thrombocytopenia, leukopenia, circulating immune-complexes, anti-beta-2-glycoprotein I, non-criteria APS clinical manifestations**

## KEY MESSAGES

- Prevalence of B2-CIC (IgG or IgM isotypes) in APS patients is 19.29%.
- B2-CIC are strongly associated with livedo reticularis and ophthalmic sicca.
- Thrombocytopenia, leukopenia and complement consumption are related with presence of B2-CIC.
- The quadruple aPL positivity (triple aPL positivity in the presence of B2-CIC) is highly associated with ophthalmic sicca and thrombocytopenia.

## INTRODUCTION

Antiphospholipid Syndrome (APS) is an autoimmune disorder characterized by thrombosis and/or pregnancy morbidity in presence of persistent antiphospholipid antibodies such as lupus anticoagulant (LA), isotype IgG and/or IgM for anti-beta-2-glycoprotein I (aB2GPI) and anti-cardiolipin antibodies (aCL) (1–4).

The current APS international classification criteria require the presence of at least one laboratory criterion on two or more occasions, at least 12 weeks apart as well as the presence of a minimum of one clinical criterion (5). APS could manifest as primary (without other concomitant morbidity), as associated to other autoimmune disease, mainly Systemic lupus erythematosus (SLE) (6) or as catastrophic-APS (multiple organ thrombosis with a high mortality rate) (7).

The APS clinical spectrum goes far beyond the classification criteria (8). There are other clinical manifestations associated with the syndrome such as heart valve disease (9), neurological manifestations (headache, migraine, or epilepsy) (10), livedo reticularis (LR) (11), thrombocytopenia (12), and nephropathy (13). Several studies have supported the strong association between APS and these clinical manifestations. However, these manifestations have not been included as clinical criteria because of their low specificity (14, 15). Similarly, other aPL antibodies as aB2GPI of IgA isotype (16), anti-Annexin A2 and A5 (17), anti-vimentin and anti-phosphatidilserin/prothrombin (18) have been associated with APS but also have not been included as laboratory criteria because there is still not sufficient evidence to include them (14).

Currently, several authors have been emphasizing the need for new APS biomarkers to improve sensitivity and specificity in the diagnosis of the syndrome (19, 20). Presence of immune complexes of IgA aB2GPI antibodies bound to B2GPI (B2A-CIC) has been described recently in the blood of patients with clinical thrombotic manifestations for APS (21, 22). The prevalence of B2A-CIC has been found to be significantly higher in patients with acute thrombotic event (22). The risk of developing thrombosis immediately after undergoing transplant surgery is significantly higher (HR: 6.72) in patients with B2A-CIC (23). Transplant surgery is a known second hit for the triggering of an APS event (24).

The immune complexes of aPL have been described previously (25) and they have recently been visualized with high resolution microscopy (26). Presence of immune complexes was associated with the occurrence of acute thrombotic events (27–29). However, prevalence in APS patients of circulating immune complexes between aPL and B2GPI (B2-CIC) and the relationship of these complexes with APS-associated clinical manifestations have still not been described.

This study has aimed to determine the prevalence of B2-CIC of IgG and/or IgM isotypes (B2G-CIC and B2M-CIC, respectively) in patients with clinical and laboratory classification criteria of APS patients and the relationship between presence of these biomarkers and the APS-related clinical manifestations.

## PATIENTS AND METHODS

### Study Design

A cross-sectional study developed to determine the prevalence of B2-CIC in APS patients and their association with the APS-related clinical manifestations of these subjects.

### Patients

A total of 57 patients with a diagnosis of thrombotic APS who met revised criteria for APS (5) were consecutively enrolled in the year 2000 in the University Hospital Center Bezanijska Kosa. All patients were examined by a combined group of rheumatologist, neurologist, cardiologist, radiologist, hematologist, and ophthalmologist. Of these, 35 had PAPS while 22 had APS in association with SLE. All the patients had thrombosis and some had gestational morbidity consistent with APS in accordance with the 2006 revised criteria for APS (5). Mean age was 47.6 ± 1.6 years; 36 (63.2%) were women. Main population characteristics are shown in **Table 1** (additional data are shown in **Supplementary Table 1**). Subjects were divided into two groups according to positivity for B2-CIC (group-1) and negativity for CIC (group-2). Diagnosis of APS was made by presence of aPL and other diagnostic criteria (Doppler ultrasound, computed tomography, heart ultrasound or others for arterial and/or venous thrombosis,

**Abbreviations:** OR, Odds ratio; CI, Confidence interval; aB2GPI, Anti-Beta-2 glycoprotein-I antibodies; aCL, Anti-cardiolipin antibodies; CIC, Circulating immune complexes; B2-CIC, Circulating immune-complexes of IgG or IgM bounded to Beta-2 glycoprotein-I.

**TABLE 1 |** Population description and clinical characteristics of the 57 APS patients included in the study.

| Condition | *N* = 57 Mean/Number of patients | % / SEM |
|---|---|---|
| Age (years) | 47.6 | ±1.6 |
| Sex (women) | 36 | (63.2%) |
| Catastrophic APS | 4 | (7.0%) |
| Primary APS | 35 | (61.4%) |
| Disease duration (years) | 5.4 | ±0.7 |
| Diabetes Mellitus | 2 | (3.5%) |
| Hypertension | 5 | (8.8%) |
| Dyslipidemia | 5 | (8.8%) |
| Smoker | 21 | (36.8%) |
| **ANTIPHOSPHOLIPID ANTIBODIES (APL) POSITIVE** | | |
| Anti-cardiolipin IgG antibodies | 24 | (42.1%) |
| Anti-cardiolipin IgM antibodies | 25 | (43.9%) |
| Anti-beta2-glycoprotein I IgG antibodies | 26 | (45.6%) |
| Anti-beta2-glycoprotein I IgM antibodies | 29 | (50.9%) |
| Lupus anticoagulant | 38 | (66.7%) |
| Triple aPL positivity | 15 | (26.3%) |
| **APS PATHOLOGY** | | |
| ***Gestational morbidity*** | | |
| Women in fertile age | 34 | (59.6%) |
| Women with fetal loss | 21 | (61.8%) |
| Mean fetal loss | 1.6 | ±0.2 |
| Women with late fetal loss | 15 | (44.1%) |
| Women with early fetal loss | 6 | (17.6%) |
| ***Thrombotic events*** | | |
| Arterial thrombosis | 44 | (77.2%) |
| Venous thrombosis | 27 | (47.4%) |
| Inferior extremity deep vein thrombosis | 12 | (21.1%) |
| Superior extremity arterial thrombosis | 3 | (5.3%) |
| Pulmonary embolism | 14 | (24.6%) |

*SEM, standard error of the mean. Early fetal loss: miscarriage during the first 13 weeks, the first trimester, Late fetal loss: miscarriage after the first 13 weeks of gestation.*

and multiple and recurrent fetal losses). All SLE-diagnosed patients met the American College of Rheumatology (ACR) classification criteria (30). Disease activity was assessed at the time of enrolment in the study using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score, and only those patients with stable disease were enrolled (31). Clinical data and medication were obtained from the patient's clinical database and records. Exclusion criteria included acute or chronic infection, marked renal and liver impairment or data of present or treated malignancy. The presence of comorbidities, such as arterial systemic hypertension (blood pressure $\geq$ 140 x 90 mmHg or use of anti-hypertensive drugs) and diabetes (glycated hemoglobin 7% or use of medication) and data on smoking habit (any person who smokes every day, occasional smoking and having quit smoking <1 year ago) and drugs used were also evaluated. Four of the patients showed the presence of catastrophic APS (CAPS) and were included in the international registry of catastrophic APS patients (CAPS Registry) created in 2000 by the European Forum on Antiphospholipid Antibodies.

## Ethical Issues

The "Belgrade APS cohort" observational and non-interventional study was approved by the Ethics Committees of the Bezanijska Kosa, University Medical Center of Belgrade. Informed consent was obtained from all individual participants included in the study.

After blood sampling, a blind code was assigned to serum of each patient to assure anonymity throughout the process. The blind numerical code associate each serum with the corresponding clinical data. The sera that were sent to the Spanish group did not contain DNA and, so according to Spanish legislation, were not considered as human biological samples that require ethical authorization for their use (article 3 of the Spanish Biomedical Research Law of 2007).

As it is a non-interventionist observational study initiated in Belgrade, the clinical data were anonimized, the sera were obtained before 2007 and do not contain information about the characteristic genetic endowment of a person (DNA), Spanish legislation does not require special ethical treatment or additional informed consent for this type of study.

## Definitions

*Thrombotic event:* thrombosis in any arterial or venous blood vessel. The diagnosis was confirmed using objective validated criteria such as imaging techniques (5).

*Gestational Morbidity*: deaths of a morphological normal fetus, premature births or spontaneous abortions defined in accordance with the International Consensus Statement for Antiphospholipid Syndrome (5).

*Leukopenia*: number of blood white cells <4,000 per microliter of blood.

*Thrombocytopenia*: number of platelets <140,000 per microliter of blood.

*Ophthalmic sicca (sicca):* multifactorial disease characterized by insufficient tear production resulting in instability of the tear film, discomfort, sensation of visual dryness and visual disturbance. The patients with sicca were positive for Schirmer's test, performed without anesthesia (<5 mm in 5 min) and Rose Bengal score or another ocular dye score (>4 according to van Bijsterveld's scoring system).

*Triple positivity of aPL:* patients who are positive for the three laboratory markers associated with APS: LA, aCL and aB2GPI antibodies (IgG or/and IgM isotypes) (32).

Quadruple positivity of aPL or aPL "poker": patients with triple positivity of aPL who also are positive for B2-CIC.

### Triple Only aPL Positivity: Patients With aPL Triple Positivity Who Are B2-CIC Negative

*Systemic lupus erythematosus (SLE):* Diagnosis of SLE is based upon ACR classification criteria (31). The proposed classification is based on 11 criteria. Person shall be said to have systemic

lupus erythematosus if any 4 or more of the 11 criteria are present, serially or simultaneously, during any interval of observation.

## Laboratory Determinations

LA, aCL, and aB2GPI antibodies (IgG and IgM) were measured using the plasma (LA) and serum samples (aCL and aB2GPI) obtained at the moment of the patient's enrolment in the study, this being more than 3 months after the APS event. The serum samples were stored frozen at −30°C.

LA was based on the use of two different screening tests: diluted activated partial thromboplastin time and sensitive activated partial thromboplastin time according to the International Society on Thrombosis and Hemostasis (ISHT) recommendations (33). LA tests were performed while the patients were not receiving anticoagulant therapy.

IgG/IgM aCL and aBGPI antibodies were measured by an enzyme-linked immunosorbent assay (Binding Site Group Ltd, Birmingham, UK). Antibodies aCL levels were expressed in GPL or MPL phospholipids units (GPL-U and MPL-U). Antibodies aB2GPI levels were expressed in U/ml.

Positivity of aCL and anti-B2GPI of IgG and IgM isotypes was confirmed by BioPlex 2200 multiplex immunoassay system using BioPlex 2200 APLS IgG and APLS IgM panels (Bio-Rad, Hercules CA, USA). Antibody levels higher than 20 U/mL were considered positive following the manufacturer's guidelines, this coinciding with the 99th percentile of the European population determined in the laboratory.

Antibodies Against dsDNA, Chromatin, SSA-52 kDa (Ro52), SSA-60 kDa (Ro60), SSB (La), Sm, Sm/RNP, RNP-A, RNP-68 kDa, Scl70, Centromere B, Jo-1, And P Ribosomal Proteins Were Evaluated by BioPlex 2200 BioPlex® 2200 ANA Screen Panel (Bio-Rad, Hercules CA, USA).

Complement factors C3 and C4 levels were measured using Beckman Coulter IMMAGE Immunochemistry System (Beckman Coulter Inc. Pasadena, CA, USA). The range of normality for C3 levels was 88–225 mg/dL and for C4 levels 12–75 mg/dL.

Quantification of B2G-CIC and B2M-CIC levels was performed as previously described (21). Briefly, 96 wells Nunc maxisorp™ plates (A/S Nunc, Kamstrup, Roskilde, Denmark) were coated overnight at 4°C with mouse monoclonal antibody anti-human B2GP1 H219 (Mabtech AB, Nacka Strand, Sweden) at 2 μg/mL in PBS pH 7.4. Plates were washed (PBS 0.1% tween 20), blocked with PBS containing 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) 30 min at room temperature (RT) and washed (PBS 0.1% tween 20). Serum diluted at 1:100 in PBS were dispensed (100 μL/well; duplicates) and incubated 2 h at RT.

Anti-human IgG HRP-conjugate was used to detect B2G-CIC and anti-human IgM HRP-conjugate was used to detect B2M-CIC, (both from INOVA Diagnostics Inc., San Diego, CA, USA). The concentration of CIC-G or CIC-M (U/mL) of each serum was obtained by interpolating the mean optical density values with a calibration curve.

Sera with B2-CIC levels (IgG or IgM) higher than 21 AU were considered positive (99th percentile of healthy population). All the procedures were performed in a Triturus® Analyzer (Diagnostics Grifols, S.A. Barcelona, Spain).

## Statistical Methods

Results were expressed as absolute frequency, percentage or mean ± standard error. The Pearson $\chi^2$ test (or Fisher's exact test, when appropriate) was used to determine the association between qualitative variables. Odds ratio (OR) was used to measure the strength of association between the presence of a risk factor and an outcome. OR and 95% confidence interval were calculated by logistic regression.

Student's *t*-test was used for comparisons in scaled variables with two previous categories of assessment of normality with Kolmogorov-Smirnov test. Mann-Whitney *U*-test was used for comparisons when the outcome was not normally distributed. Probabilities $<0.05$ were considered significant. A box-and-whisker plot represents the values from the lower to upper quartile (25 to 75 percentile) in the central box. The median is represented as the middle line in the box. Adjustment of *p*-values for multiple comparisons were obtained by the false discovery rate method (34).

Data were processed using Medcalc for Windows version 17.9 (Medcalc Software, Ostend, Belgium) and the "R" programming language (R Foundation for Statistical Computing, Vienna, Austria) (35).

# RESULTS

## Prevalence of B2-CIC in APS Patients

Eleven (19.3%) out of the 57 patients enrolled in the study had B2-CIC in the serum samples (group-1); 8 with B2M-CIC and 3 with B2G-CIC. None of the patients had both CIC formed. The remaining 46 patients had no CIC (group-2). The box and wisher data graph of B2M-CIC and B2G-CIC values is represented in **Supplementary Figure 1**.

No differences were observed in distribution of sex, age, proportion of women in fertile age and proportion of PAPS between both groups. The proportion of positivity for aPL was similar in individuals with and without CIC. No significant correlation was observed between levels of B2-CIC and aPL (not shown). LA was more frequent in patients in group-1 (90 vs. 60.9%,) but the difference was not statistically significant ($p = 0.079$). No significant differences were observed between both groups in the main APS-symptomatology (thrombotic events or gestational morbidity) presented by patients (**Supplementary Table 2**).

## APS-Related Clinical Manifestations

The prevalence of LR was significantly higher in individuals with B2-CIC in the serum sample than those without B2-CIC (63.6 vs. 23.9%, OR = 5.57, 95% CI: 1.37–22.65).

Patients with B2-CIC had a significantly higher incidence of sicca than group 2 (54.5 vs. 8.7%, OR: 12.6, 95% CI:2.63–60.48). All the patients were negative for rheumatoid factor (RF), anti-Ro60, anti-Ro52, and anti-La autoantibodies.

In patients with B2-CIC a significantly higher proportion of thrombocytopenia (54.5 vs. 17.4%, OR:5.7, 95% CI: 1.39–23.36) and leukopenia (45.5 vs. 13%, OR: 5.56, 95% CI: 1.28–24.03) was observed (**Table 2**).

## Complement Levels

The mean levels of C3 and C4 complement factors were within the normal range in both groups. However, the C3 levels were significantly lower in group-1 than in group-2 (115.6 ± 9.2 vs. 140.9 ± 4.3 mg/dL; $p = 0.014$; **Figure 1A**). Also, C4 levels were decreased in patients with B2-CIC (22.0 ± 3.4 vs. 30.8 ± 1.6 mg/dL; $p = 0.022$; **Figure 1B**).

The levels of C3 factor in patients with sicca was significantly lower than in the individuals without this clinical manifestation related to APS (115.1 ± 6.9 vs. 140.1 ± 4.5 mg/dL; $p = 0.018$, **Figure 2A**). Furthermore, C4 levels were lower in sicca (23.1 ± 3.4 vs. 30.4 ± 1.7) patients but the difference was not statistically significant ($p = 0.073$, **Figure 2B**).

Patients with sicca who were B2-CIC positive showed much lower C3 complement factors levels than patients without sicca (107.9 ± 24.8 vs. 140.5 ± 31.1 mg/dL; $p = 0.013$; *T*-test). C4 complement levels were also significantly lower in patients with sicca and B2-CIC (median: 20.6, interquartile range: 8.3–23.2 vs. median 28.7, interquartile range 23.1–36.0; $p = 0.049$). The C4 levels in patients with sicca and B2-CIC did not follow a normal distribution and they were analyzed with the Mann-Whitney test (**Figures 2C,D**).

## Triple and Quadruple aPL Positivity

Fifteen out of the 57 patients involved in the study were triple positive for aPL. The prevalences of pulmonary embolism (46.7 vs. 16.7%; $p = 0.022$) and catastrophic APS (20 vs. 2.4%; $p = 0.023$) were significantly higher in patients with triple aPL positivity than in individuals with single or double aPL positivity (**Table 3**). However, the remaining clinical manifestations did not show significant differences between both groups of patients (**Table 3** and **Supplementary Table 3**).

Four of the 15 patients with triple positivity were also positive for B2-CIC, a situation which was considered as "quadruple aPL positivity" or "aPL poker" (triple positivity plus the presence of B2-CIC). The 11 patients with triple positivity who were B2-CIC negative were considered as "triple-only" aPL positive.

Presence of LR, leucopenia and thrombocytopenia is significantly higher in patients with quadruple aPL positivity than in the patients with single or double aPL positivity (75, 75, 100 vs. 20, 14, 17; odds ratio: 12, 18, and 40.8; *p*-values:

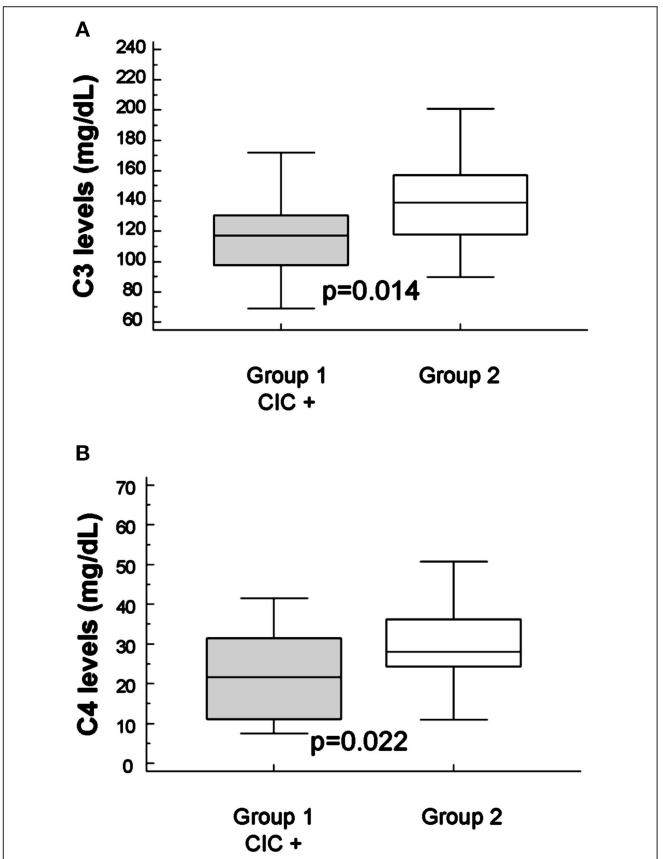


**FIGURE 1 |** Mean levels of C3 **(A)** and C4 **(B)** complement in groups. Mean levels of C3 (115.6 ± 9.2 and 140.9 ± 4.3 mg/dL, group-1 and group-2, respectively) and mean levels of C4 (140.9 ± 4.3 and 30.8 ± 1.6 mg/dL, group-1 and group-2, respectively).

**TABLE 2 |** Extra-criteria clinical manifestations of antiphospholipid syndrome.

| Condition | Group 1 | | Group 2 | | | | |
|---|---|---|---|---|---|---|---|
| | B2-CIC + *N* = 11 | % | B2-CIC – *N* = 46 | % / SEM | *P* | *P* adjusted | OR(95%CI) |
| Valve thickening and dysfunction | 0 | (0%) | 2 | (4.3%) | 0.481 | 0.561 | |
| Livedo reticularis | 7 | (63.6%) | 11 | (23.9%) | 0.011 | 0.025 | 5.57 (1.37-22.65) |
| Ophthalmic sicca | 6 | (54.5%) | 4 | (8.7%) | 0.002 | 0.014 | 12.6 (2.63-60.48) |
| Thrombocytopenia | 6 | (54.5%) | 8 | (17.4%) | 0.010 | 0.025 | 5.7 (1.39-23.36) |
| Autoimmune hemolytic anemia | 1 | (9.1%) | 1 | (2.2%) | 0.351 | 0.491 | |
| Leukopenia | 5 | (45.5%) | 6 | (13%) | 0.014 | 0.025 | 5.56 (1.28-24.03) |
| Chorea | 1 | (9.1%) | 2 | (4.3%) | 1.0 | 0.990 | |

*Group 1: patients with circulating immune complexes of IgG or IgM bound to beta-2-glycoprotein I (B2-CIC +). Group 2: patients without circulating immune-complexes (B2-CIC –). P-value < 0.05 was considered statistical significant, OR, odd ratio; CI, confidence interval. P adjusted: P-values adjusted for multiple comparisons.*

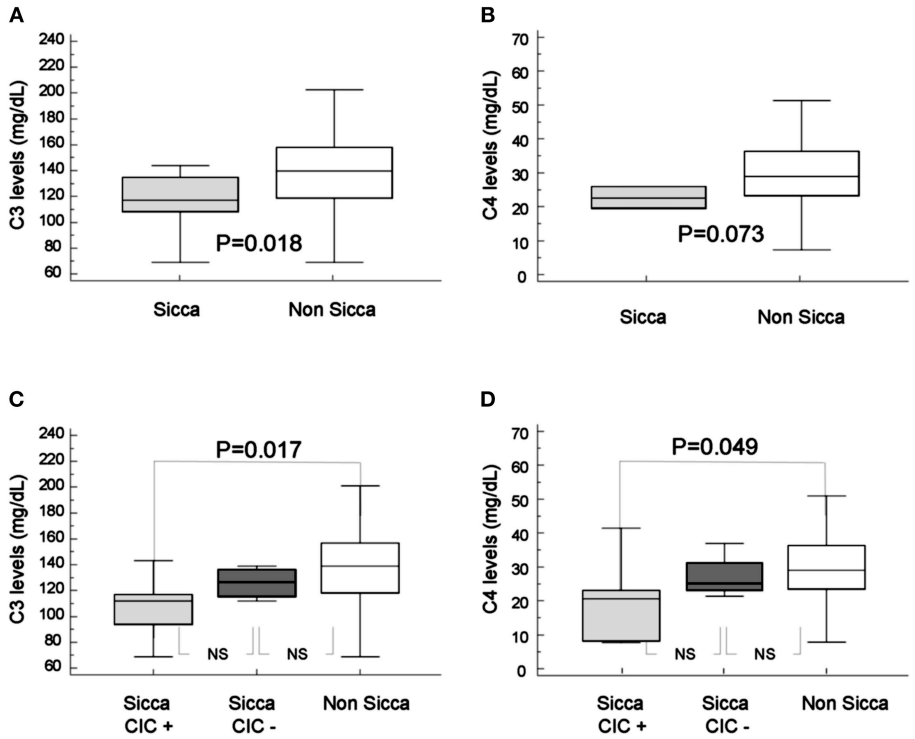


**FIGURE 2 |** Mean levels of C3 **(A)** and C4 **(B)** complement in patients with ophthalmic sicca (gray box) and without sicca (white box). Mean levels of C3 **(C)** and C4 **(D)** complement in patients with sicca and circulating immune-complexes (gray box), with sicca and without circulating immune-complexes (dark box) and without sicca (white box). Mean levels of C3 and C4 in 10 patients with sicca (115.1 ± 6.9 and 23.1 ± 3.4 mg/dL, respectively) and mean levels of C3 and C4 in 47 patients without sicca (140.5 ± 4.5 and 30.4 ± 1.7 mg/dL, respectively). Levels of C3 were significantly lower in patients with sicca ($p = 0.013$), levels of C4 were lower but the difference was not statistically significant ($p = 0.073$).

0.043, 0.021, 0.018; Fisher's exact test). When the presence of these clinical characteristics was compared in patients with triple-only aPL positivity vs. those of single or double positivity, the differences were not significant (6, 9, 18 vs. 20, 14, 17; *p*-values: 0.274, 0.658, 0.937) (**Figure 3** and **Table 4**).

## Sicca

The quadruple aPL positivity is also highly associated with sicca. Sicca was found in 50% of patients with quadruple positivity vs. 9% of patients with single or double aPL positivity ($p = 0.043$). Patients with triple-only aPL positivity presented a similar prevalence as those with single or double positivity (9.1 vs. 9%).

Mean levels of C3 and C4 complement factors in patients with sicca and quadruple aPL positivity were significantly lower than in patients with sicca and single/double aPL positivity (**Figure 4**).

# DISCUSSION

Prevalence of B2-CIC in the APS Serbian cohort is similar to that previously described for B2A-CIC in a Spanish cohort of patients with APS-clinical events (27, 28). Up to date, only the classical clinical APS manifestations (thrombosis and gestational morbidity) have been studied in patients with immune-complexes (28). For the first time, the present study has established the association between the two new biomarkers (B2G-CIC and B2M-CIC) and the clinical manifestations related to APS.

Despite the fact that B2GPI is a protein that is permanently present in the blood, most of the patients who were positive for anti-B2GPI antibodies did not form B2-CIC. The absence of B2-CIC could be explained because B2GPI circulates mainly in a "circular" conformation where the interactions between domains 1 and 5 would result in the shielding of the domain 1 epitopes (36) that would not be accessible for aPL. The CIC would form when β2GPI acquires a "hook" conformation where the epitopes are exposed (37).

The immune complexes clearance system (based on FC receptors and complement receptors) would immediately withdraw B2-CIC from circulation. B2-CIC would only be detectable in patients in whom the immune complex clearance system is insufficient to eliminate them.

This hypothesis is supported by the fact that immunocomplexes formed with IgG are more infrequent than those formed by IgM. Both immunoglobulins activate complement by classical pathway and would benefit from the immune complex clearance by complement receptors. However, B2-CIC can also be removed by the Fc gamma receptors system present in several cells of the immune system (38). This additional clearance mechanism is not present in the IgM immune complexes. Only a single receptor for IgM Fc is known

**TABLE 3 |** Clinical characteristics of APS patients with triple aPL positivity vs. single or double aPL positivity.

| Condition | Triple positivity *N* = 15 | % / SEM | Single / double positivity *N* = 42 | % / SEM | *p*-value |
|---|---|---|---|---|---|
| Age (years) | 46 | ±3.0 | 48.2 | ±1.9 | 0.548 |
| Sex (women) | 11 | (73.3%) | 25 | (59.5%) | 0.341 |
| Catastrophic APS | 3 | (20%) | 1 | (2.4%) | 0.023 |
| Primary APS | 7 | (46.7%) | 28 | (66.7%) | 0.172 |
| B2GPI Immune complexes (IgG or IgM) | 4 | (26.7%) | 7 | (16.7%) | 0.400 |
| B2GPI Immune complexes IgG | 1 | (6.7%) | 2 | (4.8%) | 0.777 |
| B2GPI Immune complexes IgM | 3 | (20%) | 5 | (11.9%) | 0.439 |
| **APS PATHOLOGY** | | | | | |
| ***Gestational morbidity*** | | | | | |
| Women in fertile age | 9 | (60%) | 25 | (59.5%) | 0.974 |
| Mean fetal loss | 1.6 | ±0.3 | 1.6 | ±0.2 | 0.972 |
| ***Thrombotic events*** | | | | | |
| Arterial thrombosis | 13 | (86.7%) | 31 | (73.8%) | 0.308 |
| Venous thrombosis | 8 | (53.3%) | 19 | (45.2%) | 0.590 |
| Pulmonary embolism | 7 | (46.7%) | 7 | (16.7%) | 0.022 |
| Inferior extremity deep vein thrombosis | 3 | (20%) | 9 | (21.4%) | 0.907 |
| Superior extremity arterial thrombosis | 2 | (13.3%) | 1 | (2.4%) | 0.103 |
| **VASCULAR RISK FACTORS** | | | | | |
| Diabetes Mellitus | 0 | (0%) | 2 | (4.8%) | 1.000 |
| Hypertension | 1 | (6.7%) | 4 | (9.5%) | 0.737 |
| Dyslipidemia | 2 | (13.3%) | 3 | (7.1%) | 0.467 |
| Trauma | 1 | (6.7%) | 2 | (4.8%) | 0.777 |
| Smoke | 5 | (33.3%) | 16 | (38.1%) | 0.743 |
| **OTHER IMMUNOLOGICAL MARKERS** | | | | | |
| Reactive protein C elevated | 4 | (26.7%) | 9 | (21.4%) | 0.678 |
| CH50 | 0 | (0%) | 1 | (2.4%) | 0.547 |
| C3 complement factor low | 0 | (0%) | 3 | (7.1%) | 0.288 |
| C4 complement factor low | 1 | (6.7%) | 0 | (0%) | 0.263 |
| Anti-DNA antibodies | 3 | (20%) | 3 | (7.1%) | 0.164 |
| Antinuclear autoantibodies | 5 | (33.3%) | 19 | (45.2%) | 0.423 |
| Rheumatoid Factor | 0 | (0%) | 2 | (4.8%) | 1.000 |
| Anti-Ro antibodies | 0 | (0%) | 2 | (4.8%) | 1.000 |
| Anti-La antibodies | 0 | (0%) | 1 | (2.4%) | 1.000 |

and although its functions are still not completely known, they seem to be related to the regulation of the immune response and have no relation with the clearance of immune complexes (39, 40).

LR is the most frequent cutaneous manifestation in APS patients, although it is not included as classification criteria. This can be the first clinical manifestation in primary APS in up to 40% of the patients and in up to 70% in patients with SLE and APS (41). In the first description of the syndrome, G. Hughes included LR as part of APS. LR was associated with venous and arterial thrombosis, spontaneous abortions, neurological manifestations and thrombocytopenia (42). Our study has established the association between LR and CIC for the first time. At present, the pathophysiology of LR is not completely known (43, 44). CIC could be deposited in the venous territory with slow blood circulation. The deposition would cause the formation of microthrombosis, thus hindering the return circulation that would, in consequence, lead to the appearance of LR.

Dry eye syndrome (DES) is a disorder affecting the tears and ocular surface accompanied by dryness, irritation, foreign body sensation, light sensitivity, and itching. It is more prevalent in patients with autoimmune diseases (45). Humoral immunity plays an important role in the pathogenesis of DES. B cell hyperactivity is related with the presence of autoantibodies, hypergammaglobulinemia, and the clinical/serological phenotypes mediated by immune complexes. The pathogenic role of autoantibodies in DES has been demonstrated by the autoantibodies transferred (46). Sjögren's syndrome (SjS) is characterized by the combination of DES and dry mouth (xerostomia) with a progressive lymphocytic infiltration on the lachrymal and salivary glands. Autoantibodies

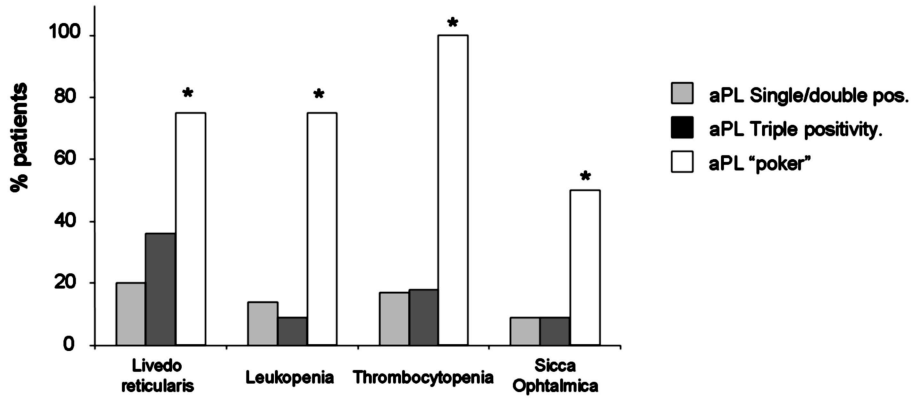


**FIGURE 3 |** Prevalence of aPL simple/double positivity (gray bar), triple aPL positivity (dark bar), and quadruple aPL positivity or poker aPL positivity (white bar) in patients with livedo reticularis, ophthalmic sicca, leukopenia and thrombocytopenia. *$p < 0.05$ respect simple/double positivity.

**TABLE 4 |** Clinical characteristics of APS patients with quadruple (left) and triple-only (right) aPL positivity vs. patients with single or double positivity.

| Condition | Quadruple aPL positivity | | | Triple-only aPL positivity | | |
|---|---|---|---|---|---|---|
| | OR | 95% CI | *p* | OR | 95% CI | *p* |
| Livedo reticularis | 12 | 1.1 to 133.6 | 0.043 | 2.3 | 0.5 to 10.1 | 0.274 |
| Leukopenia | 18 | 1.6 to 209 | 0.021 | 0.6 | 0.1 to 5.8 | 0.658 |
| Thrombocytopenia | 40.8 | 2.0 to 856 | 0.018 | 1.1 | 0.2 to 6.3 | 0.937 |
| Ophthalmic sicca | 10.7 | 1.1 to 105.3 | 0.043 | 1.1 | 0.1 to 11.4 | 0.958 |

*OR, odds ratio; CI, coefficient interval; aPL, antiphospholipid antibodies; p value < 0.05 was considered statistical significant.*

present in serum samples of SjS patients are autoantibodies to antigens Ro(SSA), La(SSB), or both (47, 48).

DES has been previously described as a common ocular manifestation in patients with antiphospholipid syndrome (49) that had already been described in the first publications in which APS was considered as an entity independent of other systemic autoimmune diseases (50). Most of these APS patients were positive for dry Schirmer's tests but did not fulfill the criteria to be qualified as having Sjogren' syndrome (51).

The prevalence of sicca in our cohort is high (17.5%), although it is within the range of prevalence described for the adult population (5–30%) (52). However, the prevalence of sicca was significantly higher (54.5%) when only patients positive for B2-CIC were considered. All the patients with APS and sicca in our cohort were negative for anti-SSA and anti-SSB autoantibodies, Schirmer's test positive and did not suffer dry mouth, suggesting that we are facing an autoantibody-mediated non-Sjogren Dry-Eye syndrome.

Thrombocytopenia, the most common non-criteria manifestation of APS (53), was significantly more frequent in patients with B2-CIC. The association between presence of aPL CIC and thrombocytopenia has also been described for B2A-CIC (21). Platelet activation is the first step in thrombus formation (54–56). The immune-complexes are bound exclusively to the platelet thrombus (54) and this phenomenon leads to the amplification of platelets activation and aggregation mediated by platelet membrane receptors. This mechanism would explain the thrombocytopenia in patients with CIC. Leukopenia was also more frequent in patients with CIC. Leukopenia is a typical feature in SLE patients and is associated with thrombocytopenia (57–59).

Complement activation plays an important role in the pathogenesis of thrombosis induced by aPL. Lower C3 and C4 complement factors levels in patients with B2-CIC reported in this study suggest a consumption of complement. Autoantibodies within the immune complexes could activate the classical pathway of the complement cascade. The complement activation and consumption could be mediated by CIC, as has already been described in other pathologies mediated by immune-complexes (60–63).

In contrast, complement is not consumed in patients with B2A-CIC. IgG and IgM isotype antibodies can fix the complement by the classic pathway, but not IgA isotype antibodies (21).

Triple aPL positivity is an increased risk factor for thromboembolic events in APS patients (64). In this study, patients with triple aPL positivity showed a higher prevalence of pulmonary embolism and infarction, and also catastrophic APS.

In our cohort, leukopenia, livedo reticularis, sicca, and especially thrombocytopenia were more frequent in patients with quadruple aPL positivity. These findings suggest that the presence of B2-CIC would be related to the pathogenesis of livedo reticularis, thrombocytopenia, leukopenia, and sicca.

The main limitation of the study is the low number of patients and the non-inclusion of asymptomatic controls. A new confirmatory study with a higher number of individuals is mandatory. Despite the low number of APS patients, the

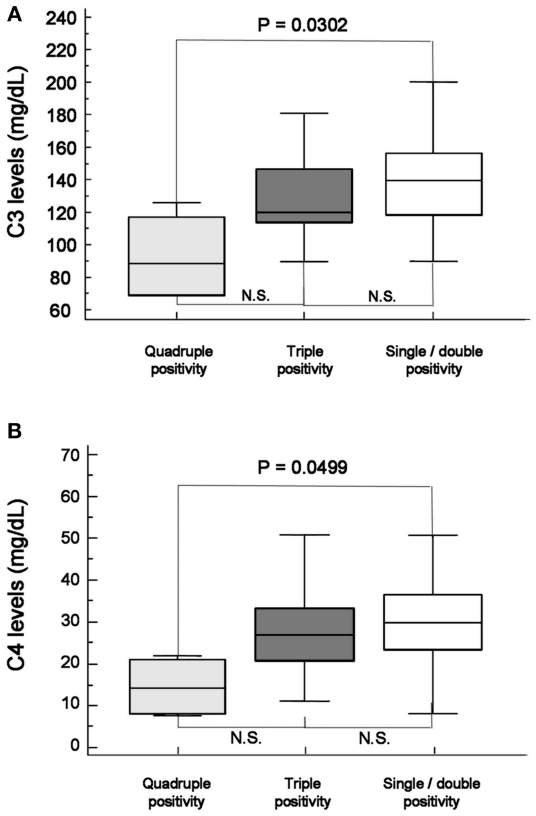


**FIGURE 4 |** Mean levels of C3 **(A)** and C4 **(B)** complement factor in patients with sicca and quadruple aPL positivity (gray) were significantly lower than patients with sicca and single/double aPL positivity(white). When they were compared with patients with triple positivity (dark), they were also lower, but did not become significant.

results are very strong. As the present work is retrospective and the serum samples were obtained after the patients had suffered the thrombotic events, it was not possible to determine whether patients with quadruple positivity had a higher risk of experiencing thrombosis or cardiovascular events than the rest and whether this risk was due to triple positivity or the presence of immunocomplexes. Further prospective studies with a large number of participants to help resolve these issues are necessary.

In summary, for the first time, B2-CIC (IgG and IgM) has been described and its presence has been associated with clinical manifestations related to APS as thrombocytopenia, LR, sicca ophthalmic, and leukopenia as well as higher complement consumption.

## AUTHOR CONTRIBUTIONS

AS and LS conceived the project, designed the research, discussed the results and wrote the manuscript. LS, NS, and GB were responsible for the patients' care and clinical data collection. DP, LN, and MS performed the determinations of antiphospholipid antibodies. MS and LN made the quantification of immune complexes. AS and DP were responsible for the database and the statistical analysis. All authors contributed to the data interpretation, reviewed the manuscript and agreed with the final version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02644/full#supplementary-material

**Supplementary Figure 1 |** Box and whiskers box of the distribution of the B2G-CIC and B2M-CIC values.

**Supplementary Table 1 |** Complementary clinical characteristics of the 57 APS patients included in the study.

**Supplementary Table 2 |** Comparison of the clinical characteristics of the two groups of patients. Group 1: patients with circulating immune complexes of IgG or IgM bound to beta-2-glycoprotein I (B2-CIC +), group 2: patients without circulating immune-complexes (B2-CIC –). *SEM, standard error of the mean, p-value < 0.05 was considered statistical significant, OR, odd ratio, CI, confidence interval.*

**Supplementary Table 3 |** Additional clinical characteristics of APS patients with triple aPL positivity vs. single or double aPL positivity.

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# Prevalence and Significance of Non-conventional Antiphospholipid Antibodies in Patients With Clinical APS Criteria

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**Background:** The biological diagnostics of antiphospholipid syndrome (APS) takes into account the persistent positivity for anticardiolipin and/or anti-β2GP1 antibodies and/or presence of lupus anticoagulant (LA). However, some non-conventional antiphospholipid antibodies have emerged that could help in the diagnosis of APS.

**Objectives:** To study the potential usefulness of non-conventional antiphospholipid antibodies in clinical practice.

**Methods:** Eighty-seven patients, aged from 15 to 92 years were included and classified in following groups: 41 patients positive for the conventional antibodies with clinical criterion of APS (31 with primary APS and 10 secondary), 17 seronegative APS (SNAPS) patients (i.e., persistent negativity for the conventional antibodies with a strong clinical suspicion of APS), 11 asymptomatic antiphospholipid antibodies carriers (i.e., persistent positivity for the conventional antibodies without clinical evidence of APS), and 18 patients presenting with a first thrombotic or obstetrical event. IgG and IgM were detected to the following antigens: phosphatidylserine/prothrombin (PS/PT) by ELISA, and phosphatidic acid, phosphatidyl-ethanolamine, phosphatidyl-glycerol, phosphatidyl-inositol, phosphatidylserine, annexin V, prothrombin by immunodot. Anti-β2GP1 IgA, and anti-β2GP1 domain 1 IgG were detected by chemiluminescence.

**Results:** Positivity for the non-conventional antibodies was correlated with APS severity; patients with catastrophic APS (CAPS) being positive for 10.7 (Median, Range: 5–14) non-conventional antibodies. 9/17 seronegative patients were positive for at least one of non-conventional antibodies. A study of non-supervised hierarchical clustering of all markers revealed that anti-PS/PT antibodies showed high correlation with the presence of LA. All patients with APS triple positivity (highest risk profile) exhibited also persistent positivity for anti-PS/PT antibodies.

**Conclusions:** Our data obtained from a prospective cohort constituted mainly by patients with primary APS, suggest that non-conventional APS antibodies may be useful

for patients classified as SNAPS. They demonstrate the potential value of aPS/PT antibodies as a strong marker of APS. We propose that anti-PS/PT antibodies could be a surrogate APS biological marker of LA to classify in high-risk profile patients treated by direct oral anticoagulants (DOACs), in whom LA detection cannot be achieved.

**Keywords: antiphospholipid syndrome, autoantibodies, anti-phosphatidylserine-prothrombin antibodies, lupus anticoagulant, thrombosis**

## INTRODUCTION

The antiphospholipid syndrome (APS) is an autoimmune disorder characterized by thrombotic and/or obstetrical manifestations associated with the persistent positivity for at least one of three markers: lupus anticoagulant (LA), anti-cardiolipin antibodies (aCL) or anti-beta2-glycoprotein I (aβ2GP1) antibodies of either IgG or IgM isotype (1, 2).

The prevalence of APS is estimated at around 40–50 cases per 100,000 persons (3). APS can be primary, i.e., without any other definable disease, or secondary, i.e., associated with other diseases, the most often being systemic lupus erythematosus (4). According to the site of thrombosis and the number and size of vessels involved, APS has different kinds of clinical manifestations such as peripheral thrombosis, neurological, pulmonary, cardiac or obstetric manifestations (recurrent fetal losses or *intra utero* growth retardation) (5). The most severe form of APS, called catastrophic APS (CAPS) is characterized by multiorgan failure due to diffuse thrombotic microvasculopathy (6, 7), and counts for <1% of APS (5).

Secondary prevention of thrombosis is based on a long-term anticoagulation therapy (8). In some severe cases, rituximab may be indicated (9). The treatment of CAPS requires an aggressive therapy using anticoagulation associated with high-dose steroids, plasma exchange or intravenous immunoglobulin (10).

Considering its high rate of thrombotic recurrence that may be efficiently prevented by prophylactic anticoagulant therapy, an accurate identification of patients with APS is crucial. Also, treatment of APS women during pregnancy leads to a very significant improvement of fetal and maternal outcomes (11, 12).

Despite progresses in the treatment, patients with APS still develop significant morbidity and mortality (13, 14), and rapid diagnosis and treatment as well as the determination of reliable prognostic markers are of ultimate importance.

Antiphospholipid antibodies (APA) are constituted by a heterogeneous group of autoantibodies directed against anionic phospholipids, phospholipid-binding plasma proteins or protein-phospholipid complexes (15). Apart from conventional biological markers (LA, aCL and aβ2GP1), numerous other markers of APS have been studied such as antibodies against phosphatidyl-ethanolamine (16), phosphatidylserine-prothrombin (17), that could be helpful in the diagnosis of so-called seronegative APS (SNAPS) (18, 19), which defines a group of patients with clinical manifestations of APS but with persistently negative aCL, aβ2GP1 antibodies and LA. Furthermore, the study of the autoantibodies' epitope specificities revealed that the β2GP1 domain I is the most specific target of APS (20). In addition, antibodies of IgA isotype, mainly against β2GP1, have been suggested as a new marker in SNAPS patients (21, 22).

In recent years, direct oral anticoagulants (DOACs) therapies directly inhibiting thrombin or factor Xa are used more frequently and many studies have shown that these drugs may influence LA testing. DOACs induce false positive results of LA detection even when their concentration is very low. Therefore, LA testing should not be performed during treatment with DOACs (23). LA is the conventional biological marker associated with the strongest risk for thrombosis and other clinical APS manifestations. Moreover, it has been demonstrated that triple positivity for conventional markers in APS patients or in asymptomatic APA carriers is associated with significantly higher risk of thrombosis than single or double positivity (24, 25). LA testing in patients treated by DOACS would necessitate discontinuing the treatment for at least 3 days but it may be unsafe. Interestingly, presence of anti-phosphatidylserine/prothrombin antibodies has been shown to be closely associated with presence of LA (26).

In this study, we aimed to explore the usefulness of several non-conventional APS markers for the diagnosis of patients with clinical manifestations of APS and SNAPS. We also wondered if anti-phosphatidylserine-prothrombin antibodies could replace the use of LA testing in patients treated with DOACs. Presence and persistence of 10 non-conventional APS markers have been studied prospectively in patients presenting with APS or thrombosis and in healthy donors.

## MATERIALS AND METHODS

### Patients

Eighty seven patients were included prospectively between September 2015 and May 2017. Patients were aged from 15 to 92 years old and comprised of 50 women and 37 men (**Table 1**). Additionally, a group of 30 healthy donors (HD) was used as controls. The study was done in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. No additional sample from the patients was collected for the study. All patients were seen in the context of their routine care, clinically evaluated and informed about the study and about the computerization of personal health data by the same physician (LD) during the follow up of the study. The patients gave oral informed consent in accordance with French legal standards for observational studies. This study has been approved by the Institutional Review Board according to standards currently applied in France (*Commission Nationale*

**TABLE 1 |** Patients' characteristics.

| | Frequency (number) |
|---|---|
| **AGE (YEARS)** | |
| 15–30 | 15% (13) |
| 31–60 | 66% (57) |
| 61–92 | 19% (17) |
| **SEX** | |
| Females | 57% (50) |
| Males | 43% (37) |
| **DISEASE GROUP:** | |
| 1. APS | 47% (41) |
| *According to clinical signs*: | |
| Thrombosis: | 88% (36) |
| CAPS | (3) |
| Arterial | (10) |
| Venous | (21) |
| Both | (2) |
| Obstetrical morbidity only | 12% (5) |
| *According to number of conventional biological markers:* | |
| With 1 biomarker | 32% (13) |
| With 2 biomarkers | 24% (10) |
| With 3 biomarkers | 44% (18) |
| *Primary or secondary*: | |
| Primary | 76% (31) |
| Associated with lupus | 22% (9) |
| Associated with Autoimmune Hepatitis | 2% (1) |
| 2. SNAPS | 20% (17) |
| Thrombosis: | 56% (9) |
| Arterial | (1) |
| Venous | (7) |
| Both | (1) |
| Obstetrical complications | 44% (8) |
| 3. Asymptomatic APA carriers | 13% (11) |
| 4. First Thrombotic/obstetrical event: | 22% (18) |
| Arterial | (6) |
| Venous | (10) |
| Both | (1) |
| Obstetrical | (1) |

*APS, Anti-phospholipid syndrome; CAPS, Catastrophic anti-phospholipid syndrome; SNAPS, Seronegative anti-phospholipid syndrome; APA, Anti-phospholipid antibodies.*

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Blood samples were collected and sera were analyzed. According to the clinical data and to the biological parameters, the patients were classified into the following groups: (1) APS; (2) SNAPS, i.e., patients with strong clinical suspicion of APS but persistently negative for conventional biological markers; (3) asymptomatic antiphospholipid antibodies (APA) carriers, i.e., clinically asymptomatic individuals presenting with persistent antiphospholipid antibodies positivity (discovered fortuitously, mostly during preoperative assessment of hemostasis); (4) first thrombotic event and/or obstetrical morbidity group, i.e., patients presenting with a first thrombotic or obstetrical event.

Patients were classified according to the type of clinical feature: arterial thrombosis, venous thrombosis, both, obstetrical complications or CAPS (**Table 1**).

Our APS cohort is composed by a majority (76%, $n = 31$) of patients presenting with primary APS whereas 24% had an APS secondary to lupus (9 patients) or other autoimmune disease [autoimmune hepatitis: 1 patient] (**Table 1**).

## Methods

The two different coagulation tests used to detect lupus anticoagulant (LA) according to ISTH recommendations (27) were: dilute Russell venom viper time (dRVVT) using LA1 reagents (Siemens, Germany) and aPTT using Automated APTT (Trinity Biotech, Ireland).The phospholipid dependence was confirmed by positive phospholipid-neutralizing assays for both tests with a dRVVT screen (LA1 reagent)/confirm (LA2 reagent, Siemens, Germany) ratio and a ratio of aPTT with silica (LA sensitive reagent)/Kaolin PTT (LA insensitive reagent, CK Prest, Stago, France).

Anti-CL and aβ2GP1 antibodies of IgG isotype were detected in the serum by the routinely used ELISA methods: Cardiolisa, (Theradiag, Croissy-Beaubourg, France) and QUANTA Lite® $\beta_2$ GP1 IgG (Inova Diagnostics Werfen, Les Lilas, France). Anti-CL and aβ2GP1 antibodies of IgM isotype were detected by the immunodot technique (Cf below).

Anti-phosphatidylserine-prothrombin (aPS/PT) antibodies of IgM and IgG isotypes were measured in the serum by ELISA (Quanta Lite, INOVA Diagnostics, Werfen, Les Lilas, France).

The immunodot technique (Anti-Phospholipid 10 Dot, Generic Assays, Eurobio, Les Ulis, France) was performed in the serum using the BlueDiver Instrument (D-Tek, Ingen, Chilly Mazarin, France) allowing the detection of IgG and IgM antibodies directed against the following antigens: CL, β2GP1, phosphatidic acid (PA), phosphatidyl-ethanolamine (PE), phosphatidyl-glycerol (PG), phosphatidyl-inositol (PI), phosphatidylserine (PS), annexin V (A5), and prothrombin (PT).

Anti-β2GP1of IgA isotype and aβ2GP1 domain I antibodies were measured in the serum by BIO-FLASH Chemiluminescent Immuno Assay technology (QUANTA Flash® $\beta_2$ GP1 IgA Inova Diagnostics Werfen, Les Lilas, France).

Prism and Medcalc Softwares were used for the statistical analysis and Genesis software (version 1.8.1) was used to perform the hierarchical clustering of APS biomarkers. The titers of APL antibodies were compared using Mann-Whitney-Wilcoxon test. Correlation between the presences of different antibodies was measured by Kendall correlation using the software R version 3.4.4 [R (28)] and the package ggcorrplot version 0.1.1 (29).

# RESULTS

## Results of Classical APS Markers

The distribution and prevalence of the classical APS markers in our cohort are depicted in the **Supplementary Figure 1**.

Presence of aCL antibodies was detected in 95% patients with APS and in 45% in the group of asymptomatic APA carriers but was not detectable in the group of SNAPS and thrombosis/obstetric (**Supplementary Table 1**). Their levels were significantly higher in the APS patients with three conventional biomarkers than in the other patients' groups (**Supplementary Figure 1A**).

Presence of aβ2GP1 was detected in 51% of patients with APS and in 18% of patients from the group of asymptomatic

APA carriers and was not detectable in the group of SNAPS and thrombosis/obstetric. IgG aβ2GP1 positivity was observed almost only in the group of APS patients with 3 biomarkers (**Supplementary Figure 1B**). The patients presenting with CAPS exhibited the highest titers of aCL and aβ2GP1 antibodies (**Supplementary Figures 1C,D**).

LA positivity was observed in 68% of APS patients and in 100% of patients from the group of asymptomatic APA carriers and was not detectable in the group of SNAPS and thrombosis/obstetric. Its prevalence in different groups of patients is shown in the **Supplementary Table 1**.

## Results of aPS/PT Antibodies

Anti-PS/PT antibodies of IgG and IgM isotypes were measured in all groups of patients and controls.

Presence of IgG aPS/PT was detected in 43.9% of patients in the group of APS, 5.6% in the group of SNAPS, 18.2% in the group of asymptomatic APA carriers. It was not detectable in the group of thrombosis/obstetric and in HD. Their levels were significantly higher in the APS patients with two and three biomarkers than in the other groups of patients (**Figure 1A**).

Presence of IgM aPS/PT was detected 65.8% in the group of APS, 16.7% in the group of SNAPS, 81.8% in the group of asymptomatic APA carriers, 5.3% in the group of thrombosis/obstetric, and 6.7% in HD. Their levels were significantly higher in the groups of APS with two and three biomarkers but also in the group of asymptomatic APA carriers as compared to the other groups (**Figure 1B**).

The comparison of the levels of aPS/PT antibodies in APS patients according to the different types of thrombosis, showed significant differences between HD and APS patients with venous thrombosis, obstetrical morbidity and CAPS for IgG isotype (**Figure 1C**). For IgM isotype significant differences were observed between HD and APS patients with venous thrombosis and CAPS (**Figure 1D**). Patients with CAPS had statistically significant higher levels of IgG aPS/PT antibodies than patients with arterial thrombosis ($p = 0.029$, **Figure 1C**).

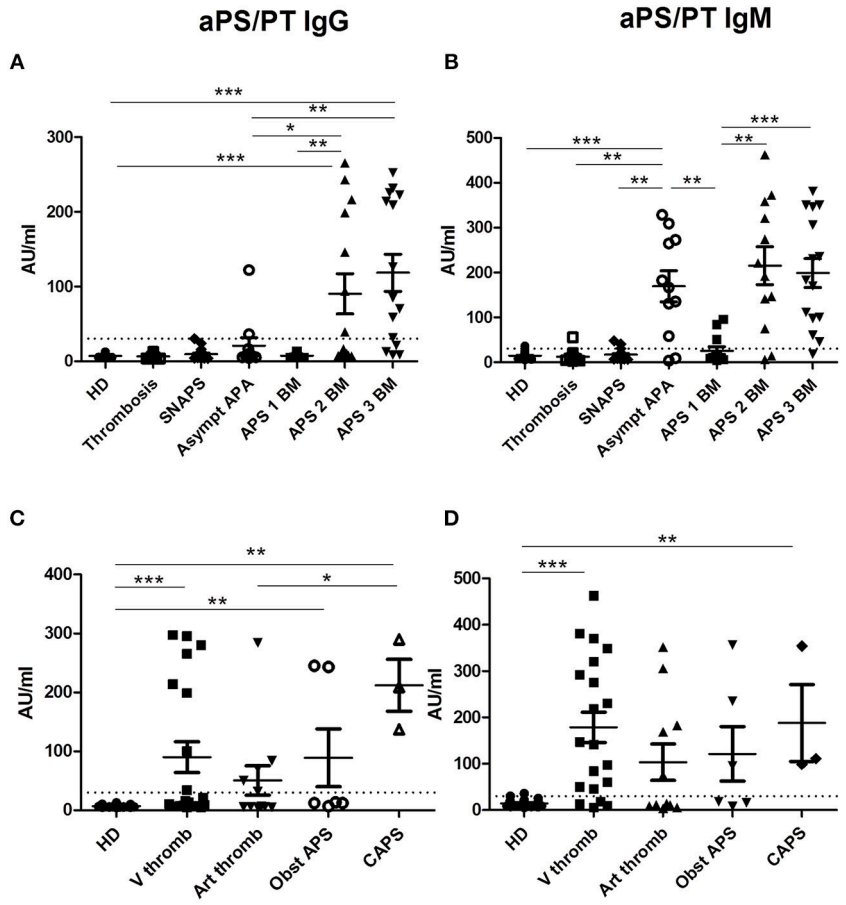


**FIGURE 1 |** Results of anti-PS/PT antibodies. The distribution of anti-PS/PT IgG or IgM antibodies is shown in all groups of patients and in HD (**A,B** according to the disease group; **C,D** according to the clinical type of APS). The positive thresholds (30 AU/mlAU/ml) are shown by dotted lines. **(A,C)** aPS/PT antibodies of IgG isotype, **(B, D)** aPS/PT antibodies of IgM isotype. The data are presented as mean ± s.e.m. with all individual dots shown. The *p*-values were calculated using Mann-Whitney test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. *HD*, Healthy Donors; *SNAPS*, seronegative anti-phospholipid syndrome; *Asympt APA*, asymptomatic carriers of antiphospholipid antibodies; *APS 1 BM*, APS with 1 biomarker; *APS 2 BM*, APS with 2 biomarkers; *APS 3 BM*, APS with 3 biomarkers; *V thromb*, venous thrombosis; *Art thromb*, arterial thrombosis; *Obst APS*, obstetrical APS; *CAPS*, Catastrophic anti-phospholipid syndrome.

## Results of aβ2GP1 Antibodies of IgA Isotype and aβ2GP I Domain I Antibodies of IgG Isotype

IgA aβ2GP1 were found only in patients with APS with two (31.6%) or three biomarkers (60.9%) and were negative in the other groups (**Figure 2A**). The levels of IgA aβ2GP1 were significantly higher in the group of APS patients with three biomarkers than those with two biomarkers ($p = 0.0002$).

IgG aβ2GP1 domain I (aβ2GP1 D1) were detected in 50% in the group of APS and in 11.1% in the group of asymptomatic APA carriers but were not detectable in the groups of SNAPS, thrombosis/obstetric and in HD (**Figure 2B**). Their levels were higher in the APS patients with triple positivity than those observed in patients with two biomarkers (mean = 571 vs. mean = 82 AU/ml, $p = 0.0027$). Of note, one patient from the asymptomatic APA carriers' group was highly positive for aβ2GP1 D1 (314 AU/ml, normal values <20 AU/ml).

No correlation was found between the clinical type of APS and level of these antibodies but in CAPS patients the level of IgA aβ2GP1 was significantly higher than in patients with obstetrical APS (**Figure 2C**) and the level of aD1 β2GP1 in CAPS was significantly higher than in patients with venous thrombosis (**Figure 2D**).

## Frequency and Distribution of Other Non-conventional APS Antibodies Tested by Immunodot

Presence of other non-conventional markers of APS was searched in serum from all groups of patients and from healthy donors by a semi-quantitative analysis (immunodot).

A higher frequency of positivity or titer in patients compared to HD was observed for IgG directed against PG, PA, PI, PS, and for IgM anti-PI, PS, PA, and PT (**Supplementary Figure 2**). No positivity was observed for anti-PE IgG and IgM.

The **Figure 3** shows the mean number of positive markers per patient for each group, compared to the HD. In the group of thrombosis, in patients with SNAPS and in the asymptomatic patients with APA, the number of positive markers was inferior or equal to one per patient whereas in APS patients it was from 2 to 6 per patient. The highest number of positive markers was

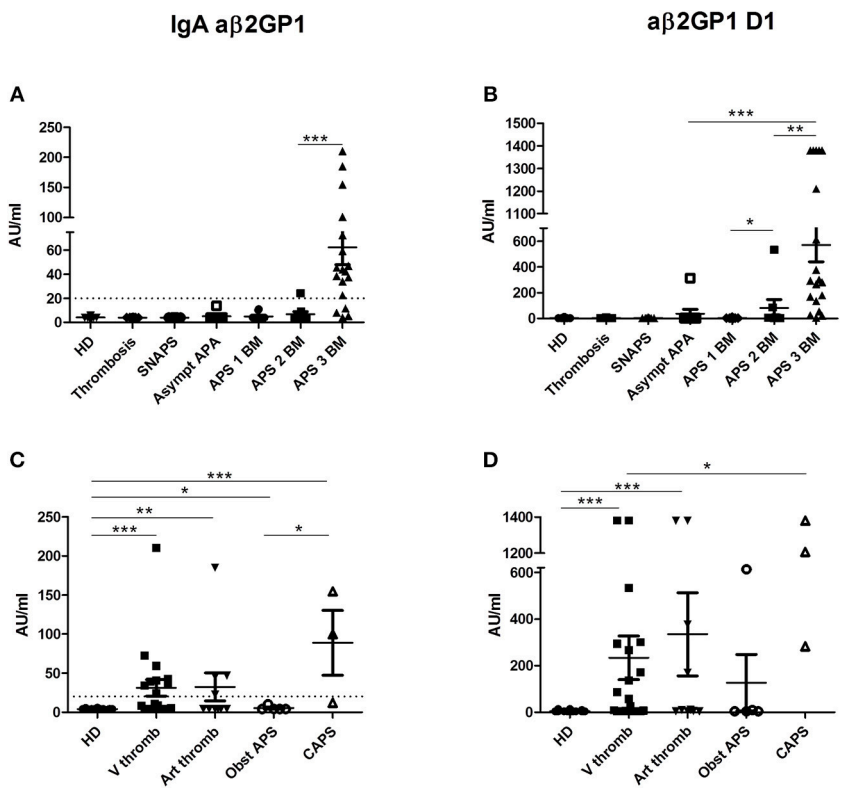


**FIGURE 2 |** Results of anti-β2GP1 IgA and anti- β2GP1 D1 IgG antibodies. The distribution of IgA aβ2GP1 and aβ2GP1 D1 antibodies is shown in all groups of patients and in HD (**A,B** according to the disease group; **C,D** according to the clinical type of APS). The data are presented as mean ± s.e.m. with all individual dots shown. The *p*-values were calculated using Mann–Whitney test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. The limit of positivity for IgA aβ2GP1 (20 AU/ml) is shown by the dotted line **(A,C)**. The limit of positivity for aβ2GP1 D1 is 20 IAU/ml (not shown) **(B,D)**. **(A)** Statistical differences are significant between APS 3 BM and all other groups ($p < 0.001$). **(B)** In addition to differences shown in the graph, statistical differences are significant between APS 3 BM, and APS 2 BM ($p < 0.01$) and between APS 3 BM and all other groups ($p < 0.001$). *HD*, Healthy Donors; *SNAPS*, seronegative anti-phospholipid syndrome; *Asympt APA*, asymptomatic carriers of antiphospholipid antibodies; *APS 1 BM*, APS with 1 biomarker; *APS 2 BM*, APS with 2 biomarkers; *APS 3 BM*, APS with 3 biomarkers; *V thromb*, venous thrombosis; *Art thromb*, arterial thrombosis; *Obst APS*, obstetrical APS; *CAPS*, Catastrophic anti-phospholipid syndrome.

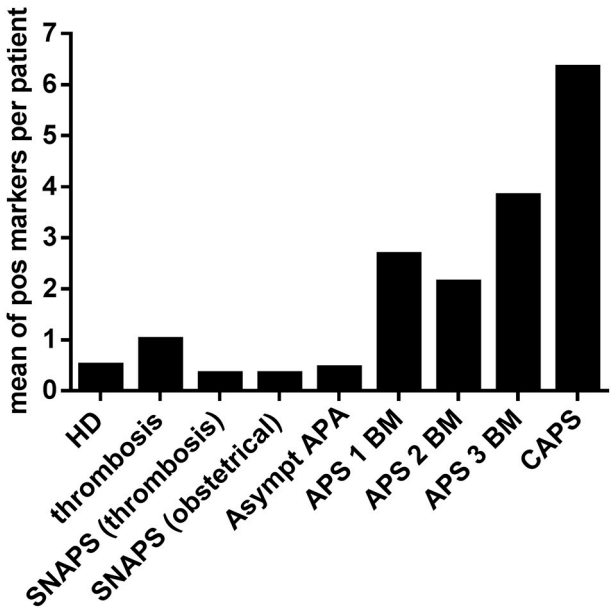


**FIGURE 3 |** Frequency of other APS non-conventional markers in the different groups of patients and HD. Non-conventional APS markers of IgG and IgM isotypes were detected by immunodot technique in sera of different groups of patients and in HD. Y ax shows a mean of positive markers (PA, PG, PI, PS, A5, and PT of IgG and IgM isotypes) per patient in each group. *HD*, Healthy Donors*; SNAPS*, seronegative anti-phospholipid syndrome*; Asympt APA*, asymptomatic carriers of antiphospholipid antibodies; *APS 1 BM*, APS with 1 biomarker; *APS 2 BM*, APS with 2 biomarkers; *APS 3 BM*, APS with 3 biomarkers; *V thromb*, venous thrombosis; *Art thromb*, arterial thrombosis; *Obst APS*, obstetrical APS; *CAPS*, Catastrophic anti-phospholipid syndrome; *Pos*, positive.

observed in the group of CAPS with a mean of 6.3 IgG or IgM markers per patient when aPS/PT antibodies are excluded, and 10.7 (range from 5 to 14) markers per patient when aPS/PT are included in the analysis. All patients with CAPS were positive for anti-PA and anti-PS IgG antibodies and 2 from 3 patients for anti-PG and anti-PI IgG antibodies. Antibodies of IgM isotype were less frequent in CAPS patients.

## Correlation Between the Different Non-conventional Markers

In APS patients, the highest correlation with conventional markers was observed for IgG directed to PA and PS. We observed a significant correlation between the presence of anti-PA and anti-PS antibodies (Spearman's coefficient of correlation is 0.78 for IgG and 0.75 for IgM), and between anti-PG and anti-PI antibodies (Spearman's coefficient of correlation: 0.84 for IgG and 0.6 for IgM). A significant correlation was also observed between anti-PS, anti-PT, and anti-PS/PT IgM positivity but at lower degree (Spearman test $< 0.5$, **Supplementary Figure 3**).

## Clinical Performances of the Tests

The **Table 2** summarizes the results of positive and negative predictive values for clinical APS obtained for the aPS/PT, aB2GP1 IgA and aβ2GP1 D1 antibodies. The calculations were performed using a control group composed by the HD and asymptomatic APA carriers.

**TABLE 2 |** Clinical performances of non-conventional APS markers.

| | a/β2GP1 IgA | a/β2GP1 domain I | a/PS PT IgG | a/PS PT IgM | PS/PT IgG+IgM |
|---|---|---|---|---|---|
| Sensitivity (%) | 38.5 (23.4–55.4) | 50 (33.4–66.6) | 43.9 (28.5–60.2) | 65.8 (49.4–79.9) | 43.9 (28.5–60.2) |
| Specificity (%) | 100 (90.7–100) | 97.4 (86.5–99.9) | 95.1 (83.5–99.4) | 73.2 (57.1–85.8) | 97.6 (87.1–99.9) |
| PPV (%) | 100 | 95 (72.8–99.3) | 90 (68.8–97.3) | 71 (58.6–81) | 94.7 (71.6–99.2) |
| NPV (%) | 61.3 (55.3–67) | 66.7 (59.2–73.4) | 62.9 (56.2–69.2) | 68.2 (57.4–77.3) | 63.5 (56.9–69.6) |
| PLR | NA | 19.5 (2.74–138.5) | 9 (2.23–36.33) | 2.45 (1.41–4.26) | 18 (2.52–128.6) |
| NLR | 0.62 (0.48–0.79) | 0.51 (0.37–0.71) | 0.59 (0.45–0.78) | 0.47 (0.29–0.74) | 0.58 (0.44–0.76) |

*Values are shown with 95% CI. For the calculations of predictive values the control group included HD, thrombosis/obstetric and asymptomatic APA groups. PPV, positive predictive value; NPV: negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio.*

The aPS/PT antibodies of IgM isotype had the highest sensitivity, comparable to the LA sensitivity, while all antibodies of IgG isotype had high specificities. Negative likelihood ratio of aPS/PT antibodies of IgM isotype was the lowest (0.47) while the positive likelihood ratio value was the highest for aβ2GP1 D1 (19.5) and the combination of aPS/PT antibodies of IgG and IgM isotypes (18) (**Table 2**). The positive likelihood ratio could not be calculated for aβ2GP1 IgA antibodies as their specificity was 100%.

## Non-supervised Hierarchical Clustering of Conventional and Non-conventional APS Markers

Genesis software v 1.8.1 (30) was used to perform a hierarchical clustering of all tested APS biomarkers in all groups of patients. We used a complete linkage approach and the distances of Pearson correlation. By this method we observed that aPS/PT antibodies of IgM isotype and LA were clustered together (**Figure 4**). The aPS/PT antibodies of IgG isotype and LA were also clustered in close proximity. In the group of asymptomatic APA carriers only aPS/PT antibodies of IgM isotype and LA were present, whereas in APS group aPS/PT antibodies of IgM isotype and LA were accompanied by aPS/PT antibodies of IgG isotype.

A very strong correlation between the positivity for aPS/PT antibodies of IgM isotype and LA presence was observed (**Table 3**). Indeed, 35 out of 40 patients with LA (87.5%) were positive for aPS/PT of IgM isotype. When taking into account also IgG aPS/PT, 36 out for 40 patients with LA, were also positive for aPS/PT antibodies (90%).

## Non-conventional Markers in SNAPS

Among the 17 patients with SNAPS, 9 were positive for at least 1 marker and 4 for more than 1 marker (**Supplementary Table 2**). Both IgG and IgM isotypes were detected in these patients. The

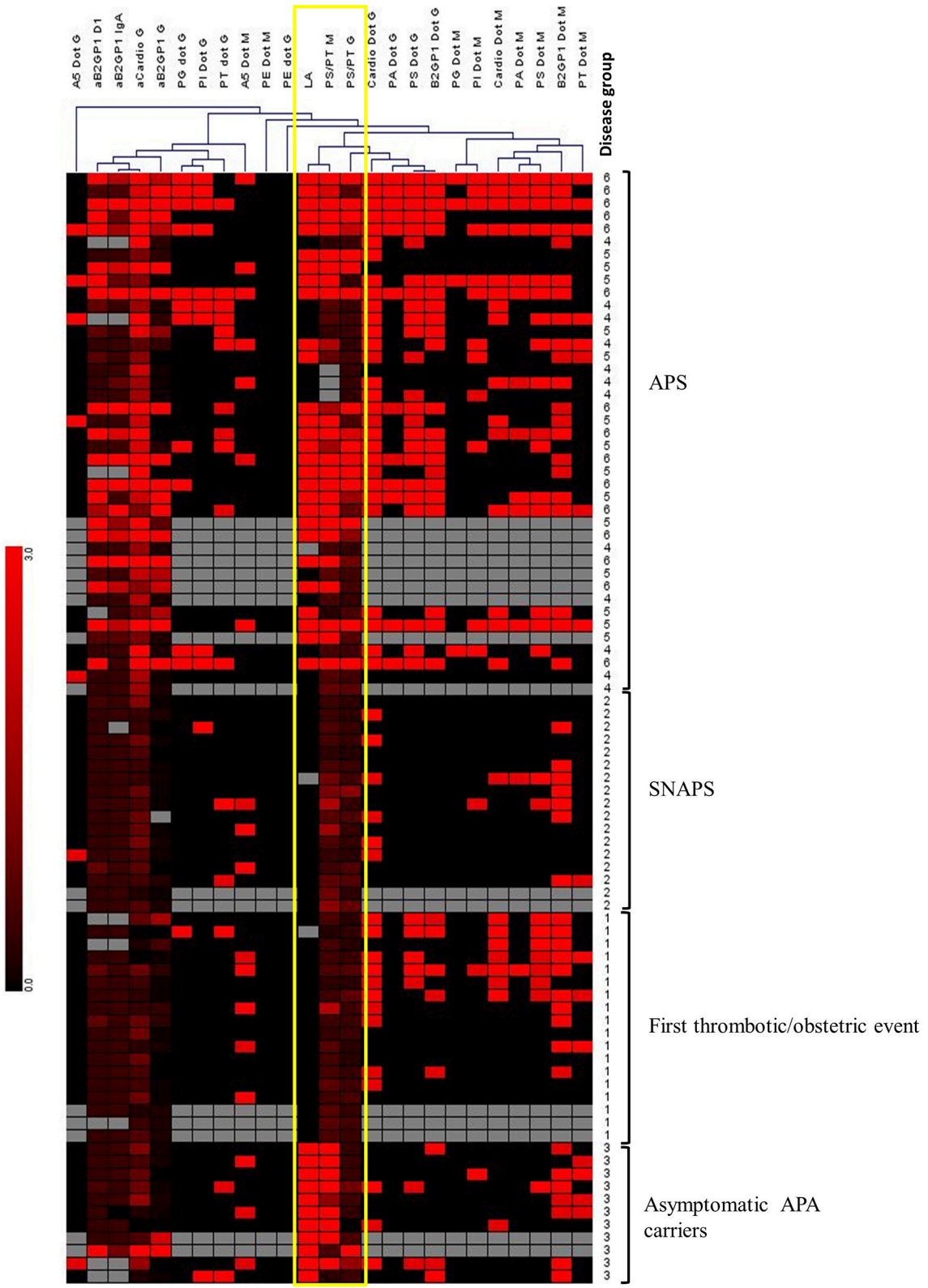


**FIGURE 4 |** Hierarchical clustering of APS biomarkers using Genesis software. Unsupervised hierarchical clustering was performed, using Pearson correlation and complete linkage analysis. Each column corresponds to an APS marker. Each line corresponds to a patient with a number corresponding to the "disease group": 1, Thrombosis; 2, SNAPS; 3, asymptomatic APA carrier; 4, APS with 1 biomarker; 5, APS with 2 biomarkers; 6, APS with 3 biomarkers. Colors are correlated with the positivity of the markers from negative (in black) to highly positive (in bright red). Gray boxes correspond to absence of data. This analysis reveals the close clustering of LA with anti-PS/PT IgG and IgM among APS patients, and with anti-PS/PT IgM alone among asymptomatic APA carriers (framed in yellow).

**TABLE 3 |** Correlation between LA and anti-PS/PT antibodies.

| | IgG anti-PS/PT | IgM anti-PS/PT | IgG and/or IgM anti-PS/PT |
|---|---|---|---|
| Sensitivity (%) | 50 (33.8–66.2) | 87.5 (73.2–95.8) | 90 (76.3–97.2) |
| Specificity (%) | 100 (92.1–100) | 91.1 (78.8–97.5) | 91.1 (78.8–97.5) |
| PPV (%) | 100 | 89.7 (77.3–95.7) | 90 (77.8–95.8) |
| NPV (%) | 69.2 (62.3–75.4) | 89.1 (78.2–94.9) | 91.1 (80.1–96.3) |
| PLR | – | 9.84 (3.83–25.27) | 10.12 (3.95–25.95) |
| NLR | 0.50 (0.37-0.68) | 0.14 (0.06–0.31) | 0.11 (0.04–0.28) |
| Chi2 | 27.4 | 52.7 | 55.9 |

*The performances of anti-PS/PT antibodies were calculated for all patients positive for LA. PPV, positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio; $Chi^2$, chi square test.*

markers found in SNAPS patients were PS/PT, PI, A5, and PT for IgG isotype and PS/PT, PA, PI, PS, A5, and PT for IgM isotype.

IgG aPS/PT were found in 1 patient but they did not persist 12 weeks later. IgM aPS/PT were detected in 3 patients. However, in one of them the antibodies were not persistent; in the two others the persistence was not assessed.

## DISCUSSION

This study aimed to explore the usefulness of several non-conventional APS markers for the diagnosis of APS, to assess whether they might play an additional role for the disease classification or for estimation of disease severity its severity. We have studied the presence and the persistence of IgA aβ2GP1 and IgG aβ2GP1 domain 1 and IgG and IgM directed against the PS/PT complex, and against phosphatidic acid (PA), phosphatidyl-ethanolamine (PE), phosphatidyl-glycerol (PG), phosphatidyl-inositol (PI), phosphatidylserine (PS), annexin V (A5), and prothrombin (PT) in a cohort of patients prospectively constituted. It comprised patients presenting with clinical APS, patients with a first episode of thrombosis and/or APS obstetric criterion but also patients classified as SNAPS and patients with at least one persistent positive APS marker without clinical manifestation.

To date, aPS/PT antibodies are not included in the APS laboratory criteria but their positivity has been recently proposed as a part of the global APS score (GAPSS) (31, 32), and has been shown to be a strong prognostic factor for both arterial and venous thrombosis (33). Our data confirm the high prevalence of aPS/PT antibodies in APS patients (34). We found IgM and IgG aPS/PT in 65.8 and 43.9% of our APS patients, respectively, and their levels were correlated with the number of positive classical markers. All CAPS patients were found positive for IgG and IgM aPS/PT. However, we failed to correlate these antibodies positivity with the type of thrombosis presented by the patients.

Interestingly, there was a striking positivity for IgM aPS/PT in the group of asymptomatic patients with APA (81.8%) whereas a positivity for IgG isotype was not frequent (18.2%). The majority of APA carriers in our study was detected by a pre-surgery assessment of coagulation tests and all were discovered positive firstly for LA. We found a very strong correlation between LA and aPS/PT IgM antibodies (chi-square test = 52.7) and this association was confirmed by our analysis by hierarchical clustering. Previously, it was shown that co-existence of these two parameters is frequent in APS patients (26). Atsumi et al have shown a strong correlation between the presence of aPS/PT antibodies of IgG and IgM isotypes and the presence of LA, whatever the detection method used (34). Our data confirm these findings but also reveal their co-existence in the group of asymptomatic APA carriers in which LA is mainly correlated with presence of IgM aPS/PT. This association might suggest that aPS/PT of IgM isotype may be less pathogenic than those of IgG isotype suggesting that the management of these patients in term of thrombosis prevention and follow up might be less strict.

LA detection method is not accurate for patients treated by DOACs even at low concentration (35). Treatment with DOACs could give false positive results of LA with dilute Russel viper venom time (DRVVT) assay whereas activated partial thromboplastin time (APTT) assay is less influenced (23). It is especially important to be able to replace LA testing, when it could not be measured, with other suitable and similar marker as LA has been shown to be the strongest risk factor of thrombosis comparing to aCL and aβ2GP1 (36). Conventional APA (i.e., LA, aβ2GP, and aCL) triple positivity allows to identify the APS group of patients with persistence of APA (37) and especially with the highest risk of thrombosis or obstetric morbidity recurrence (38, 39). Thus, LA detection is crucial in case of thrombosis' recurrence during the treatment or if suspension of oral anticoagulation is considered (23). Unlike LA detection, anti-PS/PT antibodies are detectable by immunological assay which can be performed on small sample volume of serum or plasma and is not influenced by anti-thrombotic treatments.

So, in these situations, aPS/PT antibodies (IgM and IgG) measuring could be considered to help to confirm or rule out the presence of LA (40).

Despite technical progresses a consistent group of patients with clinical symptoms remains classified as "seronegative." Recently, even a case of CAPS, has been reported in a patient negative for conventional biological markers (41). This emphasizes the persistent need of other biomarkers of APS. In this objective, we have tested a large panel of other autoantibodies. In contrast to previous studies (20–22, 42), we found aβ2GP1 D1 IgG and aβ2GP1 IgA positivity only in confirmed APS patients with double or triple positivity for conventional markers. These antibodies displayed an excellent specificity but quite low sensitivity and haven't shown any additional benefit as they were not positive in our group of SNAPS patients. One asymptomatic APA carrier exhibited a high positivity for aβ2GP1 D1 antibody. However, this patient had also a triple positivity for conventional APS markers and it was the only patient in this group positive for aPS/PT of IgG isotype. The

clinical evolution of this specific patient needs to be carefully monitored.

In our study, all patients positive for aβ2GP1 D1 IgG were positive for aPS/PT antibodies, confirming the correlation between these two markers previously shown by Nakamura et al. in APS patients (43).

The absence of IgA aβ2GP1 positivity in our SNAPS patients contrary to the previous publications (21, 22, 44) may be explained by the small size of our cohort due to the design of our study which was prospective and monocentric, and the quite short period of observation. These antibodies were found only in a small number of studied patients: in about 3% of patients together with conventional antibodies but in <1% of patients in an isolated manner (44). This may suggest that detection of these antibodies could be helpful in quite limited cases.

We found statistically significant difference in a frequency of non-conventional APS antibodies between patients and HD. These markers were particularly present in APS patients: from 2 to 7 markers per patient (or from 3 to 14 markers per patient if aPS/PT antibodies were taken into account, data not shown), with the highest number of markers in patients with CAPS. As a triple-positive APS characterizes a more severe disease comparing to the double or single positive APS, the positivity for multiple non-conventional markers could be another sign of the disease severity.

We performed a hierarchical clustering of APS markers in patients to study possible association between some markers and patient profiles. As previously suggested, we observed a clusterization of LA and aPS/PT antibodies of IgM isotype in asymptomatic APA group and a clusterization of LA and aPS/PT antibodies of IgG isotype in APS patients. This emphasizes the potential pathogenicity of IgG aPS/PT antibodies. These data are in accordance with recently published study (40) which postulates the pathogenicity of the aPS/PT antibodies of IgG and not of IgM isotype in patients with APS secondary to lupus.

In conclusion, our data obtained from a prospective cohort constituted mainly by patients with primary APS, demonstrate the potential value of aPS/PT antibodies as a strong marker of APS.

## AUTHOR CONTRIBUTIONS

EL performed the experiments and wrote the manuscript. LD designed the study, included the patients, and wrote the manuscript. AK performed the statistical analysis. YB performed some experiments and reviewed the manuscript. GdL included some patients and reviewed the manuscript. M-AD-D designed the study and wrote the manuscript.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02971/full#supplementary-material

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# Reliability of Lupus Anticoagulant and Anti-phosphatidylserine/prothrombin Autoantibodies in Antiphospholipid Syndrome: A Multicenter Study

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**Background:** Is it well-known that one of the major drawbacks of Lupus Anticoagulant (LA) test is their sensitivity to anticoagulant therapy, due to the coagulation based principle. In this study we aimed to assess the reproducibility of LA testing and to evaluate the performance of solid assay phosphatidylserine/prothrombin (aPS/PT) antibodies.

**Methods:** We included 60 patients that fulfilled the following inclusion criteria: (I) diagnosis of thrombotic antiphospholipid syndrome (APS); (II) patients with thrombosis and (a) inconstant previous LA positivity and/or (b) positivity for antiphospholipid antibodies (aPL) at low-medium titers [defined as levels of anti-β2Glycoprotein-I or anticardiolipin (IgG/IgM) 10–30 GPL/MPL] with no previous evidence of LA positivity. aPL testing was performed blindly in 4 centers undertaking periodic external quality assessment.

**Results:** The 60 patients enrolled were distributed as follows: 43 (71.7%) with thrombotic APS, 7 (11.7%) with thrombosis and inconstant LA positivity and 10 (16.7%) with low-medium aPL titers. Categorical agreement for LA among the centers ranged from 0.41 to 0.60 (*Cohen's kappa* coefficient; moderate agreement). The correlation determined at the 4 sites for aPS/PT was strong, both quantitatively (Spearman rho 0.84) and when dichotomized (*Cohen's kappa* coefficients = 0.81 to 1.0). Discordant (as defined by lack of agreement in ≥3 laboratories) or inconclusive LA results were observed in 27/60 (45%) cases; when limiting the analysis to those receiving vitamin K antagonist (VKA), the level of discordant LA results was as high as 75%(15/20). Conversely, aPS/PT testing showed an overall agreement of 83% (up to 90% in patients receiving VKA), providing an overall increase in test reproducibility of +28% when compared to LA,

becoming even more evident (+65%) when analyzing patients on VKA. In patients treated with VKA, we observed a good correlation for aPS/PT IgG testing (*Cohen's kappa* coefficients = 0.81–1; Spearman rho 0.86).

**Conclusion:** Despite the progress in the standardization of aPL testing, we observed up to 45% of overall discrepant results for LA, even higher in patients on VKA. The introduction of aPS/PT testing might represent a further diagnostic tool, especially when LA testing is not available or the results are uncertain.

**Keywords: antiphosphospholipid syndrome, Antiphospholipid Antibodies, Lupus Anticoagulant, aPS/PT, thrombosis, laboratory, diagnostic performance, reliability**

# INTRODUCTION

Since clinical features of Antiphospholipid Syndrome (APS) (thrombosis and pregnancy complications) are common in the general population and often related to other underlying factors, the diagnosis of APS requires next to clinical assessment the detection of persistently positive Antiphospholipid Antibodies (aPL). Thus, reliable laboratory tests with good clinical and analytical performance reproducibility are required. There is a large variety of assays available to assess aPL, but despite progress, standardization is still not optimal (1–3).

Lupus anticoagulant (LA) has been shown to be the strongest risk factor for aPL-related clinical manifestations (4), and the correct interpretation of this functional assay is crucial for diagnosis of APS. However, testing patients during treatment with vitamin K antagonists (VKA) or other oral anticoagulants remains a contentious issue and has been discouraged by official guidelines (5–7) because of interpretational problems affecting the mixing test. Besides, the clinical significance of low aPL titer and/or weak LA positivity, especially when detected in patients receiving anticoagulation [either VKA or direct anticoagulant agents (DOAC)], remains uncertain and certainly needs a more thorough evaluation.

More recently, the family of aPL has been expanded to include a heterogeneous group of autoantibodies whose specificity is directed to proteins involved in coagulation or to a complex of these proteins with phospholipids (8). Among others, autoantibodies that recognize a phosphatidylserine/prothrombin (aPS/PT) complex have been reported to be associated with APS and may have diagnostic relevance in these settings (9, 10). However, since aPS/PT antibodies are not currently included in the current APS classification criteria (11), aPS/PT antibodies are not assessed in all patients suspected to suffer from APS. Given the importance of aPL confirmation to improve the interpretability of laboratory test results for clinical trials and research studies, the objective of this study was to assess the reproducibility of LA and aPS/PT antibody testing when performed in different expert centers and to assess the diagnostic performance of these tests in different clinical settings of APS.

# METHODS

## Patients

We chart-reviewed patients with thrombotic events who tested persistently positive for at least one aPL (more than two occasions over a time of more than 12 weeks) that presented at San Giovanni Bosco Hospital in the last 5 years. The study was performed in compliance with the Declaration of Helsinki; approval from the ethic committee was not required according to the local and national guidelines. We enrolled 60 patients who met one of the following inclusion criteria:

1) Fulfilled the diagnosis of thrombotic APS defined as per Sydney criteria (11).
2) Patients with thrombosis and suspected APS not completely fulfilling the laboratory criteria (11), as follows: (a) inconsistent previous LA positivity; and/or (b) low-medium aPL titers [defined as levels of anticardiolipin (aCL) IgG/IgM or anti-β2-glycoprotein I (aβ2GPI) IgG/IgM antibodies 10–30 GPL/MPL]. Clinical and laboratory characteristics were retrospectively collected.

## Previous Autoantibody Detection

The aPL profile, at the diagnosis, included aCL, LA, and aβ2GPI antibodies.

The aCL and aß2GPI (IgG and IgM) were detected by commercial ELISA (Inova Diagnostics, Inc., San Diego, CA, US). Plasma samples were tested for the presence of LA according to the recommended criteria from the International Society on Thrombosis and Haemostasis (ISTH) Subcommittee on Lupus Anticoagulant/Phospholipid-Dependent Antibodies (12, 13).

## Study Design

LA and aPS/PT testing was performed in a blind fashion in four centers of the "Antiphospholipid Antibodies Regional Consortium" in northwest Italy: San Giovanni Bosco Hospital, Turin, Italy, A.O.U. Città della Salute e della Scienza, Turin, Italy, A.O. Ordine Mauriziano, Turin, Italy, and A.O.U. Maggiore della Carità, Novara, Italy (14).

LA was tested with the detection of two different reagents, used as screening and confirmatory tests, Silica Clotting Time HemosIL and dRVVT Screen and Confirm HemosIL, respectively (Instrumentation Laboratory, Bedford, MA, USA). Both tests were automated on ACL TOP 750 LAS instruments and results were normalized by means of plasma pools obtained from healthy donors without any deficit in coagulation factors, as per the current criteria from the ISTH Subcommittee on LA-Phospholipid-dependent antibodies (12, 13).

Both IgG and IgM aPS/PT were assayed using commercial ELISA kits (QUANTA Lite®, Inova Diagnostic), in accordance

with manufacturer's instructions. Samples were considered positive for aPS/PT IgG/IgM if tested >30 U.

Agreement was defined when all four laboratories had a concordant binomial result (positive/negative), both for LA and aPS/PT IgG/IgM testing.

## Statistical Analysis

Categorical variables are presented as number (%) and continuous variables are presented as mean (S.D.). Categorical agreement and degree of linear association was analyzed. The significance of baseline differences was determined by the chi-squared test, Fisher's exact test or the unpaired *t*-test, as appropriate. A two-sided *p*-value $< 0.05$ was statistically significant. All statistical analyses were performed using SPSS version 19.0 (IBM, Armonk, NY, USA).

# RESULTS

Demographic, clinical and laboratory characteristics of the 60 patients enrolled in the study are summarized in **Table 1**.

Briefly, mean age at data collection was 49.9 years old (SD ± 10.9) (females: males = 71.7%: 28.3%). Forty-three patients (71.7%) had a confirmed diagnosis of thrombotic APS (arterial 58.1%; venous 56.3%), and 17 patients presented with thrombosis and inconsistent LA positivity [7/17 (41.2%)] and/or with low-medium titers [10/17(58.8%)]. In the latest, 10/17 patients with suspected APS were tested positive (titer > 30 UI) for aPS/PT, IgG and/or IgM.

**TABLE 1 |** Characteristics of the patients included in the study.

| | APS patients (43; 72%) | Suspected APS (17; 28%) |
|---|---|---|
| **ANAGRAPHIC** | | |
| Mean age (±S.D.) at data collection | 45.7 (±11.9) | 51.9 (±7.3) |
| Females | 30 (69.8%) | 11 (64.7%) |
| **CLINICAL MANIFESTATIONS** | | |
| Arterial thrombosis | 21 (48.8%) | 5 (29.4%) |
| Venous thrombosis | 26 (60.5%) | 12 (70.6%) |
| **aPL PROFILE AT DIAGNOSIS** | | |
| LA (positive, *n*)* | 37 (86%) | 11 (64.7%) |
| aCL (IgG/M)* | 22 (51.2%) | 7 (41.2%) |
| aβ2GPI (IgG/M)* | 23(53.5%) | 6 (35.3%) |
| **ANTICOAGULANT THERAPY AT THE MOMENT OF TESTING** | | |
| VKA (warfarin) | 18 (41.9%) | 2 (11.8%) |
| LMWH | 8 (18.6%) | 2 (11.8%) |
| DOAC | 13 (30.2%) | 0 |
| Anti-platelets therapy | 17 (39.5%) | 13 (76.5%) |

*SD, Standard Deviation; APS, Antiphospholipid Syndrome; aPL, Antiphospholipid Antibodies; LA, Lupus Anticoagulant; aCL, Anticardiolipin Antibodies; anti-β2GPI, Anti-β2Glycoprotein I antibodies; VKA, Vitamin K antagonists; LMWH, Low Molecular Weight Heparins; DOAC, Direct Anticoagulants. *When considering patients with suspected APS: defined as inconsistent LA positivity and/or low levels of ACA IgG/IgM or anti-β2GPI IgG/IgM antibodies 10–30 GPL/MPL.*

Overall, categorical agreement for LA among all the four centers, as expressed by Cohen's *kappa* coefficients, ranged from 0.41 to 0.60 (corresponding to moderate agreement). The correlation among quantitative results for aPS/PT IgG/IgM was strong (Spearman *rho* 0.84; when dichotomizing for positive vs. negative results, Cohen's *kappa* coefficients = 0.81–1.00).

Overall categorical agreement is resumed in **Figure 1**.

We observed 27 (45.0% of the total) cases (15/20, 75% patients on VKA) in which LA results were discordant (defined by lack of agreement) or inconclusive. Conversely, in those cases, we observed a good correlation for aPS/PT IgG/IgM testing (Cohen's *kappa* coefficients = 0.81–1.00, Spearman *rho* 0.86).

When considering previous LA testing, we observed a statistically significant higher agreement among centers of LA testing if LA testing was previously positive [LA previously positive testing vs. negative: full agreement among centers 74.5% vs. 30.7% (*chi Square* test $p < 0.05$)]. Interestingly, the level of agreement of aPS/PT IgG/IgM among centers was similar regardless of previous LA testing [LA previously positive vs. negative: full agreement among centers 85.1% vs. 92.3% (*chi Square* test $p = 0.49$)].

When stratifying patients according to the inclusion criteria, we observed that in patients with confirmed diagnosis of APS, LA, and aPS/PT (IgG/IgM) agreements were 24/43 (55.8%) and 40/43 (93.0%), respectively. Conversely, in patients with thrombosis not completely fulfilling the Sydney laboratory criteria, we found aPL testing agreement among the four centers as follows: LA 9/17 (52.9%) and aPS/PT IgG/IgM 11/17 (64.7%).

# DISCUSSION

The diagnosis and consequent management as well as the classification of APS relies on the identification of persistent aPL positivity in patients with thrombosis and/or pregnancy morbidity (11). Among aPL tests, LA has been shown to be the strongest risk factor for thrombotic events (15) and LA testing should always be performed in parallel with aCL and aβ2GPI (3, 16–18) when a patients is investigated for APS.

However, despite significant progress in LA testing thanks to the updated guidelines of the ISTH (12, 13), LA testing still suffers from some shortcomings and remain much more labor intensive and complicated to perform compared to immunoassays.

In our study, when testing for LA in a blind fashion in four centers all undergoing regular external quality assessment (EQA) (14), we observed that up to 45% of LA positive samples were not unanimously identified. When limiting the analysis to patients with VKA, the observed level of agreement dropped to 55%.

Is it well-known that one of the major drawbacks of LA tests is their sensitivity to anticoagulant therapy (such as VKA, and DOAC), due to the coagulation based principle. Preferably, tests should be postponed until therapy is stopped; however, in the real world, requests during therapy still occur very frequently with potentially false-positive or false-negative results (13, 19). In addition, it might be logistical inconvenient for the patients to switch (or stop) anticoagulant therapy for LA testing purpose.

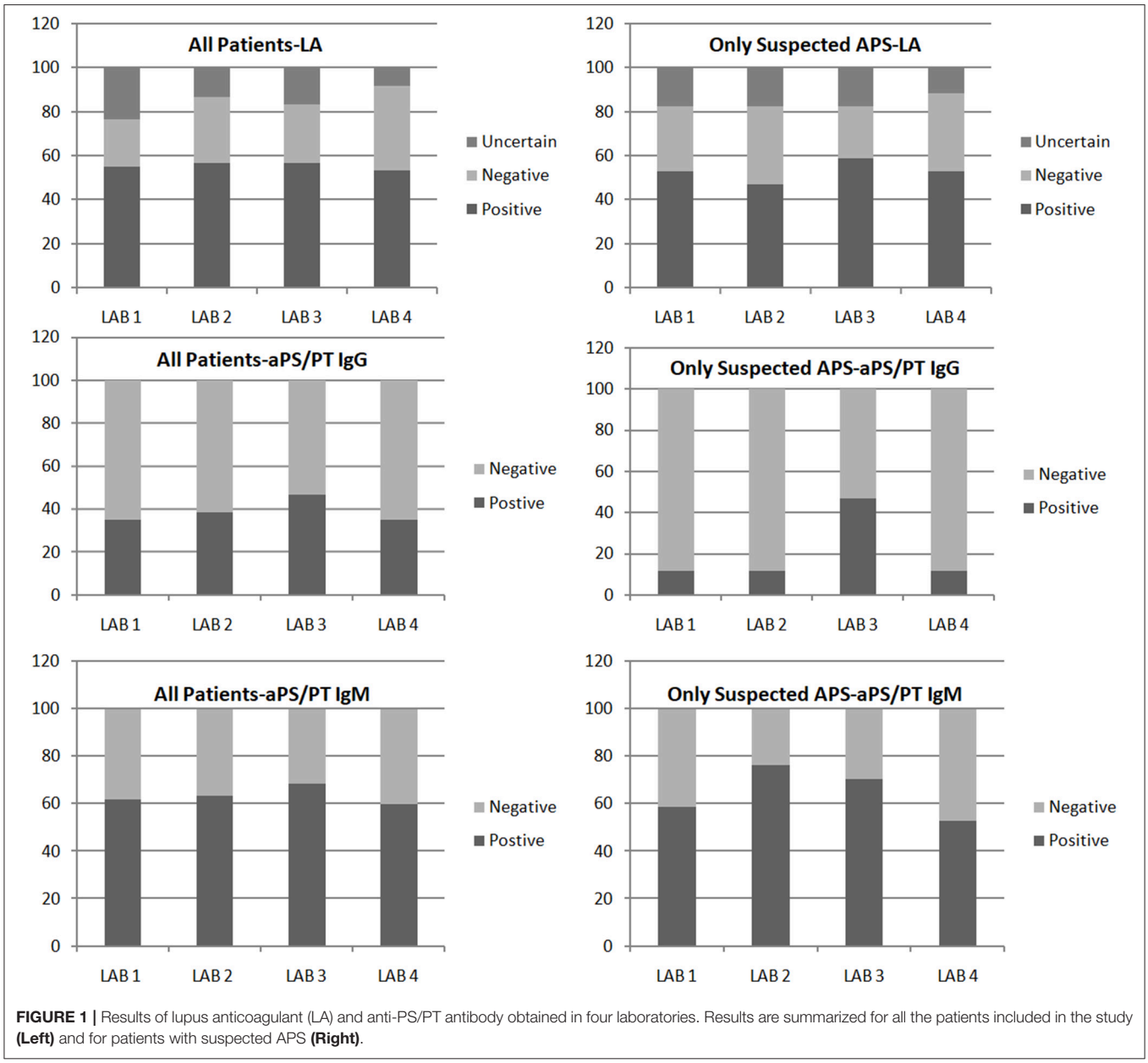


**FIGURE 1 |** Results of lupus anticoagulant (LA) and anti-PS/PT antibody obtained in four laboratories. Results are summarized for all the patients included in the study **(Left)** and for patients with suspected APS **(Right)**.

When a thrombotic event occurs in patients suspected for APS with inconsistent LA positivity and/or with low-medium aPL titers, clinical management can be challenging, as no consensus exists on the choice and, more critically, the duration of anticoagulation in this setting. In this study, when analyzing patients not completely fulfilling the criteria for APS, we observed a level of LA agreement of only 53%, supporting the need of further diagnostic tool to help physicians in the management of these patients.

Autoantibodies directed toward PS/PT complexes have been extensively studied for their diagnostic and prognostic utility in patients with suspected APS (9). Due to the observation that anti-prothrombin antibodies associate significantly with LA (20–24), several studies have sought to define the diagnostic relevance of these antibodies in APS (9, 23, 25, 26). Recent evidence support that while aPS/PT are frequently found in patients with LA, their association with thrombosis seems to be independent of the presence of LA (27).

Among the so-called extra-criteria aPL tests, besides aPS/PT, antiβ2GPI-domain1 antibodies have been also proposed to potentially improve the diagnostic accuracy in patients with suspected APS (28, 29), especially when assessing the risk for both thrombosis and pregnancy morbidity. Other antibody specificities, such as anti-annexin A5 and anti-vimentin antibodies, might be considered for thrombotic risk assessment only in selected patients, particularly when other aPL tests are negative and in the presence of clinical signs and/or symptoms strongly suggestive for APS (26, 30).

In our cohort, aPS/PT testing showed an overall agreement of 83% (up to 90% in patients receiving VKA), providing an overall increase in test reproducibility of +28% when compared to LA, becoming even more evident (+65%) when analyzing patients on VKA. These observations have important implications. On the one hand, LA testing remains a cornerstone for APS diagnosis. On the other, ongoing efforts to reduce the LA testing interlaboratory/interassay variations remain important. Taking into account the methodological shortcomings of LA, aPS/PT might represent a reliable and reproducible test, even during VKA or when APS diagnosis in uncertain. Besides the diagnosis, these findings also might have significant implications for classification criteria and therefore for clinical trials of new treatments.

Besides, albeit investigating the impact of aPS/PT testing on the management of patients with suspected APS was out of the scope of this study, one might note that up to nearly 60% of the patients with suspected APS were found positive for aPS/PT. From a speculative point of view, this observation might support a role for aPS/PT testing when APS is suspected but currently classification criteria aPL are not fully informative/reliable.

Although our investigation suffers for some limitations (cross-sectional approach limiting the analysis of the longitudinal fluctuation in aPL positivity; limited sample size; no further analysis on the level of agreement for aCL and aβ2GPI), the strengths of this study relies on the blind approach of aPL testing, performed in four different centers all undergoing periodic EQA. Besides, in this investigation, we evaluated the robustness of aPS/PT ELISA testing in different clinical settings, including patients suspected for APS but tested negative/low-titers for aCL and aβ2GPI antibodies. In such cases, a further diagnostic tool for APS with reliable performances might be crucial to guide the diagnostic process and to avoid under/over treatment (31). Finally, testing for aPS/PT by a commercial kit was proven to be a reproducible and accurate test for the detection of aPS/PT, bringing the added advantage of shorter running times when compared to in-house assays (32).

In conclusion, despite the progress in the standardization of aPL testing, we observed up to 45% of overall discrepant results for LA, even higher in patients on VKA. Our findings showed that the persistence of significant discordance in the reliability of LA testing. The introduction of aPS/PT antibodies in the diagnostic process of APS might represent a further valuable diagnostic tool, especially when LA is not available or reliable. In addition, detection of aPS/PT antibodies provides another tool which can complement and support current testing with aCL and aβ2GPI assays, and further help guiding clinical management.

## DATA AVAILABILITY

This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations for rare diseases in Piedmont Region, Northwest Italy with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

## AUTHOR CONTRIBUTIONS

SS, MR, IC, ER, MM, and DR drafted the manuscript, figures, and tables and critically reviewed the manuscript. AS, RR, BM, PP, GM, EMu, SB, MF, AV, and EMe participated in laboratory testing and critically reviewed the manuscript.

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# Altered Th17/Treg Ratio in Peripheral Blood of Systemic Lupus Erythematosus but Not Primary Antiphospholipid Syndrome

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**Introduction:** The role of the immune response in the pathogenesis of antiphospholipid syndrome (APS) remains elusive. It is possible that differences in the frequencies of Th17 cells and/or defects in the immunoregulatory mechanisms are involved in the pathogenesis of APS. Our aim was to determine the peripheral blood Th cells phenotype and the circulating cytokine profile in patients with primary APS (pAPS) and compare it with systemic lupus erythemathosus (SLE) as disease control group.

**Methods:** The frequencies of circulating regulatory T cells (Tregs) were determined in PBMCs from 36 patients with pAPS by flow cytometry. As control groups we included 21 age- and gender-matched healthy controls (HC) and 11 patients with SLE. The suppressive capacity of Tregs was evaluated *in vitro* by coculture assay. On the other hand, intracellular cytokine production was assessed in Th1, Th2, and Th17 cells and circulating IL-6, IL-10, and IL-35 were measured by Cytometric Bead Array and ELISA. The quantification of Th master gene expression levels was performed by real time quantitative PCR.

**Results:** pAPS patients and SLE patients did not show differences in the percentage or number of Tregs compared to HC. The suppressive capacity of Tregs was also similar in the three study group. Instead, we found higher FoxP3·mRNA expression levels in pAPS patients and HC than SLE patients. Regarding the Th17 response, patients with pAPS and HC showed a significantly lower frequency of circulating Th17 cells than SLE. However, no differences were observed in the Th1 response between patients and controls. Thus, increased Th17/Th1 and Th17/Treg ratios were found in SLE patients but not in pAPS patients. pAPS and SLE patients had higher serum IL-6 levels than HC but there was not difference between both disease groups. Besides, a significant increase in the immunosuppressive cytokine levels was observed only in pAPS as compared to HC.

**Conclusions:** Our data demonstrate an increased inflammatory profile of peripheral blood CD4$^+$ T cells from SLE as compared with pAPS mostly due to an increased Th17 response. In conclusion, there seems not to be a direct pathogenic role for Th cells in pAPS but in SLE.

**Keywords: pAPS, SLE, Th1, Th17, Treg, inflammation**

## INTRODUCTION

The antiphospholipid syndrome (APS) is a systemic autoimmune inflammatory disease characterized by the presence of serum antiphospholipid antibodies (aPL) and clinically by vascular thrombosis and/or obstetric events (1). Several lines of evidence indicate that aPL contribute to the inflammatory response that plays an important role in the pathogenesis of APS (2, 3). Thus, aPL have also emerged as triggers of innate immune inflammatory pathways in APS, through the activation of toll like receptors (TLR) (4). Besides, some authors have described that aPL might induce *in vitro* Th2 and Th17, but not Th1 or Treg differentiation (5). Although a clear association between aPL and clinical manifestations in APS has been established, the pathogenesis of this syndrome is poorly understood and it is generally considered multifactorial with an inflammatory background (6).

On the other hand, the clinical distinction between primary APS (pAPS) and APS secondary to other autoimmune disorders is sometimes difficult, especially with APS secondary to systemic lupus erythematosus (SLE) (7). APS and SLE share some clinical features, such as multiorgan manifestations, and the aPL profile and complement activation. At the same time, there are differences in the serum autoantibodies detected in both syndromes and we have recently demonstrated that the B cell phenotype differs between pAPS and SLE (8). From a pathogenic point of view, both entities are inflammatory disorders in which the activation of the innate immune response triggers an exacerbated acquired immune response (9). SLE and pAPS share an IFN-inducible gene expression signature (10). Besides, peripheral blood mononuclear B cells from pAPS patients show an additional monocyte-elicited inflammatory response demonstrated by increased expression of genes such as TLR8 and CD14 (11). In any case, both are inflammatory processes that drive autoantibodies production with some differences, and it is possible that the different expression of inflammatory genes may induce different T cells responses (7). However, there are not studies addressing differences in the circulating numbers or function of all the main T CD4+ subsets in pAPS, such as Th1, Th2, Th17, and Tregs, which play important roles in autoimmune diseases. Differences in the distribution of these cells may favor the hypotheses for differentiating SLE from pAPS.

Apart from the differences between pAPS and SLE, there is another important issue regarding the two main clinical variants of APS: the thrombotic and the obstetric APS (12). The differentiation between both variants may give and therapeutic potential. Since, no different autoantibodies have been found to distinguish vascular and obstetric events, differences in the circulating T cell phenotype might help. Although there are scarce works evaluating the peripheral blood T cell phenotype in pAPS (13, 14), to our knowledge there is no study comparing it between the vascular and obstetric variants.

In this study, we analyzed in the peripheral blood compartment the frequencies of Th1, Th2, Th17, and Tregs in patients with pAPS and compared them with SLE patients and healthy controls (HC). Besides, we addressed whether there was any difference for these cells between pAPS with thrombotic manifestations and obstetric disorders.

## MATERIALS AND METHODS

### Patients

The present study included 36 patients with pAPS (17 patients with obstetric complications and 19 patients with thrombotic phenomena), 11 patients with SLE, and 21 age-matched healthy controls (HC) without a previous history of infectious, neoplastic or autoimmune disease. Patients with pAPS were diagnosed according to the Sydney classification criteria (1). aPL were considered positive when medium and high titers of serum aPL were confirmed in two separate determinations at least 12 weeks. The cut off value was set at our laboratory in 20 GPL and MPL for anti-cardiolipin antibodies and 20 U/ml for anti-β2GPI antibodies (Aeskulab, Wendelsheim, Germany). pAPS patients were considered as a whole and divided into vascular and obstetric subtypes. The obstetric complications were those of classification APS criteria (9 patients had one or more early pregnancy losses, 12 patients had late pregnancy loss, and three patients had a live birth with prematurity) and in two cases non-classification criteria were found (intrauterine growth restriction). Patients with SLE had to satisfy the ACR 1997 and SLICC 2012 classification criteria (15, 16). All SLE patients were in remission or presented low disease activity defined by a score $\leq$4 in the systemic lupus erythematosus disease activity index (SLEDAI). The main demographic, clinical, and laboratory characteristics of the study population are shown in **Table 1.** All the patients and controls gave signed informed consent in accordance with the Declaration of Helsinki, and the study was approved by the Regional Ethics Committee.

### Detection of Intracellular Cytokines in Circulating Lymphocytes and Monocytes by Flow Cytometry Analysis

Intracellular cytokine staining is able to detect the production and accumulation of cytokines in the endoplasmic reticulum following stimulation. Cells collected in sodium heparin tubes

**TABLE 1 |** Demographic and main clinical features of patients with primary antiphospholipid syndrome (pAPS), systemic lupus erythematosus (SLE), and healthy controls (HC).

| | HC | pAPS | Obstetric APS | Thrombotic APS | SLE |
|---|---|---|---|---|---|
| Number of patients, *n* | 21 | 36 | 17 | 19 | 11 |
| Age, years (mean ± SD) | 40.29 ± 11.6 | 36.36 ± 9.8 | 34.5 ± 4.2 | 38.0 ± 12.8 | 32.8 ± 13.1 |
| Females, *n* (%) | 15 (71.4) | 30 (83.3) | 17 (100) | 13 (68.4) | 11 (100) |
| Follow-up, months (mean ± SD) | – | – | 66.3 ± 22.5 | 77.7 ± 65.0 | 121.1 ± 72.5 |
| Clinical manifestations associated with APS, *n* (%) | 0 | 36 (100) | 17 (100) | 19 (100) | 0 |
| Obstetrical events, *n* (%) | – | 17 (47.2) | 17 (100) | 0 (0) | – |
| Arterial thrombosis, *n* (%) | – | 12 (33.3) | 0 (0) | 12 (63.2) | – |
| Venous thrombosis, *n* (%) | – | 7 (19.4) | 0 (0) | 7 (36.8) | – |
| Positive aPL Serology, *n* (%) | 0 | 36 (100) | 17 (100) | 19 (100) | 6 (54.5) |
| Positivity for one Ab, *n* (%) | – | 19 (52.8) | 11 (64.7) | 8 (42.1) | 4 (36.3) |
| Positivity for two Ab, *n* (%) | – | 11 (30.5) | 5 (29.4) | 6 (31.6) | 0 (0) |
| Positivity for three Ab, *n* (%) | – | 6 (16.7) | 1 (5.9) | 5 (26.3) | 2 (18.2) |
| **SEROLOGICAL PROFILE** | | | | | |
| aCL, *n*(%) | – | 29 (78.4) | 15 (88.2) | 14 (73.7) | 5 (45.5) |
| aβGPI, *n* (%) | – | 17 (45.9) | 7 (41.2) | 10 (52.6) | 2 (18.2) |
| Lupus Anticoagulant, *n* (%) | – | 15 (40.5) | 3 (17.6) | 12 (63.2) | 3 (27.3) |
| **TREATMENT** | | | | | |
| Antiaggregant *n* (%) | – | 23 (63.9) | 16 (94.1) | 7 (36.8) | 4 (36.4) |
| Anticoagulant *n* (%) | – | 15 (41.7) | 0 (0) | 15 (78.9) | 1 (9.1) |
| Corticosteroids *n* (%) | – | 3 (8.3) | 0 (0) | 3 (15.8) | 3 (27.3) |
| Antimalarials *n* (%) | – | 4 (11.1) | 1 (5.9) | 3 (15.8) | 9 (81.8) |

*Patients with pAPS were divided into the two groups considered: obstetric and thrombotic.*
*APS, antiphospholipid syndrome; aPL, antiphospholipid; SLE, Systemic lupus erythematosus; SD, standard deviation; aCL, anticardiolipin; aB2GPI, anti beta 2 glycoprotein; Ab, antibody.*

were polyclonally stimulated for 4 h with phorbol 12- myristate 13-acetate (PMA) (Sigma Aldrich, St Louis, Missouri, USA) and ionomycin (Calbiochem, Gibbstown, New Jersey, USA) in polystyrene tubes (lymphocytes) in the presence of brefeldin A (Sigma Aldrich).

After culture, cells were stained with PerCP-conjugated anti-CD3 (Clone SK7) antibody (BD Biosciences) to identify T lymphocytes. Thereafter, the red blood cells were lysed with FACS lysing solution (BD Biosciences), and the mononuclear cells were permeabilized using FACS Permeabilizing Solution (BD Biosciences) and intracellularly stained with FITC- or PE-conjugated cytokine-specific monoclonal antibodies (BD Biosciences) for IL-2, IFNγ, and IL-4 for lymphocytes (Clones: IL-2: 5344.11; IFN-γ: 25723.11; IL-4: 3010.211). For characterization of Th17 cells, cells were stimulated under the same conditions, followed by surface staining with anti-CD4 APC (clone SK3), anti-CD161 PerCP-Cy 5.5 (clone DX12), and/or anti-CCR6 PE (clone 11A9) and intracellular staining with anti-IFNγ-PE (clone 25723.11) and anti-IL-17 FITC (clone eBio64Dec17). BD Biosciences provided all the antibodies except Alexa Fluor 488-conjugated anti-IL-17 monoclonal antibody that was provided by eBiosciences.

The quantification of circulating regulatory T cells ($CD4^+$ $CD25^{hi}$ $CD127^{-/low}$ $CD27^+$ $CD62L^+$ $CD45RO^+$ $FoxP3^+$, and $CD8^+CD28^-CD27^+$) was performed by flow cytometry with the following specific monoclonal antibodies: CD3 (clone SK7), CD4 (SK3), CD8 (SK1), CD25 (clone 2A3), CD27 (clone M-T271), CD28 (clone, L293), CD45RO (clone UCHL1), CD62L (clone SK11), CD127 (clone hIL-7R-M21) (BD Biosciences), and FoxP3 (Clone PCH101) (eBiosciences). FoxP3 expression was analyzed by flow cytometry after intracellular staining using the APC-anti-human FoxP3 staining set (eBiosciences) and following the protocol recommended by the manufacturer.

Levels of intracellular cytokine-producing cells together with surface expression were determined by FACS Canto II Flow Cytometer (BD Biosciences) and analyzed using FACS Diva software (BD Biosciences) to quantify the numbers of the different T $CD4^+$ subsets. Percentages of Th subsets were referred to the $CD4^+$ T cells.

## Detection of Soluble Cytokines in serum

### Cytometric Bead Array

The serum was isolated from 4 ml of blood obtained in tubes without additives from each individual and stored at −80°C until analysis. The quantitative determination of inflammatory cytokines in serum was performed using the Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine kit (BD Biosciences; San Diego, California, USA). This kit allows quantitatively measure interleukin (IL) 2, IL-4, IL-6, IL-10, Tumor Necrosis Factor (TNF) α, Interferon (IFN) γ, and IL-17A protein levels in a single sample. The fluorescence produced by CBA beads was measured on a FACS Canto II Flow Cytometer (BD Biosciences) and analyzed using FCAP array software (Soft Flow Inc; New Brighton, MN, USA). Detection limits were 2.6 pg/ml for IL-2, 4.9 pg/ml for IL-4, 2.4 pg/ml for IL-6, 4.5 pg/ml

for IL-10, 3.8 pg/ml for TNF-α, 3.7 pg/ml for IFN-γ, and 18.9 pg/ml for IL-17A.

### ELISA

Serum levels of IL-35 were determined using the commercial human ELISA kit (USCN Life Science Inc, Wuha, China) in accordance with the manufacturer's instructions. The sensitivity of the ELISA kit for IL-35 was 6.0 pg/ml.

## Proliferation Assays

To evaluate the *in vitro* suppressive capacity of Treg toward effector T (Teff) cells, FACS-sorted Treg and Teff cells of HC ($n = 9$), pAPS ($n = 12$), and SLE patients ($n = 5$) were setup in a coculture assay. PBMCS from sodium-heparinized peripheral blood were obtained by Ficoll Histopaque 1077 (Sigma Aldrich) gradient centrifugation. PBMCs were stained with specific monoclonal antibodies (BD Biosciences): anti-CD25-PE (Clone 2A3), anti-CD4-PerCP (Clone SK3), and anti-CD127-Alexa fluor 647 (Clone hIL-7R-M21) for isolation by FACS sorting of two populations: regulatory T cells and effector T cells, defined as $CD4^+$ $CD25^{hi}$ $CD127^{-/low}$ and $CD4^+$ $CD25^-$ $CD127^+$, respectively. Purity of FACS-sorted $CD4^+$ $CD25^{hi}$ $CD127^{-/low}$ T cells was routinely >97%. Following sorting, effector T cells were labeled with 10 μM CFSE (Vybrant CFDA SE Cell Tracer Kit; Molecular Probes, Eugene, Oregon, USA) in PBS-1% FBS buffer for 10 min at 37°C. Cells were washed and resuspended in culture medium at $5 \times 10^5$ cells/ml. CFSE-labeled effector T cells were cultured *in vitro* (96-well round bottom plates) alone and cocultured with unlabelled regulatory T cells (1:1). Cells were either unstimulated or stimulated with aCD3+aCD28 (Dynabeads human T-activator CD3/CD28 (Invitrogen Dynal, Oslo, Norway) at a bead to-cell ratio 1:1 and incubated at 37°C and 5% $CO_2$. After 4 days of culture, cells were harvested, and stained for surface markers and CFSE signal of gated lymphocytes was analyzed by flow cytometry using FlowJo Software. Cells were acquired in a FACS Canto II Flow Cytometer (BD Biosciences). The suppressive capacity of Tregs toward effector T cells in culture [ratio (1:1)/ Effect] was expressed as the proliferation index.

## Real Time Quantitative PCR

Quantification of *T-bet, GATA-3*, *FoxP3*, and *RORγt* gene expression levels in PBMCs from patients and healthy subjects was performed by real time quantitative PCR. Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and phenol/chloroform extractions method. The RNA concentration was quantified with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and RNA integrity was tested by electrophoresis on 1.5% agarose gel. cDNAs were obtained by retrotranscription using iScript cDNA synthesis Kit (Bio-Rad Laboratories, CA, USA) according to the manufacturer's recommendations in a Master Cycler Pro S thermal cycler (Eppendorf, Hamburg; Germany).

The primer sequences are the following:

T-***bet*** forward 5′-GATGCGCCAGGAAGTTTCAT-3′,
reverse 5′-GCACAATCATCTGGGTCACATT-3′;

***GATA-3*** forward 5′-CCCTCATTAAGCCCAAGCGA -3′,
reverse 5′-GTCTGACAGTTCGCACAGGA -3′;

***FoxP3*** forward 5′-TTCAAGTTCCACAACATGCG ACCC -3′,
reverse 5′-GCACAAAGCACTTGTGCAGACTCA-3′;

***RORγt*** forward 5′-CAGTCATGAGAACACAAATTGA AGTG -3′,
reverse 5′-CAGGTGATAACCCCGTAGTGGAT -3.

The housekeeping gene β-actin was used as an endogenous control,

forward 5′-ACCAACTGGGACGACATGGAGAAA -3′,
reverse 5′-TAGCACAGCCTGGATAGCAACGTA-3′.

Real-time quantitative PCR was carried out using the Sso Fast Evagreen Supermix (Bio-Rad) according to the manufacturer's recommendations. PCR reactions were conducted in duplicate in a CFX96 detection system (Bio-Rad) and raw data were converted into the threshold cycle (Ct) values for each sample. Relative quantification was analyzed by the comparative Ct method, also referred to as $2^{-\Delta\Delta Ct}$ method, described by Schmittgen and Livak (17). The *p*- values were calculated based on a Student's *t*-test for each gene in patients and healthy subjects.

## Statistical Analysis

The normality was assessed using the Shapiro Wilk test. The data from the healthy controls and patient groups were first analyzed by Kruskall-Wallis test. The statistical comparisons of data between different pathologies and healthy controls were performed using the Mann-Whitney *U*-test. Correlations were assessed using Spearman's rank correlation coefficient. Differences were considered significant when *p* values were $< 0.05$. All the statistical analysis of data was carried out with the SPSS 15.0 software (Chicago, Illinois, USA).

# RESULTS

## Circulating T Cells With Regulatory Phenotype in Patients With pAPS and SLE

We first looked at the main circulating regulatory T cells subtype (Tregs), identified as $CD4^+CD25^{hi}CD127^{low}FoxP3^+$ in fresh peripheral blood samples by flow cytometry and compared pAPS and SLE patients. No differences neither in the percentages or numbers of these circulating Tregs were found between the pAPS patients and SLE patients and neither between both disease groups and HC (**Figure 1A**). However, we found peripheral blood frequencies of other regulatory T cell subset, defined as $CD8^+CD28^-CD27^+$, significantly decreased in pAPS compared to SLE patients ($p = 0.019$) but not to HC (**Figure 1B**). SLE patients showed no differences in the $CD8^+CD28^-CD27^+$ compared to HC.

We subdivided pAPS patients in two groups according to their main clinical features, thrombotic or pregnancy complications. Thus, those patients with thrombotic APS had a significant decreased frequency of $CD8^+CD28^-CD27^+$cells as compared to SLE patients ($p = 0.026$). In the case of patients with obstetric pAPS, differences did not reach statistical significance ($p = 0.055$) (**Figure 1B**). Frequencies and absolute numbers of peripheral blood $FoxP3^+$ Tregs did not differ between the two subgroups of pAPS (**Figure 1A**).

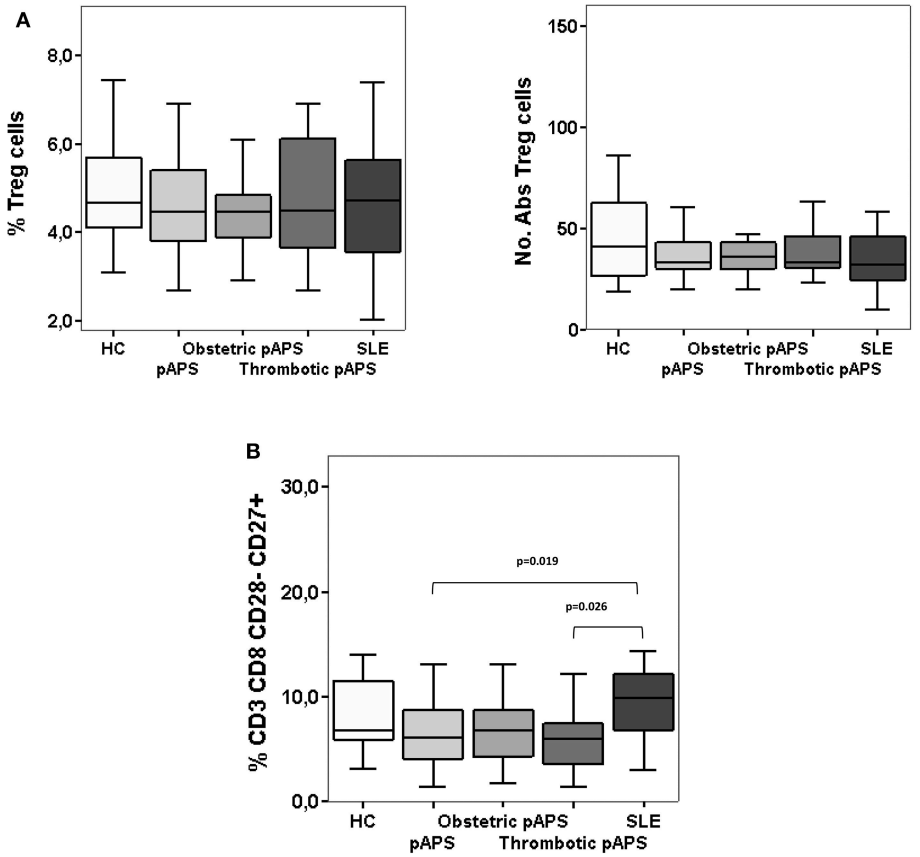


**FIGURE 1 |** Frequencies and numbers of peripheral blood regulatory T cells: $CD4^+$ $CD25^{hi}$ $CD127^{-/low}$ $CD27^+$ $CD62L^+$ $CD45RO^+$ $FoxP3^+$ **(A)** and $CD8^+CD28^-CD27^+$ **(B)** cells in patients with primary antiphospholipid syndrome (pAPS, $n = 36$) and systemic lupus erythematosus (SLE, $n = 11$) and healthy controls (HC, $n = 21$). Data of pAPS patients were also analyzed according to the two pAPS variants (lower panel of figures): obstetric ($n = 17$) and thrombotic ($n = 19$) variants. Frequencies of $CD8^+CD28^-CD27^+$ cells in pAPS patients were significantly lower than in SLE patients and mainly due to the thrombotic patients **(B)**, calculated by Mann-Whitney *U* test. There was no significant difference for $CD4^+$ Tregs **(A)**. Data are expressed as the median and interquartile range. HC, healthy controls; pAPS, primary antiphospholipid syndrome; SLE, systemic lupus erythematosus.

## Patients With pAPS Display Decreased Frequencies of Peripheral Blood Th17 Cells Than Patients With SLE

Th17 and Th1 cell responses in peripheral blood from patients and healthy subjects were analyzed by measuring intracellular cytokine production by CD4+ T cells. The frequencies of Th17 cells, defined as $CD4^+CD161^+CCR6^+$ $IL17^+IFN\text{-}\gamma^-$, were significantly decreased in pAPS patients, and also in HC, compared to SLE patients ($p = 0.001$ and $p = 0.005$, respectively), although no differences were found between APS vs. HC (**Figure 2A**). Interestingly, both patients with obstetric and thrombotic pAPS showed a lower frequency of Th17 cells compared to SLE ($p = 0.002$ and $p = 0.005$, respectively) (**Figure 2B**). In addition, as shown in **Figure 2C**, patients with pAPS had a lower frequency of $CD4^+IL\text{-}17^+$ $IFN^+$ cells than SLE patients ($p = 0.021$). More specifically, those cells were also significantly decreased in patients with obstetric pAPS compared to HC, thrombotic pAPS and SLE ($p = 0.015$, $p = 0.032$, and $p = 0.006$, respectively) (**Figure 2D**).

Regarding Th1 response, there was a similar proportion of $CD4^+IL17^-IFN\text{-}\gamma^+$ T cells in both patient groups and HC (**Figure 2C**). No difference was found for Th2 cells, quantified as producers of IL-4 (**Figure 2D**). Additionally, we also measured intracellular cytokine-producing monocytes in peripheral blood and did not observe differences in the percentages between patients and controls (**Supplementary Figure 1**).

## Altered Th17/Th1 and Th17/Treg Balance in SLE Patients but Not in pAPS Patients

The relation between Th17 cells to Th1 or regulatory T cells was determined in patients and controls by calculating the Th17/Th1 and Th17/Treg ratios. Th17/Th1 cell ratio was significantly lower in pAPS than in SLE patients ($p = 0.015$), although similar compared to HC (**Figure 3A**). We also noticed significant differences in the Th17/Th1 cells ratio in patients with obstetric and thrombotic pAPS compared to SLE ($p = 0.021$ and $p = 0.046$, respectively). In addition, HC had a lower Th17/Th1 cell ratio than SLE patients ($p = 0.033$) (**Figure 3A**).

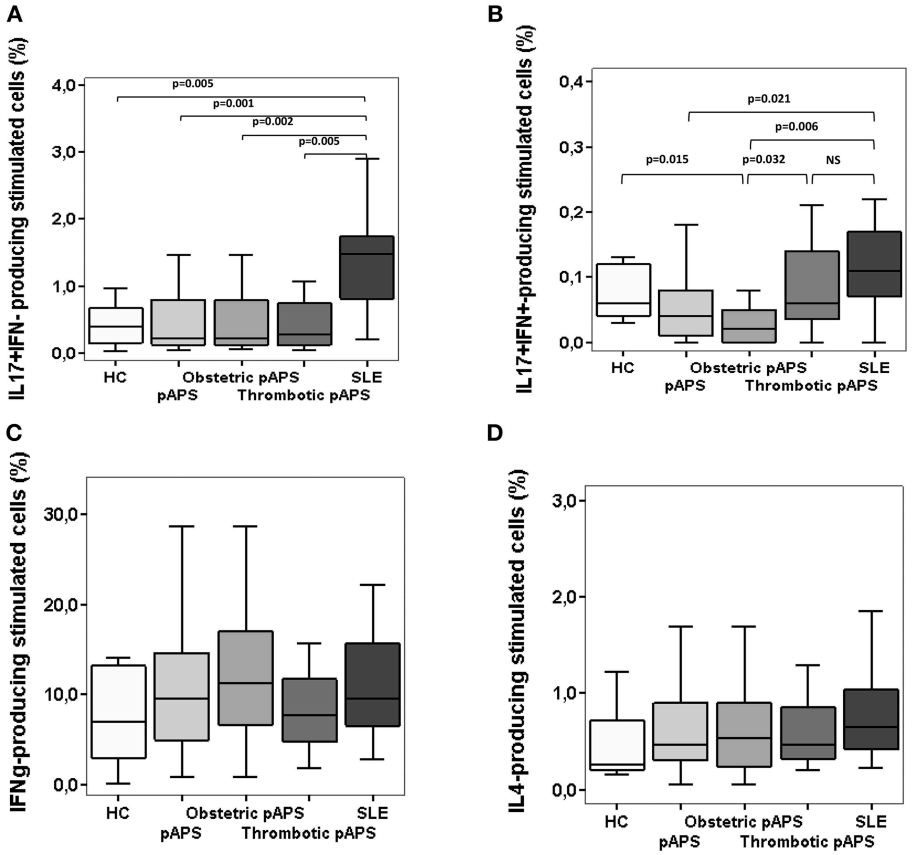


**FIGURE 2 |** Frequencies of Th17 cells **(A,B)**, Th1 **(C)**, and Th2 **(D)** cells in patients with primary antiphospholipid syndrome (pAPS, $n = 37$) and systemic lupus erythematosus (SLE, $n = 11$) and healthy controls (HC, $n = 21$). Th17 cells were divided in two subtypes: conventional Th17 (IL-17$^+$ IFN-$\gamma^-$) and Th17Th1 (IL-17$^+$ IFN-$\gamma^+$). Data from pAPS patients were analyzed globally or separately by obstetric ($n = 17$) or thrombotic ($n = 19$) pAPS were analyzed. Only significant differences are displayed when $p$ value was $< 0.05$ by Mann-Whitney $U$-test. Data are expressed as the median and interquartilic range and referred to the gate of CD4$^+$ T cells. HC, healthy controls; pAPS, primary antiphospholipid syndrome; SLE, systemic lupus erythematosus.

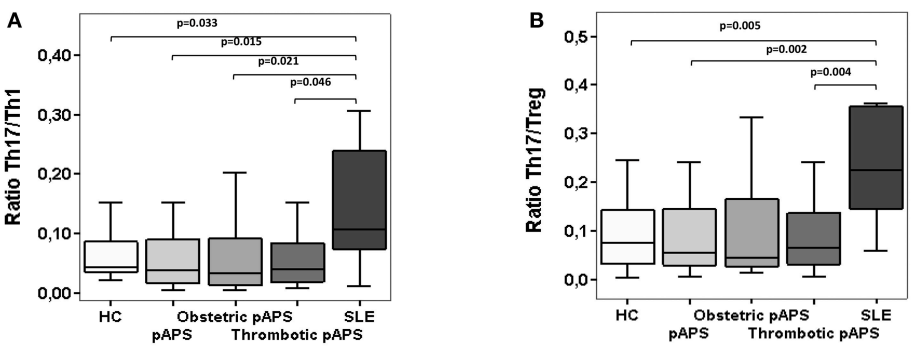


**FIGURE 3 |** Ratios of circulating Th17/Th1 **(A)** and Th17/Treg **(B)** cells in patients with primary antiphospholipid syndrome (pAPS, $n = 36$) and systemic lupus erythematosus (SLE, $n = 11$) and healthy controls (HC, $n = 21$). Th17 were considered as T CD4+ cells IL-17$^+$ IFN-$\gamma^-$) whereas the Th1 cells were IL-17$^-$ IFN-$\gamma^+$). Treg cells were defined as CD4$^+$ CD25$^{hi}$ CD127$^{-/low}$ CD27$^+$ CD62L$^+$ CD45RO$^+$ FoxP3$^+$. Data of pAPS patients were also analyzed according to the two pAPS variants: obstetric ($n = 17$) and thrombotic ($n = 19$) variants. The level of significant differences between groups are indicated only when $p$ value was $< 0.05$ by Mann-Whitney $U$-test. Data are expressed as the median and interquartilic range. HC, healthy controls; pAPS, primary antiphospholipid syndrome; SLE, systemic lupus erythematosus.

Similarly, Th17/Treg cell ratio was significantly increased in SLE patients compared to HC and pAPS patients ($p = 0.005$ and $p = 0.002$, respectively) (**Figure 3B**), particularly with obstetric pAPS patients ($p = 0.004$) (**Figure 3B**). Again, these differences were not found in the pAPS patients compared to HC.

We also measured the expression of specific Th transcription factors (**Figure 4**). SLE patients had lower FoxP3 mRNA expression levels compared to HC and pAPS ($p = 0.007$ and $p = 0.008$, respectively). In agreement with cell ratios described above, those patients with obstetric pAPS had higher FoxP3 mRNA expression levels than SLE patients ($p = 0.004$) (**Figure 4**). On the other hand, RORγt mRNA levels were significant reduced in SLE patients compared to HC ($p = 0.018$) but not with pAPS. Besides, pAPS patients, especially obstetric patients, had also significant reduced RORγt mRNA expression levels compared to HC ($p = 0.006$).

## Patients With APS Have Higher Serum Concentration of Immunosuppresive Molecules Than Healthy Controls

IL-6 plays a major role in the differentiation to Th17 cells. Possibly, an excess of IL-6 could tip the balance toward the induction of Th17 cells. As shown in **Figure 5**, pAPS and SLE patients had higher IL-6 levels than HC ($p = 0.048$ and $p = 0.036$, respectively) but no differences were found between APS and SLE. Besides, both obstetric and thrombotic APS displayed similar IL-6 levels and not different from SLE (**Figure 5**). No differences in other soluble inflammatory cytokine levels such as IL-2, IL-4, TNF-α, INF-γ, and IL-17 were observed among three groups (**Supplementary Figure 2**).

One of the Treg-mediated suppression mechanisms is the production of immunosuppressive cytokines, such as IL-10 and IL-35. Circulating serum levels of IL10 were significantly higher in patients with pAPS than HC ($p = 0.002$). These differences were markedly increased in those patients with thrombosis ($p = 0.001$) (**Figure 5**). Likewise, IL-35 serum levels were also significantly increased in pAPS compared HC ($p = 0.045$) (**Figure 5**). Importantly, patients with SLE did not show any significant difference in these immunosuppressive molecules, although showed a trend to have lower serum levels (**Figure 5**).

## Normal Suppressive Capacity of Tregs in Patients With pAPS and SLE

According to ours results, we investigated the *in vitro* suppressive capacity of Tregs toward effector T cells, by co-culture assay, and the cytokine producing into culture supernatants (**Figure 6**).

The suppressive capacity of Tregs was similar in three study groups (**Figure 6**). In addition, patients with pAPS or SLE did not show differences in the concentration of suppressive IL-10 nor IL-35 (**Figure 5**).

# DISCUSSION

The hypothesis of the present work is that pAPS and SLE pathogenesis may be differentiated by a distinct balance between Th17 and Tregs in peripheral blood that could result in an increased inflammatory response in SLE as compared to pAPS. There are a number of studies showing an increased inflammatory effector $CD4^+$ T cells response in SLE (18–23). Treg cells have been extensively studied in peripheral blood of SLE patients, although with contradictory results (24–27). However, data of different Th subsets in pAPS are very scarce and with limited methodological approach (13, 14). Simonin et al. (28) studied the peripheral naïve, memory and effector B and T cell compartments but not Th subsets in 22 patients with pAPS and 49 controls. They found disturbances in the B cell compartment but not in T cells, which only showed a general reduction in comparison to controls. In the present work, we investigated the balance between immunoregulatory and effector immune response by measuring the number of circulating Th1, Th17, and Tregs cells as well as serum levels of cytokines in pAPS and compared them with SLE. As a limitation, the SLE patient number was low as compared with pAPS (11 vs. 37) and all of them were female what can add a bias to the analysis but it was not possible to recruit SLE male patients for the study. More importantly, we selected two well-established groups of pAPS and SLE because they had a long-term follow-up to exclude a possible progression to secondary APS from SLE, although we are aware that some authors consider pAPS and SLE as one single disease (29). Furthermore, numbers of pAPS patients were balanced between obstetric and thrombotic manifestations in the present work, in contraposition to previous studies that even do not differentiate them. Although it is possible that obstetric patients could develop thrombotic events, the time of follow-up is long-enough to differentiate both subtypes in the present work. In our experience, patients with obstetric pAPS rarely develop thrombotic pAPS (30).

Our results showed an equivalent Th17 response in pAPS to HC but decreased as compared to SLE patients. These data differ from those found by Jakiela et al. (14) that found an increased frequency of effector T $CD4^+$ cells including Th17 in pAPS as compared to HC. This increase was even more evident in those pAPS patients with high titers of aPL IgG antibodies. We did not observe such an association between aPL titers and Th17 cells in peripheral blood. One possible explanation may have to be with the low serum aPL titers in our long-term patients in whom they can decrease. However, the strength of the present work is the comparison we made between pAPS and SLE patients since the latter were with low disease activity (SLEDAI $\leq 4$) in order to exclude any influence of the systemic inflammatory response in the immunoregulatory phenotype. We also looked at the percentages of circulating IFN-γ producing Th1 cells but there was no change between both disease groups and between any disease group and HC. For intracellular cytokine-producing $CD4^+$ T cells we used the percentages of cells referred to $CD4^+$ T cells instead of absolute numbers, since the data were obtained after *in vitro* stimulation. Besides, there was no significant difference for absolute numbers of any lymphoid cell ($CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, and $CD16^+CD56^+$) in peripheral blood (**Supplementary Figure 3**) and, consequently, we considered that frequencies and absolute numbers were equivalent.

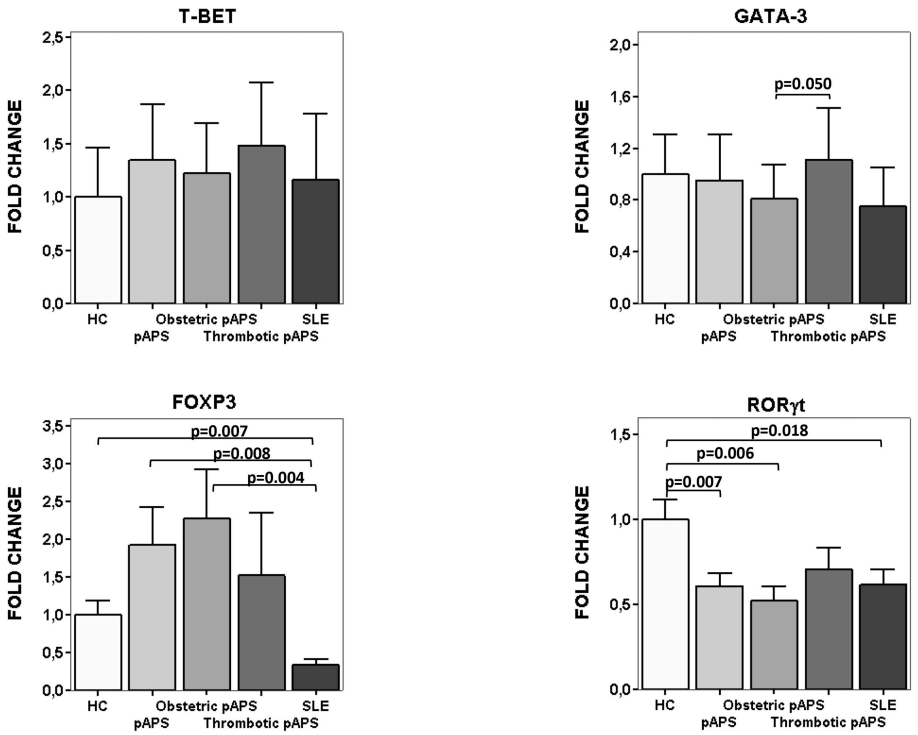


**FIGURE 4 |** mRNA expression of transcription factors for Th differentiation. Relative fold change expression of mRNA quantified by real time quantitative PCR of T-bet, GATA-3, FoxP3, and RORγt in patients (15 pAPS patients: 8 obstetric and 7 arterial thrombosis; 10 SLE patients) and healthy controls ($n$: 10) is displayed. pAPS were subdivided into the main two variants: obstetric and thrombotic. Level of significance is only shown when $p$ value was $< 0.05$ by Student-$t$ test between groups for each gene. Data are expressed as the mean value. HC, healthy controls; pAPS, primary antiphospholipid syndrome; SLE, systemic lupus erythematosus.

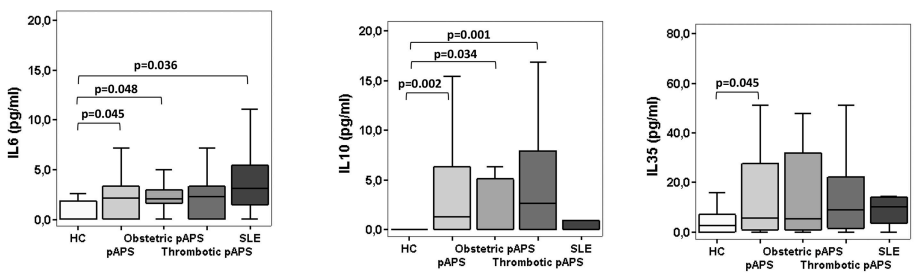


**FIGURE 5 |** Serum cytokines levels in patients with primary antiphospholipid syndrome (pAPS, $n = 37$) and systemic lupus erythematosus (SLE, $n = 11$) and healthy controls (HC, $n = 21$). Serum IL-6 and IL-10 levels were assessed by CBA whereas serum levels of IL-35 were measured by ELISA. The level of significant differences between groups are indicated only when $p$ value was $< 0.05$ by Mann-Whitney $U$-test. Data are expressed as the median and interquartile range. HC, healthy controls; pAPS, primary antiphospholipid syndrome; SLE, systemic lupus erythematosus.

Isolated analysis of Th17 cells does not give enough information about the effector immune response since regulatory T cells may balance it (31). In this regard, peripheral blood Tregs did not differ between both disease groups and HC. This is in disagreement with other authors, that found lower frequencies of Tregs in 20 pAPS patients than in HC, although the panel of antibodies to define Tregs was shorter than the one employed in our study (32). We did not find an increase in Tregs as described by other authors in SLE (25, 26), but it could be attributed to the low disease activity of our SLE cohort. Neither, there was any significant difference in the frequency of circulating Th2 cells. As a consequence, we only observed significantly higher Th17/Tregs ratios in SLE patients than in pAPS patients and HC. Again, Th17/Tregs ratios did not differ between pAPS patients and HC. Likewise, there was a similar increased Th17/Th1 ratio in SLE. These data indicate that SLE, despite our cohort was not clinically active, showed an unbalanced inflammatory state mainly due to a higher Th17 response, with no important changes in Th1 or regulatory T cell responses. This finding was relevant since our SLE patients were mostly on antimalarial treatment, which have

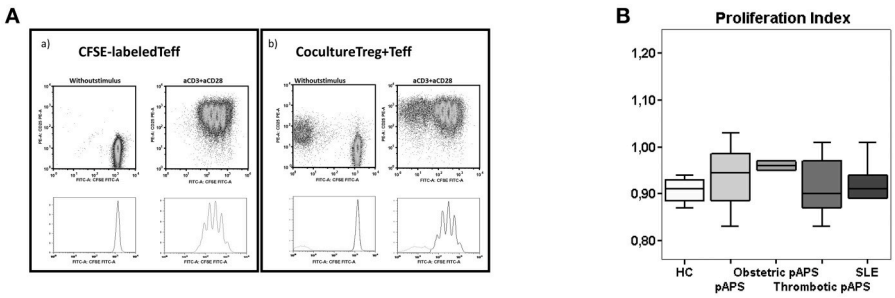


**FIGURE 6 |** Suppression function of $CD4^+$ Treg cells in *in vitro* culture with polyclonally stimulated $CD4^+$ effector T cells. FACS-sorted Treg ($CD4^+$ $CD25^{hi}$ $CD127^{-/low}$) and effector (Teff, $CD4^+CD25^-CD127^+$) T cells of HC ($n = 9$), pAPS ($n = 12$), and SLE patients ($n = 5$) were setup in a coculture assay. Following sorting, effector T cells were labeled with 10 μM CFSE. CFSE-labeled Teff cells were cultured *in vitro* alone and cocultured with unlabeled Treg cells (1:1). Cells were either unstimulated or stimulated with anti-CD3+anti-CD28 antibodies at a bead to cell ratio 1:1 and incubated for 4 days. Then, cells were harvested and analyzed by flow cytometry. **(A)** Shows a representative experiment with only Teff cells stimulated (left) or with the addition of Treg cells to stimulated Teff cells (right). The suppressive capacity of Treg toward effector T cells in culture [ratio (1:1)/ Effect] was expressed as the proliferation index with respect to the proliferation of Teff cells stimulated without Treg cells. **(B)** Represents the median proliferation index in each group and interquartilic range.

been described to decrease Th17-related cytokines in SLE (33). Our cellular findings were also observed for mRNA expression in PBMC. Thus, FoxP3 mRNA expression was significantly decreased in SLE as compared with HC and pAPS, as previously described (34). Such a decrease is even more significant since more SLE than pAPS patients were receiving corticosteroids what have been described as inducing factor of FOXP3 expression (34). On the other hand, ROR-gamma T expression was lower in SLE patients than in HC but not in pAPS. No important changes were detected for the other two Th master genes expression, T-bet or Gata-3. Regarding regulatory mechanisms, we did find decreased numbers of $CD8^+CD28^-CD27^+$ cells in pAPS patients, another type of regulatory T cells different from the most studied Tregs CD4+ cells, and that has been described in mouse models and human liver transplantation (35), as well as in organ-specific autoimmune diseases (36). These cells might be induced by peripheral mechanisms and secreted suppressive cytokines, such as IL-10 or TGF-β (37). Importantly enough, there was a significant increase of the immunoregulatory IL-10 in serum from pAPS patients as compared with HC. Such a difference was not observed for SLE. To the best of our knowledge there are not report showing such a finding. The only related finding has to be not with an increase but a decrease of another regulatory cytokine, TGF-beta (14). The increase we found in serum IL-10 might support our theory of a higher immune regulation in pAPS than in SLE.

All our data together might suggest that the adaptative immune response is not significantly altered in pAPS as compared to the hallmark systemic autoimmune disease, SLE, despite pAPS is also an autoimmune response with autoantibodies production. These data differ from Xiao et al. (5) that found a diminished Th1/Th2 ratio and increased Th17 and decreased Tregs in culture of PBMC from HC with different concentrations of aPL. However, the experimental approach was different from ours since they focused on the *in vitro* effect of aPL on Th differentiation. In opposition to our findings, other authors described that β2GPI- reactive T cells might provide T cell help to B cells to induce the production of class-switched aPL in APS which was also associated with certain HLA class II genes (38). However, such a Th cell response is not specific of APS since it has been described in APS but also in SLE and in APS-negative subjects (39, 40). The specificity seems to be specific of the β2GPI epitope used to stimulate the Th cells (38). Besides, these β2GPI- reactive T cells have been found increased in APS subjects with subclinical (41) and clinical atherosclerosis (42). Interestingly, Benagiano et al. (42) evidenced domain I β2GPI- specific Th1 infiltrates in atherosclerotic plaques of pAPS patients what supported the role of the adaptative response in the induction of aPL and the plaque formation in pAPS. However, they did not studied the possible role of T follicular helper cells that provides the signal to B cells in the follicle to generate switch class and affinity maturated autoantibodies (43). In the present study, we neither focused in T follicular helper cells. Evidences suggest a higher involvement of innate response, as demonstrated by increased gene transcription of TLR8 in pAPS (11) or β2GPI-anti-β2GPI immunocomplex stimulation of monocytes through TLR4 (44). Furthermore, APS patients demonstrated increased expression of adhesion molecules in endothelium (45). On the other hand, neutrophils of pAPS patients display a pro-inflammatory gene signature (46). All the data together establish pAPS as an inflammatory autoimmune disease, although it does not induce a pro-inflammatory Th17 response as inflammation in SLE does in the present work and previous reports (18–23).

There exists a clear debate about the inflammation associated with vascular pAPS and obstetric pAPS (12). Antiphospholipid syndrome has two clinical subtypes according to obstetric or thrombotic events. From the laboratory diagnostic criteria, they do not differ and have the same aPL profile although recent evidences show also differences. In some cases, vascular and obstetric manifestations occur in the same patient, although pAPS is a stable disease in which the patient that presents with obstetric pAPS does not develop thrombosis and vice versa. This suggests that obstetric and vascular pAPS could

be different diseases. Apart from clinic-epidemiological data, the study of immune-mediated mechanisms involved in both clinical variants may help to determine whether they are the same or different disease with therapeutic implication. Evidences suggest that vascular pAPS courses with complement activation that subsequently would induce thrombi, but with very low inflammation (12). On the contrary, obstetric pAPS would be induced by inflammatory mechanisms, although the presence of inflammatory cells in placenta of pAPS is very uncommon (47). Our data, although obtained from peripheral blood lymphocytes, point to a more inflammatory profile in thrombotic than in obstetric pAPS. Indeed, patients with thrombotic pAPS had a higher load of aPL than obstetric one (**Table 1**) and it could explain some of the differences found in Th cells between both pAPS subtypes. Furthermore, the decreased percentages of Th17Th1 cells (also considered as the subtype Th17.1) could explain the lower expression of ROR-γt, which is usually expressed in Th17Th1 cells together with T-bet (48). In any case, the number of circulating proinflammatory Th17 cells was always lower in any subtype of pAPS than in SLE. It is possible that the CD4+T cells phenotype in peripheral blood is not enough to show important differences between vascular and obstetric pAPS or, at least, does not correlate with previously described tissue cellular infiltrates in both pAPS variants.

Finally, we are aware of the limitation of our study regarding the possible effect of the different treatments in SLE and pAPS that were not considered as confounding factors. Patients with pAPS were treated with antiplatelet and/or anticoagulant therapies, whereas SLE patients were treated with antimalarials and steroids. To the best of our knowledge, we have not found any influence of treatments in pAPS on Th differentiation in the literature, although there is one study showing *in vitro* the inhibitory effect of prasugrel on effector Th cells that were induced to an inflammatory phenotype by addition of plateles (49). Patients with pAPS included in the present study did not receive prasugrel. However, the effect of antimalarials to inhibit Th17-mediated immunity is well-known (33, 50). The effect of corticosteroids in Th1 and Th17 cells has been already studied and demonstrated (51, 52). This reinforces our findings showing a stronger Th17 response in SLE than in pAPS, despite the suppressive effect of treatment.

In conclusion, our data demonstrated an increased inflammatory Th17 phenotype in patients with SLE that was not observed in pAPS patients who showed a similar phenotype to HC. In addition, thrombotic pAPS showed higher levels of peripheral blood Th17 than obstetric pAPS. Altogether, these data bring important information about the therapeutic possibilities in pAPS.

## AUTHOR CONTRIBUTIONS

LÁ-R performed most of the experiments and participated in the writing of the manuscript. VM-T participated in research design and recruited patients. JC-A and IV participated in recruitment of patients and clinical data collection. IB contributed with analytical tools. ML-H participated in research design, supervision of experiments, and writing of the manuscript. All the authors reviewed the draft and approved it.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00391/full#supplementary-material

**Supplementary Figure 1 |** Frequencies of peripheral blood monocytes secreting inflammatory cytokines in patients with primary antiphospholipid syndrome (pAPS, $n = 37$) and systemic lupus erythematosus (SLE, $n = 11$), and healthy controls (HC, $n = 21$). Data from pAPS patients were analyzed globally or separately by obstetric ($n = 17$) or thrombotic ($n = 19$) pAPS were analyzed. Monocytes from peripheral blood were intracellular stained with monoclonal antibodies specific for IL-1β, TNF-α, and IL-6, after *in vitro* stimulation with LPS for 4 hours. No significant differences were found among groups by Mann-Whitney U-test. Data are expressed as the median and interquartile range and referred to the gate of $CD4^+$ T cells. HC, healthy controls; pAPS, primary antiphospholipid syndrome; SLE, systemic lupus erythematosus.

**Supplementary Figure 2 |** Serum cytokines levels in patients with primary antiphospholipid syndrome (pAPS, $n = 37$) and systemic lupus erythematosus (SLE, $n = 11$), and healthy controls (HC, $n = 21$). Data of pAPS patients were also analyzed according to the two pAPS variants (lower panel of figures): obstetric ($n = 17$) and thrombotic ($n = 19$) variants. Serum cytokine levels (IL-2, TNF-α, IFN-γ, IL-4, IL-17) were assessed by CBA. No significant differences were found among groups. Data are expressed as the median and interquartile range. HC, healthy controls; pAPS, primary antiphospholipid syndrome; SLE, systemic lupus erythematosus.

**Supplementary Figure 3 |** Absolute numbers of peripheral blood total lymphocytes **(A)** and lymphocyte subsets (absolute numbers in **(B)** and frequencies in **(C)**: $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, and $CD16^+/CD56^+$) in patients with primary antiphospholipid syndrome (pAPS, $n = 37$) and systemic lupus erythematosus (SLE, $n = 11$) and healthy controls (HC, $n = 21$). Data of pAPS patients were also analyzed according to the two pAPS variants (lower panel of figures): obstetric ($n = 17$) and thrombotic ($n = 19$) variants. No significant differences were found among groups. Data are expressed as the median and interquartile range. HC, healthy controls; pAPS, primary antiphospholipid syndrome; SLE, systemic lupus erythematosus.

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# Alarmin HMGB1 and Soluble RAGE as New Tools to Evaluate the Risk Stratification in Patients With the Antiphospholipid Syndrome

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Antiphospholipid antibody syndrome (APS) is a systemic autoimmune disease characterized by arterial and/or venous thrombosis, pregnancy morbidity in the presence of circulating "anti-phospholipid antibodies" (aPL). One of the main target antigens of aPL is $\beta_2$-glycoprotein I ($\beta_2$-GPI). APS may occur as a primary syndrome or associated with Systemic Lupus Erythematosus (SLE). High Mobility Group Box 1 (HMGB1) is a nuclear non-histone protein which is secreted from different type of cells during activation and/or cell death and may act as a proinflammatory mediator through ligation to its receptors, including RAGE**.** There is accumulating evidence that HMGB1 contributes to the pathogenesis of inflammatory and autoimmune diseases, especially SLE. In a previous study we demonstrated increased serum levels of HMGB1 in both primary and secondary APS patients. In this work we analyzed: (i) *in vitro* whether anti-$\beta_2$-GPI antibodies from APS patients may induce both a HMGB1 cellular relocation by activation of its putative receptor RAGE in platelets and monocytes and, (ii) *ex vivo,* serum levels of HMGB1/soluble RAGE (sRAGE) in APS patients and their possible correlation with clinical manifestations. Platelets and monocytes from healthy donors were incubated with affinity purified anti-$\beta_2$-GPI antibodies. HMGB1 and RAGE expression were analyzed by Western Blot. Sera from 60 consecutive APS patients (primary or secondary), diagnosed according to the Sydney Classification Criteria, were enrolled. As a control, 30 matched healthy subjects were studied. Serum levels of HMGB1 and sRAGE were analyzed by Western Blot. *In vitro* results showed that anti-$\beta_2$-GPI antibodies were able to induce RAGE activation and HMGB1 cellular relocation in both monocytes and platelets. HMGB1 and sRAGE serum levels were significantly increased in APS patients in comparison with healthy subjects ($p < 0.0001$). Interestingly, APS patients with spontaneous recurrent abortion showed significantly higher levels of sRAGE; moreover, in

APS patients a direct correlation between serum levels of HMGB1 and disease duration was detected. Our observations suggest that anti-$\beta_2$-GPI antibodies may trigger RAGE activation and HMGB1 cellular relocation during APS. Monitoring these molecules serum levels may represent an useful tool to evaluate the pathogenesis and risk stratification of clinical manifestations in APS.

**Keywords: HMGB1, sRAGE, antiphospholipid syndrome, thrombosis, recurrent abortion**

## INTRODUCTION

Antiphospholipid antibody syndrome (APS) is a systemic autoimmune disease characterized by arterial and/or venous thrombosis, pregnancy morbidity in the presence of "anti-phospholipid antibodies" (aPL) namely lupus anticoagulant (LA), anticardiolipin antibodies (aCL), or anti-$\beta_2$–glycoprotein I antibodies (a$\beta_2$-GPI) (1). The incidence of the APS is around 5 new cases per 100,000 persons per year and the prevalence around 40–50 cases per 100,000 persons (2). APS may occur in patients having neither clinical nor laboratory evidence of another definible condition (primary APS, PAPS) or it may be associated with other diseases (secondary APS, SAPS), mainly Systemic Lupus Erythematosus (SLE) (3), but occasionally with other autoimmune conditions, infections (4), drugs (5), and malignancies (6).

There is accumulating evidence that High Mobility Group Box 1 (HMGB1), an endogenous danger signal released when immune cells are activated or cell death occurs (7), contributes to the pathogenesis of inflammatory and autoimmune diseases, especially SLE (8, 9). HMGB1 is a 30 kDa nuclear protein which was purified from nuclei for the first time in 1970 (10). In the nucleus it works as a chromatin structural protein, organizing DNA, stabilizing nucleosome formation and regulating transcription; it was also found in cytosol, mitochondria and cell plasma membrane, where it can be released to the extracellular milieu (11). In particular, HMGB1 may be secreted by immune cells, such as macrophages, monocytes, and dendritic cells, during activation and/or cell death (12, 13) and may act as a proinflammatory mediator through ligation to its receptors, Toll like receptor 2 or 4 (TLR2, TLR4) or its most interesting binding partner, Receptor for Advanced Glycation End products (RAGE) (14, 15). RAGE is a transmembrane receptor of the immunoglobulin superfamily that engages diverse ligands. Ligand-triggered RAGE-dependent cellular activation results in several effects, including activation of nuclear factor-kB (NF-kB), increased expression of cytokines and adhesion molecules, and induction of oxidative stress (16, 17). The C-truncated secretory isoform of RAGE, termed soluble RAGE (sRAGE), can be shedded by several cell types, including monocytes, and may neutralize the AGEs-mediated damage by acting as a decoy (18–20).

Level of HMGB1/sRAGE increases in plasma and serum of patients with inflammatory diseases associated with sepsis or thrombosis. In a previous study (21) we detected increased serum levels of HMGB1/sRAGE in subjects affected by pregnancy morbidity, as well as in a small group of patients with primary or secondary APS, suggesting that in these patients elevated levels of HMGB1/sRAGE may represent an alarm signal, indicating an increase of proinflammatory triggers.

Moreover, it has been demonstrated that platelet activation increases RAGE surface expression (22) and that HMGB1 may represent a master regulator of the prothrombotic cascade involved in the pathogenesis of deep venous thrombosis (23). Indeed, HMGB1 is involved in the prothrombotic cross-communication between platelets, monocytes and neutrophil granulocytes, thus promoting the thrombotic process.

In order to evaluate whether autoantibodies in APS may be able to activate HMGB1/sRAGE, in this work we have analyzed *in vitro* whether anti-$\beta_2$-GPI antibodies from APS patients can induce a relocation of HMGB1 to the cytosol and activation of its putative receptor RAGE in platelets and monocytes from healthy donors. Furthermore, in order to evaluate any correlations between HMGB1/sRAGE and different APS clinical manifestations, we analyzed serum levels of these molecules in a larger cohort of patients with APS.

## MATERIALS AND METHODS

### Isolation of Monocytes

Human peripheral blood mononuclear cells (PBMC) from buffy coat of healthy donors were isolated by Lymphoprep density-gradient centrifugation (Nycomed Pharma, Oslo, Norway). Cells were washed 3 times in phosphate buffered saline (PBS), pH 7.4, and were isolated by density-gradient separation (Lympholyte; Cedarlane, Hornby, Ontario, Canada). CD14+ monocytes were purified by incubation with anti-CD14–coated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by sorting with a magnetic device (MiniMacs Separation Unit; Miltenyi Biotec), according to the manufacturer's instructions.

The purity of the isolated monocytes was evaluated by staining with a fluorescein isothiocyanate (FITC)–conjugated anti-CD14 antibody against monocytes and analyzing stained cells by flow cytometry. The purity was higher than 95% CD14+. The viability of the monocytes was up to 99%, using the Trypan blue staining.

Before *in vitro* experiments, monocytes were cultured for 24 h, in RPMI 1640, containing 2 mM L-glutamine, 100 units/ml of penicillin, 100 mg/ml of streptomycin, and 250 pg/ml of Fungizone (Gibco, Grand Island, NY), in the absence of antioxidant agents, at 37°C in a humified atmosphere, containing 5% $CO_2$.

### Platelets Preparation

Platelets were prepared from blood samples of healthy donors, with the addition of acid citrate dextrose (ACD) as anticoagulant.

Platelet-rich plasma (PRP) was preliminary obtained from the whole blood by centrifugation at 150 × g for 15 min at 20°C.

PRP was centrifuged at 900 × g for 10 min at 20°C. Platelet-poor plasma (PPP) was removed and pellets were re-suspended in Tyrode's buffer, containing 10% (v:v) ACD. Then, after washing, pellets were re-suspended in Tyrode's buffer, containing Bovine Serum Albumine (BSA), 3 mg/ml.

Platelet counts were performed by a hemocytometer (Coulter, Beckman Coulter, Brea, California, USA); that leukocyte contamination was <1 leukocyte/$10^7$ platelets. The purity of the isolated platelets was confirmed by staining with a fluorescein isothiocyanate (FITC)–conjugated anti-CD41 mAb (Beckman Coulter) and analyzing by flow cytometry (Coulter Epics, Beckman Coulter).

## Purification of Anti-$\beta_2$-GPI Antibodies

Human anti-$\beta_2$-GPI IgG were purified by affinity chromatography, as previously reported (24), from 3 patients who had been diagnosed as affected by APS according to the Sydney Classification Criteria (1), showing a high titer of anti-$\beta_2$-GPI antibodies and, as a control, from 3 healthy donors. The antibodies displayed lupus anticoagulant (LA) activity, in all tests, the stimulatory effect of the 3 antibodies was virtually the same (data not shown).

## *In vitro* Incubation of Monocytes and Platelets With Anti-$\beta_2$-GPI Antibodies

For *in vitro* studies, monocytes were cultured at 37°C in a humified atmosphere of 5% $CO_2$ with serum-free RPMI 1640, containing 2 m*M* L-glutamine, 100 units/ml of penicillin, 100 mg/ml of streptomycin, and 250 pg/ml of Fungizone. Platelets were resuspended in Tyrode's buffer, containing BSA (3 mg/ml).

Monocytes (2 × $10^6$/ml) or platelets (300 × $10^6$/ml) were incubated at 37°C for 4 h with human affinity-purified anti-$\beta_2$-GPI IgG (200 μg/ml), according to the method of Raschi et al. (25) with normal human serum IgG (200 μg/ml), or with LPS (100 ng/ml). All materials contained <0.00025 ng of endotoxin/μg of protein, as determined by the *Limulus* amebocyte lysate test (Associates of Cape Cod, Falmouth, MA).

## Immunoblotting Analysis of HMGB1 Expression

In order to analyze HMGB1 relocation in cell compartments, monocytes, untreated or treated for 4 h at 37°C with normal human serum IgG, or with human affinity-purified anti-$\beta_2$-GPI, or with LPS were subjected to subcellular fractionation according to Manganelli et al. (26). Equal amounts of nuclear or cytosolic extracts were separated by 12% SDS-PolyAcrylamide Gel Electrophoresis (SDS-PAGE). The proteins were electrophoretically transferred onto polyvinylidene fluoride (PVDF) transfer membranes (Amersham Biosciences, Piscataway, NJ, USA) and after blocking with Tris-buffered saline, that contains 25 mM Tris-HCl, 150 mM NaCl, pH 7.4, and 0.05% Tween-20 (TBS-T) with 3% BSA, were probed with rabbit anti-HMGB1 polyclonal Ab (1:1,000; Abcam, Cambridge UK). The secondary Ab was horseradish peroxidase conjugated anti-rabbit (1:1,0000; Sigma-Aldrich, Milan, Italy). After washing, proteins were detected using ECL reagents (Amersham Biosciences, Buckinghmashire, UK). As a control, anti-actin mAb antibodies (1:1,000 Sigma-Aldrich) and anti-LMNB1 mAb (1:1,000 Santa Cruz Biotechnology, Dallas, Texas, USA) were used. Lamin B1 (LMNB1) served as nuclear contamination marker and actin as cytoplasmic contamination marker. Densitometric analysis was performed using ImageJ Software (National Institutes of Health, Bethesda, MD, USA).

To analyze HMGB1 release from monocytes, supernatants of untreated and treated cells were analyzed by immunoblotting. The cells were stimulated for 24 h with human affinity-purified anti-$\beta_2$-GPI. For Western blotting of HMGB1, supernatants were collected after 4 or 24 h of culture and concentrated by Centricon YM-10 (Millipore). The volume of the concentrated supernatants was adjusted to 70$\mu$l for equal loading. Samples and HMGB1 standard (Human recombinant HMGB1 SIGMA) were resolved in 12% SDS-PAGE under reducing conditions, as described before, using rabbit anti-HMGB1 polyclonal antibody (1:1,000; Abcam).

In parallel experiments platelets obtained from a healthy donor were incubated for 4 h at 37°C with normal human serum IgG, or with human affinity-purified anti-$\beta_2$-GPI, or with LPS. Cells were lysed in Radioimmunoprecipitation assay (RIPA) buffer containing 1 mM $Na_3VO_4$, and 75 U of aprotinin, and analyzed by immunoblotting as described above using rabbit anti-HMGB1 polyclonal antibody (1:1,000; Abcam).

## Immunoprecipitation of RAGE

Monocytes and platelets, untreated or treated for 4 h at 37°C with normal human serum IgG, or with human affinity-purified anti-$\beta_2$-GPI, or with LPS (27) were lysed in RIPA buffer 1 mM $Na_3VO_4$, and 75 U of aprotinin. To preclear non-specific binding, cell-free lysates were mixed with protein G-acrylic beads (Sigma-Aldrich) and stirred by a rotary shaker for 2 h at 4°C. The supernatants were centrifuged (500 × g for 1 min) and then immunoprecipitated with goat polyclonal anti-RAGE (1:1,000; Abcam) or with irrelevant IgG as a negative control plus protein G-acrylic beads. The immunoprecipitates were analyzed and checked by Western Blot.

## Immunoblotting Analysis of Phospho-RAGE Expression

Immunoprecipitated samples from monocytes and platelets untreated or treated as reported above, were subjected to immunoblotting analysis. After evaluation of the protein concentration by Bradford Dye Reagent assay (Bio-Rad, Segrate, Italy), the immunoprecipitates were analyzed by immunoblotting using mouse anti-Phospho-Ser mAb (1:1,000; Sigma-Aldrich), followed by a secondary Ab horseradish peroxidase conjugated anti-mouse (1:10,000; Sigma-Aldrich). Immunoprecipitation was checked by mouse anti-RAGE mAb (1:1,000; Millipore, Billerica, MA, USA). Densitometric analysis was performed using ImageJ Software (National Institutes of Health, Bethesda, MD, USA).

## Patients

Sixty consecutive adult patients classified as affected by APS according to the Sydney Classification Criteria (1),

attending the Lupus Clinic, Sapienza University of Rome were consecutively enrolled.

Written informed consent was obtained from eligible patients during the screening period, at which time physical examination and medical history were evaluated.

In addition, 30 healthy subjects (normal blood donors, mean age ± *SD* 40.2 ± 8.8 F/M 25/5) were included as controls.

Subjects (both patients and controls) carrying any other disease, such as neurodegenerative, infectious or hepatic, were excluded, as well as pregnant or breast feeding women. In addition, subjects with surgical/anesthesia trauma in the last 3 months were also excluded. For control subjects, drugs interfering with hormonal, metabolic, or immunological function were also exclusion criteria.

Anti-CL and anti-$\beta_2$-GPI antibodies were tested by enzyme-linked immunosorbent assay (ELISA) kits obtained from Inova Diagnostic Inc. (San Diego, CA, USA). Lupus Anticoagulant was studied by a dilute sensitized activated partial thromboplastin

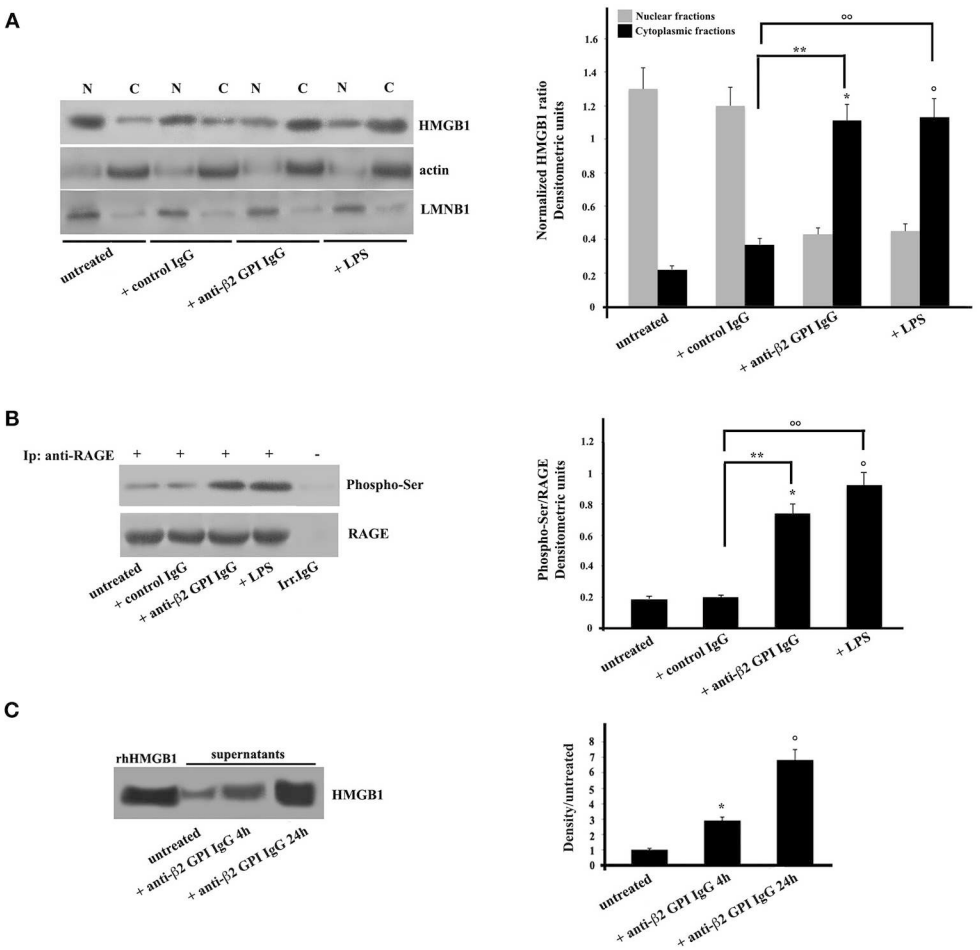


**FIGURE 1 |** Anti-$\beta_2$-GPI antibodies induce HMGB1 translocation and RAGE phosphorylation in human monocytes. **(A)** Monocytes from healthy donors, untreated or treated with control human IgG (200 μg/ml), human affinity-purified anti-$\beta_2$-GPI IgG (200 μg/ml) or LPS (100 ng/ml), for 4 h at 37°C, were subjected to subcellular fractionation. Equal amounts of nuclear (N) or cytosolic (C) extracts were analyzed by immunoblotting using rabbit anti-HMGB1 polyclonal Ab. Lamin B1 (LMNB1) served as nuclear contamination marker and actin as cytoplasmic contamination marker. HMGB1 loading within each compartment was also normalized with Lamin B1 and actin, respectively. As a control, mouse anti-actin monoclonal antibodies and anti-LMNB1 mAb were used. Right panel, densitometric HMGB1 levels are shown. Results represent the mean ± SD from 3 independent experiments. $^{*}p$ + anti-$\beta_2$_GPI IgG < 0.001 vs. untreated, $^{\circ}p$ + LPS < 0.001 vs. untreated, $^{**}p$ + anti-$\beta_2$_GPI IgG < 0.001 vs. + control IgG, $^{\circ\circ}p$ +LPS < 0.001 vs. + control IgG. **(B)** Human monocytes from healthy donors, untreated or treated with control human serum IgG (200 μg/ml), with human affinity-purified anti-$\beta_2$-GPI IgG (200 μg/ml) or LPS (100 ng/ml), for 4 h at 37°C, were immunoprecipitated with goat anti-RAGE polyclonal Ab. Immunoprecipitates were analyzed using mouse anti-Phospho-Ser monoclonal antibody. Immunoprecipitation was checked by mouse anti-RAGE mAb. Right panel, densitometric phospho-Ser /RAGE ratios are shown. Results represent the mean ± SD from 3 independent experiments. $^{*}p$ + anti-$\beta_2$-GPI IgG < 0.001 vs. untreated, $^{\circ}p$ + LPS < 0.001 vs. untreated, $^{**}p$ + anti-$\beta_2$-GPI IgG < 0.001 vs. + control IgG, $^{\circ\circ}p$ + LPS < 0.001 vs. + control IgG. **(C)** Western blot analysis of monocyte supernatants for HMGB1. The supernatants of untreated and cells treated for the indicated times with human affinity-purified anti-$\beta_2$-GPI and HMGB1 standard (rhHMGB1) were run under reducing conditions on the gel (12% SDS-PAGE) and transferred to nitrocellulose. Filters were stained with rabbit anti-HMGB1 polyclonal Ab. Right panel, density/untreated is shown. Results represent the mean ± SD from 3 independent experiments. $^{*}p$ + anti-$\beta_2$-GPI IgG 4 h < 0.001 vs. untreated, $^{\circ}p$ + anti-$\beta_2$-GPI IgG 24 h < 0.001 vs. untreated.

time (aPTT) and a dilute Russell's viper venom time (dRVVT), followed by confirm tests using reagents and instrumentation by Hemoliance Instrumentation Laboratory, Lexington, MA, USA.

This study was approved by the local ethic committees. Sera were collected at several times and stored at −20°C until use.

## Western Blot Analysis of HMGB1/sRAGE Levels in Sera

To avoid the possibility that serum/plasma components able to bind to HMGB1 may interfere with its detection, we tested HMGB1 by Western Blot, instead of ELISA (21, 28).

Briefly, sera (3 μl) from patients with APS or healthy donors were diluted with 72 μl RIPA buffer and heated at 95°C for 5 min in Sodium Dodecyl Sulfate (SDS)-loading buffer. For immunodetection, the proteins were separated by 12% SDS-PAGE and transferred onto PVDF transfer membranes. The membrane was blocked at room temperature for 1 h with TBS-T with 3% BSA. The membranes were incubated with primary Ab: anti-HMGB1 polyclonal Ab (1:1000; Abcam) or mouse anti-RAGE mAb (1:10,000; Millipore). The secondary Ab was horseradish peroxidase conjugated anti-rabbit (1:10,000; Sigma-Aldrich) or anti-mouse (1:5,000; Amersham Biosciences) IgG, which was incubated for 1 h at room temperature. After washing, proteins were detected using ECL reagents (Amersham Biosciences). Densitometric analysis was performed using ImageJ Image Software (National Institutes of Health).

## Statistical Analysis

All the statistical analysis were performed by GraphPad Prism software Inc. (San Diego, CA, USA). D'Agostino-Pearson omnibus normality test was used to assess the normal distribution of the data. Normally distributed variables were summarized using the mean ± standard deviation (SD) and non-normally distributed variables by the median and interquartile range (IQR). Frequencies were expressed by percentage. Paired *t*-test and Wilcoxon's matched pairs test were performed accordingly. $P < 0.05$ were considered statistically significant.

# RESULTS

## Anti-$\beta_2$-GPI Antibodies Induce HMGB1 Relocation and RAGE Phosphorylation in Monocytes

In order to evaluate whether autoantibodies in APS may be able to elicit the alarmin response, we preliminary analyzed *in vitro* whether anti-$\beta_2$-GPI antibodies from APS patients may induce both a HMGB1 relocation to cytosol and an activation of its putative receptor RAGE in monocytes from healthy donors.

Purified monocytes, untreated or stimulated with anti-$\beta_2$-GPI antibodies, or incubated with IgG from healthy donors or LPS as controls, were subjected to subcellular fractionation and equal amounts of nuclear or cytosolic extracts were analyzed by Western Blot. Our results revealed that the cytosolic fraction of monocytes incubated with anti-$\beta_2$-GPI antibodies showed the relocation of HMGB1 from nuclei (**Figure 1A**). Virtually no relocation was observed following treatment with control IgG. As expected (29), treatment with LPS also induced relocation of HMGB1 from nuclei. Loading control was evaluated using anti-actin mAb as a cytosol protein marker and anti-LMNB1 as a nuclear protein marker. Densitometric analysis, reported on the right panel, clearly showed the effect of anti-$\beta_2$-GPI antibodies on the HMGB1 relocation from nuclei to cytosol in monocytes.

Then, we analyzed the activation of the putative receptor of HMGB1, RAGE. Anti-$\beta_2$-GPI antibodies were able to elicit in purified monocytes the activation of RAGE, which appeared in its phosphorylated form (30) (**Figure 1B**). Similar findings were found following LPS treatment, which has been reported to being involved in RAGE regulation (27). Virtually no RAGE phosphorylation was observed following treatment with control IgG. The identity of RAGE was confirmed using an anti-RAGE mAb (**Figure 1B**).

Since extracellular HMGB1 is known to be able to interact with RAGE, and in turn participate to the cell activation, i.e., inducing proinflammatory phenotype (12), we investigated whether HMGB1 was secreted from monocytes treated with anti-$\beta_2$-GPI antibodies from APS patients. The level of HMGB1 was assessed by Western Blotting and by measuring the intensities of the immunoreactive bands at 25 kDa. As shown in **Figure 1C**, HMGB1 was significantly increased as early as after 4 h of stimulation. Densitometric analysis revealed higher concentration of HMGB1 in supernatant of cells stimulated with anti-$\beta_2$-GPI antibodies from APS patients either for 4 or 24 h than in the supernatants of the untreated cells. This indicates that HMGB1 secretion by activated monocytes is a late event, as well documentated after LPS treatment (**Figure 1C**).

We further investigated weather mTORC1 activation is involved in aPL-induced secretion of HMGB1 and RAGE. We observed that anti-$\beta_2$-GPI IgG from APS patients or starvation (HBSS) caused significant reduction in phospho-mTOR compared to untreated cells or to monocytes stimulated with control human serum IgG, $p < 0.001$ (no change was detected in the levels of total mTOR) (**Supplementary Figure 1**).

## Anti-$\beta_2$-GPI Antibodies Induce HMGB1 Expression and RAGE Phosphorylation in Platelets

In the same vein, platelets, as emblematic cells involved in the pathogenesis of thrombosis, were incubated with anti-$\beta_2$-GPI antibodies. Again, similarly to monocytes, Western Blot analysis of HMGB1 on cell lysates obtained from anti-$\beta_2$-GPI antibodies-treated platelets revealed that HMGB1 levels were significantly increased after 4 h of anti-$\beta_2$-GPI treatment, as well as after LPS treatment (**Figure 2A**), as confirmed by densitometric analysis reported on the right panel. Loading control was evaluated using anti-actin mAb.

As showed in monocytes, also in platelets the anti-$\beta_2$-GPI antibodies were able to elicit the activation of RAGE, indeed the Western Blot analysis of RAGE immunoprecipitated from platelets lysates showed the molecule in its serine phosphorylated form (**Figure 2B**). Similar findings were found following LPS treatment. Virtually no phosphorylation was observed following treatment with control IgG.

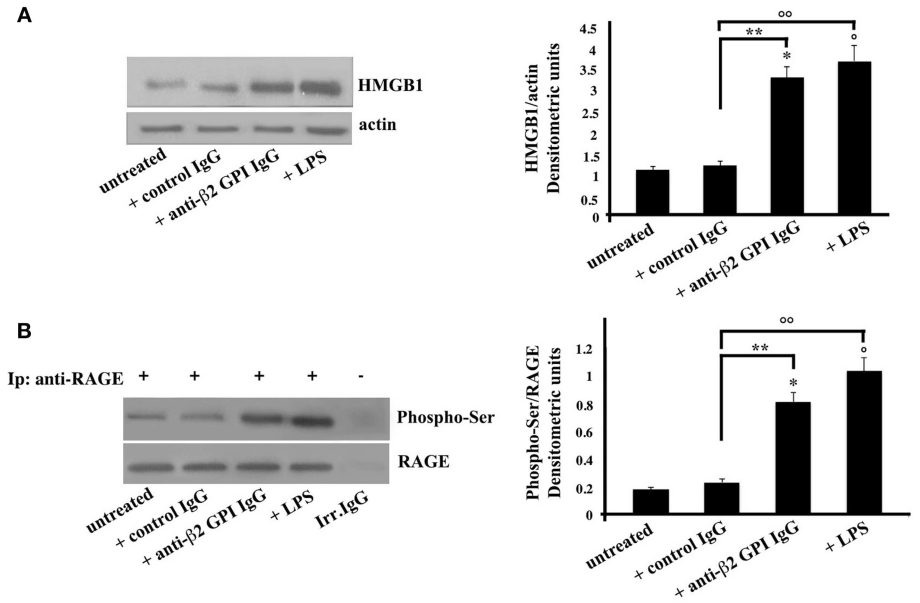


**FIGURE 2 |** Anti-$\beta_2$-GPI antibodies induce an increase of HMGB1 expression and RAGE activation in platelets. **(A)** Platelets from healthy donors, untreated or treated with control human serum IgG (200 μg/ml), human affinity-purified anti-$\beta_2$-GPI IgG (200 μg/ml) or LPS (100 ng/ml) for 4 h at 37°C, were analyzed by Western Blotting using rabbit anti-HMGB1 polyclonal Ab. Expression of HMGB1was detected and representative images of three independent experiments were shown. Right panel: the ratio of HMGB1 immunopositivity to actin was calculated. The results are expressed as mean ± SD from 3 independent experiments. *$p$ + anti-$\beta_2$-GPI IgG < 0.001 vs. untreated, °$p$ + LPS < 0.001 vs. untreated, **$p$ + anti-$\beta_2$-GPI IgG < 0.001 vs. + control IgG, °°$p$ + LPS < 0.001 vs. + control IgG. **(B)** Human platelets, untreated, or treated as above were lysed in RIPA buffer. Cell lysates were prepared and immunoprecipitated with goat anti-RAGE polyclonal Ab. Immunoprecipitates were analyzed using mouse anti-Phospho-Ser mAb. Immunoprecipitation was checked by mouse anti-RAGE monoclonal Ab. Right panel, densitometric Phospho-Ser /RAGE ratios are shown. Results represent the mean ± SD from 3 independent experiments. *p + anti-$\beta_2$-GPI IgG < 0.001 vs. untreated, °$p$ + LPS < 0.001 vs. untreated, **$p$ + anti-$\beta_2$-GPI IgG < 0.001 vs. + control IgG, °°$p$ + LPS < 0.001 vs. + control IgG.

## Patients Characteristics and Clinical Manifestations

The 60 APS patients enrolled in this study were all Caucasian, 25 were primary APS and 35 APS associated with SLE. The clinical and demographic characteristics of the enrolled patients are reported in **Table 1**. No patient had liver enzyme elevations. The absolute number of circulating monocytes was in the normal range ($0.1–1 \times 10^{-3}/\mu L$) in each patient.

## Serum Levels of HMGB1 and sRAGE in APS Patients and Clinical Outcomes

Since in a previous work we detected increased serum levels of HMGB1 in subjects affected by pregnancy morbidity, as well as in a small group of patients with APS (21), in this investigation we wanted to extend the data evaluating serum levels of HMGB1/soluble RAGE (sRAGE) in patients with APS and their possible correlation with clinical manifestations.

The results showed that the APS patients, either PAPS or SAPS, showed significantly increased serum levels of HMGB1, as compared to healthy subjects (**Figure 3A**), as revealed by densitometric analysis ($p < 0.0001$). Furthermore, no significant differences of HMGB1 levels between primary and secondary APS were found (**Figure 3B**).

In APS patients with recurrent thrombosis, a tendency to have elevated HMGB1 serum levels has been highlighted, compared to APS patients without recurrent thrombosis, although this result does not reach statistical significance (**Table 2**). In APS patients a correlation between serum levels of HMGB1 and disease duration and age was detected (**Table 3**).

As for the analysis of sRAGE serum levels, it revealed that patients with APS showed increased serum levels of protein compared to healthy controls (**Figure 4A**). This difference was highly significant ($p < 0.0001$) while no significant differences in sRAGE levels were found between primary and secondary APS (**Figure 4B**). Interestingly, in APS patients with spontaneous recurrent abortions we observed increased levels of sRAGE compared to APS patients without recurrent abortions ($p = 0.04$) (**Figure 4C**).

No significant correlation between HMGB1 levels and sRAGE levels were found.

# DISCUSSION

To analyze whether autoantibodies in APS may trigger the alarmin response, we preliminary analyzed *in vitro* whether anti-$\beta_2$-GPI antibodies from APS patients may induce both a HMGB1 relocation and activation of its putative receptor RAGE. We observed that anti-$\beta_2$-GPI can induce HMGB1 cellular relocation in both monocytes and platelets. Monocyte subpopulations in autoimmune diseases have been studied in

**TABLE 1 |** Clinical characteristics of patients studied.

| Characteristics *n* (%) | APS patients (*n* = 60) |
|---|---|
| F/M | 51/9 |
| Age (years) | |
| Mean ± SD | 43.5 ± 13.3 |
| Primary APS/APS associated with SLE | 25/35 |
| Disease duration (years) Median (IQR) | 5 (11) |
| **AUTOANTIBODIES** | |
| aCL (IgG or IgM) | 37 (61.7) |
| aβ2-GPI (IgG or IgM) | 34 (56.7) |
| LA | 35 (58.3) |
| Platelets Median (IQR) | 243 (72) |
| **TREATMENTS** | |
| Oral anticoagulants | 29 (48.3) |
| Antiaggregants | 32 (53.3) |
| Glucocorticoids | 17 (28.3) |
| Hydroxychloroquine | 19 (31.7) |
| Azathioprine | 5 (8.3) |
| Thalidomide | 1 (1.7) |
| Sulphasalazyne | 1 (1.7) |
| Pregnancy morbidity | 18 (35) |
| Spontaneous abortions* | 13 (25.5) |
| Normal Fetus Deaths** | 4 (7.8) |
| Premature births*** | 3 (5.9) |
| Vascular thrombosis# | 51 (85) |
| Arterial thrombosis | 24 (40) |
| Venous thrombosis | 37 (61.7) |
| Recurrent thrombosis | 25 (41.7) |
| **NON-CRITERIA APS FEATURES** | |
| Livedo reticularis | 18 (18.3) |
| Thrombocytopenia | 13 (30) |
| Migraine | 7 (21.7) |
| Seizures | 8 (18.3) |

**3 or + losses <10 weeks of gestation; **1 or + losses ≥10 weeks of gestation; *** preterm birth <34 weeks due to eclampsia, pre-eclampsia, or placental insufficiency. # Thrombosis (arterial, venous, or in small vessels) in any tissue, confirmed by imaging or histopathology (thrombosis without significant inflammation). aCL, anticardiolipin antibodies; aβ2GPI, anti-β2glycoprotein-I antibodies; LA, lupus anticoagulant; IQR, interquartile range.*

the last few years. In particular, M1 subtype plays an important inflammatory role in SLE pathogenesis and reduced M2a and M2c subpopulations may contribute to the lack of anti-inflammatory activity, whereas M2b subtype may play a role in causing disease directly (31). No significant difference was observed in the proportion of monocyte subsets between patients with APS and patients with SLE (32), as well as in our case in the expression of HMGB1 among monocyte subsets (data not shown). Furthermore, the anti-$\beta_2$-GPI Abs can induce the activation of RAGE, which appeared in its phosphorylated form. HMGB1 expression in monocytes may have several mechanistic implications. In particular, we observed that *in vitro* anti-β2-GPI-triggered HMGB1 release induces a decrease of phosphorylated mTOR. This finding is not surprising since it has already reported that HMGB1/RAGE knockdown is able to induce mTOR phosphorylation (33, 34), but may contribute to clarify the effect in APS patients of drugs able to induce mTOR blockade (35). Indeed, activation of the mTORC pathways plays a role in the vascular changes that are characteristic of APS nephropathy. Consequently, mTOR inhibitors such as sirolimus are currently used with a beneficial secondary effect on endothelial cells (35).

Regarding the molecular mechanisms by which anti-$\beta_2$-GPI antibodies may trigger the HMGB1/RAGE pathway in human monocytes, we can hypothesize that the antibodies may react with their target antigen strictly in association with TLR4 (24). Because of the molecular mimicry among $\beta_2$-GPI and bacterial antigens or microbial products, $\beta_2$-GPI might directly interact with a TLR (24) or, alternatively, anti-$\beta_2$-GPI may crosslink the molecule and TLR4 (36), thus inducing a proinflammatory and procoagulant phenotype, as ourselves already reported. As reported in results, HMGB1 was significantly increased in supernatant of cells stimulated with anti-$\beta_2$-GPI antibodies from APS patients either than in the supernatants of the untreated cells. It is well known that HMGB1 release by macrophages happens through the regulation of HMGB1 acetylation. In particular, in monocytes and macrophages HMGB1 can be extensively acetylated in such a way that the protein can be transferred from the nucleus to the cytoplasm and, in this form, be actively released by the secretory lysosomes (37). Thus, we suggest that most of HMGB1 in supernatant is acetylated. Alternatively, HMGB1 may be released along with NETosis, together with many other danger-associated molecular patterns in response to infections. Indeed, alarmins, including HMGB1, are detected as the initiators of NETosis (38).

Furthermore, HMGB1 was significantly increased in platelets„ with consequent RAGE activation, following anti-$\beta_2$-GPI antibody triggering. These data are not surprising since it has been already reported (39) that progenitor cells provided PLT with HMGB1, mRNA as well as protein. This gives PLTs, once stimulated, the ability to increase the expression of HMGB1 from mRNA and eventually release it. Moreover, in patients HMGB1 expression on platelets might also be achieved by fusion of HMGB1 containing microparticles with the plasma membrane of active platelets (39).

A series of studies have provided a close link between HMGB1 and platelet activation. In 2000, Rouhiainen et al. (40) detected the expression of HMGB1 in human platelets, using the Western blot and reverse transcription-polymerase chain reaction. Today, there are many data indicating that inflammation may induce thrombosis through the activation of platelets (41) and studies of recent years have shed light on the functions of platelets activated by HMGB1. Over the last decade, increasing evidence has suggested that HMGB1 could recognize its receptors on platelets, contributing to platelet aggregation and secretion (42, 43). In fact, HMGB1 has been shown to be released outside stressed or activated cells, including platelets (7, 44). Once in the extracellular space, HMGB1 can exert its biological functions by interacting with its receptors. Stark et al. (23) identified a high expression of HMGB1 as an important regulator of the prothrombotic cascade involving myeloid leukocytes and platelets, that favor the formation of occlusive deep venous thrombosis in a mouse model of venous thrombosis induced

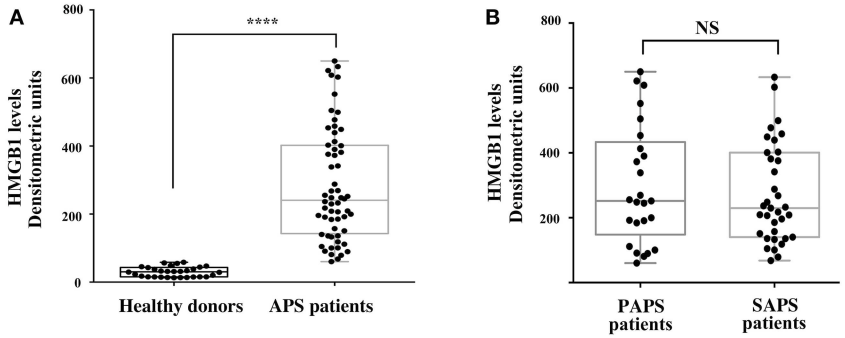


**FIGURE 3 |** Increase of serum HMGB1 levels in APS patients. **(A)** HMGB1 level were detected by Western Blot in APS patients ($n = 60$) and healthy donors ($n = 30$) sera, using rabbit anti-HMGB1 polyclonal Ab. Densitometric values of HMGB1 levels calculated in healthy subjects and APS patients are represented and summarized by boxplot statistics. A statistically significant difference between expression of HMGB1 in APS patients vs. control subjects was found (****$p < 0.0001$). **(B)** Densitometric values of HMGB1 levels calculated in PAPS patients ($n = 25$) and SAPS patients ($n = 35$) are represented and summarized by boxplot statistics. NS, not significant.

**TABLE 2 |** Treatment influence on HMGB1 and sRAGE serum levels.

| Variable no vs. yes | HMGB1 Densitometric Unit median (IQR) | *P*-value |
|---|---|---|
| Antiaggregants | 213.4 (151.5) vs. 331.93 (286.72) | 0.042 |
| Oral anticoagulants | 251.89 (265) vs. 236.55 (236.89) | 0.404 |
| Glucocorticoids | 278.52 (292.54) vs. 251.89 (265) | 0.595 |
| Hydroxychloroquine | 206.57 (292.84) vs. 375.87 (221.79) | 0.086 |
| Azathioprine | 278.52 (264.16) vs. 227.35 (254.48) | 0.538 |
| | **sRAGE Densitometric Unit median (IQR)** | ***P*-value** |
| Antiaggregants | 1012.82 (488) vs. 1036.92 (289.92) | 0.585 |
| Oral anticoagulants | 1034.73 (366) vs. 1022.78 (491.36) | 0.678 |
| Glucocorticoids | 1095 (425.57) vs. 953.83 (293.74) | 0.068 |
| Hydroxychloroquine | 952.71 (275.91) vs. 1133.87 (424.21) | 0.085 |
| Azathioprine | 1039.12 (382.5) vs. 944.27 (488.89) | 0.381 |

*HMGB1, High Mobility Group Box 1; sRAGE, serum Receptor for Advanced Glycation End products; IQR, Interquartile Range.*

**TABLE 3 |** Univariate analysis with serum HMGB1 and sRAGE.

| | Spearman *r* | 95% CI | *P*-value |
|---|---|---|---|
| HMGB1(DU) vs. sRAGE (DU) | 0.1390 | −0.1268 to 0.3861 | 0.2897 |
| HMGB1(DU) vs. Platelets (units/μl) | 0.03958 | −0.2884 to 0.3592 | 0.8109 |
| HMGB1(DU) vs. Disease duration (years) | 0.2801 | 0.01804 to 0.5061 | 0.0317 |
| HMGB1(DU) vs. Age (years) | 0.3608 | 0.1100 to 0.5684 | 0.0046 |
| sRAGE (DU) vs. Platelets (units/μl) | −0.1419 | −0.4457 to 0.1911 | 0.3888 |
| sRAGE (DU) vs. Disease duration (years) | 0.02738 | −0.2377 to 0.2887 | 0.8369 |
| sRAGE (DU) vs. Age (years) | −0.001654 | −0.2627 to 0.2596 | 0.9900 |

*HMGB1, High Mobility Group Box 1; sRAGE, serum Receptor for Advanced Glycation End products; DU, Densitometric Units.*

by reduction of flow in the inferior vena cava. These *in vivo* data are in line with what we report in this paper, in fact we suggest the possibility in the APS of a role of anti-$\beta_2$-GPI antibodies in the activation of the thrombotic cascade via the HMGB1 platelet pathway. Furthermore, again, in an *in vivo* model, Vogel et al. (45) reported that mice lacking HMGB1 in platelets showed increased bleeding times and reduced thrombus, platelet aggregation, inflammation and organ damage during experimental trauma/hemorrhagic shock. On the other hand, they showed that platelets were the main source of HMGB1 in the thrombi and that in traumatized patients, the expression of HMGB1 on the surface of the circulating platelets was markedly upregulated. Our data greatly reinforce the hypothesis considering platelet-derived HMGB1 as a potential target for the prevention of thrombosis.

In addition, following previous studies, by Shao et al. (46) in which is reported that preeclamptic sera and aPL both induced an increase in the cytoplasmic levels of the alarmin HMGB1, and by ourselves (21) in which we observed a significant increase in HMGB1 in sera from patients with APS, in the present work we confirmed and expanded these results in a larger cohort of patients with APS, demonstrating a significant correlation with the duration of the disease, without a significant difference between primary and secondary APS, which showed HMGB1 levels comparable with those detected in SLE patients (without APS) (47, 48).

Furthermore, in sera from APS patients with recurrent abortions we showed a significant increase in sRAGE respect to patients without. These findings are not surprising, since during pregnancy proinflammatory stimuli have been associated with higher risk of adverse pregnancy outcomes (49). In particular, HMGB1 may convey danger signals by triggering inflammatory patterns with extracellular signal–regulated kinases (ERKs), p38 and NF-kB activation via several cell surface receptors, including RAGE (22). Ota et al. showed that elevated levels of serum sRAGE are associated with recurrent pregnancy

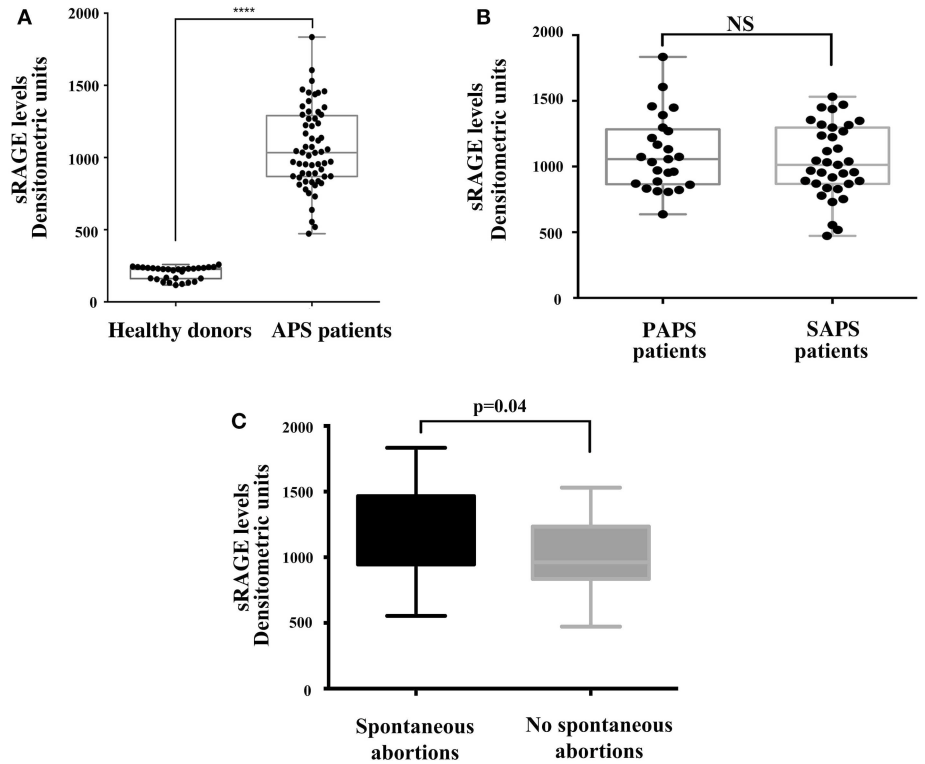


**FIGURE 4 |** Increase of serum sRAGE levels in APS patients. **(A)** sRAGE levels were detected by Western Blot in APS patients ($n = 60$) and healthy donors ($n = 30$) sera, using mouse anti-RAGE mAb. Densitometric values of sRAGE levels calculated in healthy subjects and APS patients are represented and summarized by boxplot statistics. A statistically significant difference between expression of sRAGE in APS patients vs. control subjects was found (****$p < 0.0001$). **(B)** Densitometric values of sRAGE levels calculated in PAPS patients ($n = 25$) and SAPS patients ($n = 35$) are represented and summarized by boxplot statistics. NS, not significant. **(C)** Densitometric analysis of sRAGE levels, represented and summarized by boxplot statistics, revealed that patients with spontaneous abortions had significantly higher values than patients without spontaneous abortions ($p = 0.04$).

losses (RPL) and speculated that RAGE might contribute to RPL by reducing uterine blood flow and subsequently causing ischemia in the fetus via inflammatory and thrombotic reactions (50). Interestingly, our results also showed that, at a distance of the acute clinical manifestation, a significant increase in the serum level of the soluble form of RAGE persisted. This situation indicates the involvement of this molecular system in the pathogenesis of thrombotic episodes related to APS. Furthermore, the significant increase in serum sRAGE levels was observed in patients with spontaneous recurrent abortions, which represent one of the main manifestations of the syndrome.

In conclusion, although it is already widely known that HMGB1 can induce and improve innate immunity, playing a role in the inflammatory phenomena of autoimmune diseases, our findings suggest that, in subjects with APS, not only elevated levels of HMGB1 but also of sRAGE may represent an alarm signal, indicating an increase of proinflammatory triggers. Although this finding cannot be considered highly specific (21), HMGB1/sRAGE may play a role in monitoring of recurrent abortion risk. In addition, HMGB1 may be useful in monitoring patients during particular treatments, such as antiaggregants, in which we found significantly higher levels of HMGB1. Further studies are needed to demonstrate that monitoring HMGB1/sRAGE, together with other prognostic parameters, may be a useful tool to evaluate the risk stratification of clinical manifestation(s) during APS. Finally, our *in vitro* data suggest that monitoring of these molecules can be useful to evaluate some pathogenic steps of the clinical manifestations of APS.

## ETHICS STATEMENT

Ethics committee, Sapienza Università di Roma - Policlinico Umberto I. All the patients signed an informed consent prior to enter in the study.

## AUTHOR CONTRIBUTIONS

VM, ST, MS, and RM conceived and designed the study. VM, AC, and GR performed the experiment. VM, ST, SM, FS, and FuC analyzed the data. MS and RM wrote the original manuscript. FaC, CA, GV, AL, and TG read and approved the final version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00460/full#supplementary-material

**Supplementary Figure 1 |** Anti-$\beta_2$-GPI IgG induced decrease of mTOR phosphorylation in monocytes. Cells, untreated or treated with control human serum IgG (200 μg/ml), with human affinity-purified anti-$\beta_2$-GPI IgG (200 μg/ml) or starved in total starvation medium (HBSS solution containing 1% rich medium), for 4 h at 37°C were lysed in RIPA buffer, containing protease and phosphatase inhibitors. Western blot analysis was done to detect the levels of phosphorylated (p-mTOR) and non-phosphorylated mTOR. The membrane was incubated with the following primary antibodies: rabbit polyclonal anti-p-mTOR (Ser2448; 1:1,000 Cell Signaling Technology, Inc., Boston, MA, USA) and anti-actin mAb antibodies (1:1,000 Sigma-Aldrich). The nitrocellulose membrane was stripped and re-probed for total mTOR levels using rabbit polyclonal anti-mTOR (1:1,000; Cell Signaling Technology, Inc.). Loading control was performed by evaluating actin expression in the same filter. Right panel, ratios of p-mTOR to mTOR were normalized to untreated, set at 1.0. Data are presented as the mean ± SD of three independent experiments. $*p$ + anti-$\beta_2$-GPI IgG < 0.001 vs. untreated, $^{\circ}p$ + HBSS < 0.001 vs. untreated, $**p$ + anti-$\beta_2$-GPI IgG < 0.001 vs. + control IgG, $^{\circ\circ}p$ + HBSS < 0.001 vs. + control IgG.

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REVIEW
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# Complement in the Pathophysiology of the Antiphospholipid Syndrome

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The antiphospholipid syndrome (APS) is characterized by thrombosis and pregnancy morbidity in the presence of antiphospholipid antibodies (aPL). Complement is a system of enzymes and regulatory proteins of the innate immune system that plays a key role in the inflammatory response to pathogenic stimuli. The complement and coagulation pathways are closely linked, and expanding data indicate that complement may be activated in patients with aPL and function as a cofactor in the pathogenesis of aPL-associated clinical events. Complement activation by aPL generates C5a, which induces neutrophil tissue factor-dependent procoagulant activity. Beta-2-glycoprotein I, the primary antigen for pathogenic aPL, has complement regulatory effects *in vitro*. Moreover, aPL induce fetal loss in wild-type mice but not in mice deficient in specific complement components (C3, C5). Antiphospholipid antibodies also induce thrombosis in wild type mice and this effect is attenuated in C3 or C6 deficient mice, or in the presence of a C5 inhibitor. Increased levels of complement activation products have been demonstrated in sera of patients with aPL, though the association with clinical events remains unclear. Eculizumab, a terminal complement inhibitor, has successfully been used to treat catastrophic APS and prevent APS-related thrombotic microangiopathy in the setting of renal transplant. However, the mechanisms of complement activation in APS, its role in the pathogenesis of aPL related complications in humans, and the potential of complement inhibition as a therapeutic target in APS require further study.

**Keywords: antiphosholipid antibodies, complement, thrombosis, endothelial, beta2 - glycoprotein I**

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## INTRODUCTION

Antiphospholipid syndrome (APS) is a systemic autoimmune disorder characterized by thrombosis affecting the venous or arterial vascular systems, and/or obstetrical morbidity along with the persistent presence of antiphospholipid antibodies (aPL), including lupus anticoagulant, anticardiolipin antibody and anti-beta-2-glycoprotein-I (β2GPI) (1). Rather than binding to anionic phospholipids such as cardiolipin as was previously believed, aPL are directed against phospholipid binding proteins bound to an appropriate biological surface such as a cellular membrane (2). Anti-β2GPI antibodies are the primary pathogenic antibody in APS (3–5), although aPL directed against other antigens such as prothrombin and phosphatidylserine have also been described (3, 6). The mechanisms by which aPL induce thrombosis and pregnancy loss are not fully understood. Though β2GPI is the primary antigen in APS, its inhibition does not directly have thrombotic effects as evidenced by the lack of a thrombotic phenotype in β2GPI deficient individuals (7, 8). Multiple pathogenic mechanisms have been proposed including inhibition of

the natural anticoagulant and fibrinolytic systems (9–12), activation of vascular cells including endothelial cells (13), platelets (14), and monocytes (15), procoagulant effects of extracellular vesicles (16), and disruption of the anticoagulant annexin A5 shield on cellular surfaces (17). Over the past two decades, complement activation has emerged as an attractive target for mechanistic and therapeutic investigations based on studies demonstrating evidence of complement activation in patients with APS, and murine models that indicate a critical role of complement in aPL-mediated thrombosis (18–21) and obstetric (22–24) complications (25). It remains unclear whether these are distinct mechanisms that reflect antibody heterogeneity or are linked to an as yet undefined central mechanism. Recurrent thrombosis and fetal loss are common despite standard treatment. The variability of treatment effectiveness in APS suggests that subsets of patients might benefit from treatments beyond anticoagulation.

The complement system, consisting of over 50 plasma proteins involved in host defense, is organized into three pathways; the immune complex mediated classical pathway, the lectin pathway, and the alternative pathway. These pathways converge at the level of complement component C3 and the terminal complement pathway that leads to generation of C5a, a potent pro-inflammatory molecule, and C5b-9 (the membrane attack complex) (**Figure 1**). Anti- β2GPI antibodies are associated with complement activation, and the complement and coagulation pathways are closely linked (26, 27); C5a induces neutrophil tissue factor-dependent procoagulant activity (26) and may inhibit fibrinolysis though increased activation of thrombin activated fibrinolysis inhibitor (TAFI). C3a and C5a also activate endothelial cells, inducing the expression of adhesion molecules and procoagulant activity (28–30), as well as platelets (31). Complement activation also causes placental inflammation and injury (32), a hallmark of fetal loss in APS (23). This review summarizes the current evidence supporting the role of complement in aPL associated clinical events, the interplay between complement and thrombosis in APS, therapeutic perspectives on complement targeted agents in APS, and areas of future research.

# COMPLEMENT IN OBSTETRIC APS

## Animal Models of Obstetric APS

The earliest and most compelling evidence for complement involvement in APS comes from murine models of aPL-induced pregnancy loss in which Branch et al. showed that passive transfer of IgG fractions from patients with aPL led to fetal loss (33). On histopathologic examination, IgG localized to the decidua, which showed prominent necrosis. A subsequent series of experiments confirmed this finding and elucidated the role of complement in aPL induced fetal loss. *In vivo* experiments have shown that β2GPI localizes to the decidua (34), and that aPL binding to β2GPI inhibits trophoblastic proliferation, syncytia formation and invasion into maternal decidua, which are required for successful placentation (35).

Holers et al. showed that intraperitoneal injection of IgG from patients with APS into pregnant mice led to fetal resorption in 40% of pregnancies and a 35% fetal weight reduction compared with control mice (36). Inhibition of the complement cascade with the C3 convertase inhibitor complement receptor 1–related gene/protein y (Crry)-Ig prevented aPL mediated fetal resorption. C3 deficient mice ($C3^{-/-}$) were also resistant to aPL mediated fetal loss (36). Girardi et al. later demonstrated that C5 deficiency or treatment of mice with anti-C5a monoclonal antibody protects against aPL induced pregnancy loss and growth retardation (22). Placentae from the aPL IgG treated mice showed human IgG deposition in the decidua, which demonstrated focal necrosis and apoptosis with neutrophil infiltrates (36). Neutrophils recruited by C5a expressed tissue factor that potentiated neutrophil activation and the respiratory burst leading to trophoblastic injury and fetal loss (24, 32). The absence of aPL-induced growth retardation and fetal resorption in mice deficient in C4 or C5 suggests that the classical pathway is involved in initiating these effects. However, factor B is necessary for aPL mediated fetal loss and its inhibition ameliorates these effects supporting a role of the alternative pathway in amplifying complement activation (37). Taken together, these studies suggest that C3 and C5 activation is central to aPL-mediated fetal loss in this model, with neutrophils and tissue factor playing pro-inflammatory roles. Girardi et al. have also suggested that the protective effect of heparin in APS pregnancies may reflect its inhibitory effects on complement (23).

## Complement Activation in Human Studies of Obstetric APS

Studies in humans support the role of complement in aPL mediated pregnancy complications. Hypocomplementemia, suggesting complement activation, has been observed in patients with SLE and APS (38), as well as those with primary APS and obstetric complications (39–41); however others have not found an association with hypocomplementemia and pregnancy complications in APS (42). In the PROMISSE study, which included nearly 500 pregnant women with lupus and/or aPL, adverse pregnancy outcomes were associated with increased serum levels of complement products Bb and C5b-9 early in pregnancy (43). In addition to elevated levels of complement activation products in serum, C4d was deposited at the feto-maternal interface in the placentae of women with SLE or APS, and correlated with fetal loss, decidual vasculopathy, increased syncytial knots and villous infarcts (44, 45). Interestingly, C5b-9 deposition in the trophoblast was not increased compared with control placentae, leading the authors to suggest that C5b-9 may not play a central role in aPL mediated placental injury, which is more likely to be caused by C3a and C5a mediated inflammation (45). Overall, these findings support a role for complement in aPL mediated pregnancy complications; however, the exact mechanisms of complement activation remain to be determined.

# COMPLEMENT IN VASCULAR APS

## Animal Models of Thrombotic APS

Animal models of thrombotic APS support a role for complement in aPL mediated thrombosis. Most early models of aPL induced thrombosis included passive transfer of aPL along

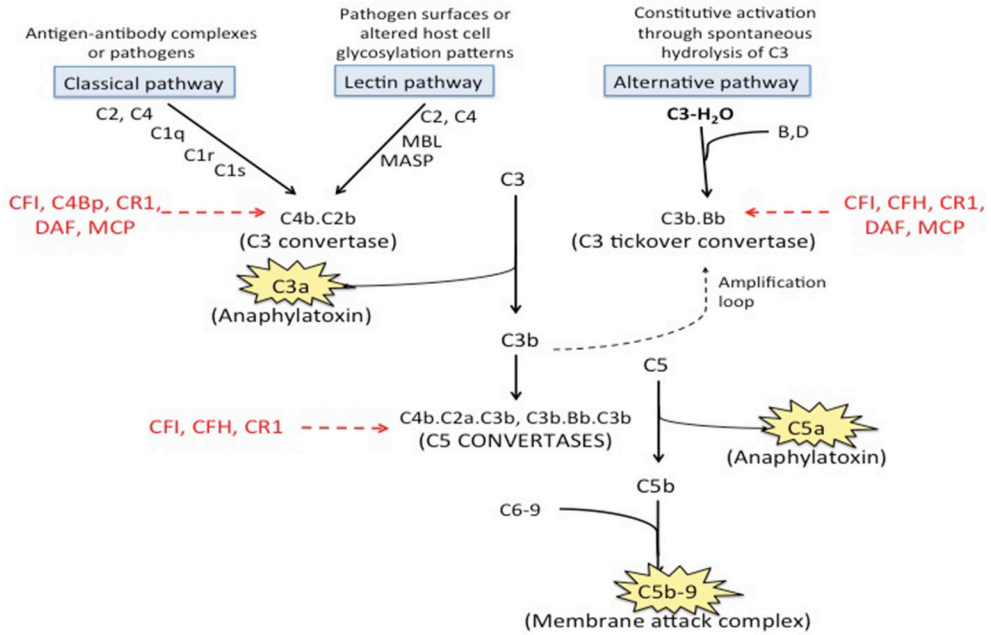


**FIGURE 1 |** Complement pathways. There are three well-recognized pathways of complement activation; (1) the classical pathway, (2) the lectin pathway, and (3) the alternative pathway. The classical and lectin pathways are activated when specific triggers are recognized by host pattern-recognition receptors while the alternative pathway is constitutively active. Activation of all there pathways ultimately leads to generation of a C3 convertase (C4b.C2a for the classical and lectin pathways and C3b·B for the alternative pathway), which cleave C3 to generate C3a and C3b. C3a is an anaphylatoxin. C3b is quickly inactivated when it lands on a healthy host cell but triggers a rapid amplification loop when it binds to a pathogen or altered host cell. C3b also complexes with the C3 convertases to form the C5 convertases (C4b·C2a·C3b and C3b·Bb·C3b) that cleave C5 into C5a (an anaphylatoxin) and C5b. C5b combines with C6-9 to form C5b-9, also called the membrane attack complex (MAC). Regulatory factors including decay accelerating factor (DAF, CD55), CD59, factor H (CFH), factor I (CFI), membrane cofactor protein (MCP) and C3b/C4b receptor 1 (CR1) act at various stages of the cascade to control complement activation.

with direct vessel injury by pinching (19, 46) or other means to induce thrombosis, which was reduced in mice with deficiencies of complement proteins C3, C5, or C6 (19), or in the presence of an inhibitory antibody against C5 (18). However, mechanical or chemical endothelial injury to initiate thrombosis that is propagated in the presence of aPL differs from the usual events in APS, in which a localized vascular insult is usually absent. Fischetti et al. used rats primed with lipopolysaccharide, which does not cause thrombosis by itself (20). Administration of aPL IgG to LPS primed mice led to thrombosis while administration of control IgG did not. Intravascular microscopy showed thrombosis in mesenteric vessels, and immunofluorescence staining confirmed co-localization of IgG and C3 in the vessel wall (20). Thrombosis was markedly attenuated in C6 deficient ($C6^{-/-}$) rats or animal treated with a C5 inhibitor (20). In another set of experiments, a recombinant single-chain fragment variable recognizing domain 1 of β2GPI induced thrombosis in wild type male Wistar rats primed with lipopolysaccharide and pregnancy loss in female mice, but these effects were blocked in C6 deficient rats or C5 depleted mice (21). A CH2 deleted version of this antibody still recognized β2GPI but failed to fix complement and did not induce thrombosis or pregnancy loss. In addition to demonstrating the critical role of complement in aPL induced thrombosis, these experiments show that unlike effects of anti-β2GPI on the placenta, the procoagulant effects of aPL require a priming factor or "second hit" provided by an inflammatory stimulus such as lipopolysaccharide (34). In these murine models of thrombotic APS, C9 is deposited on the vascular endothelium indicating the presence of the membrane attack complex (20, 21). The membrane attack complex triggers the extrinsic pathway of coagulation by inducing tissue factor expression on the endothelial surface (47).

## Complement Activation in Patients With APS

A role for complement activation in patients with thrombotic APS was first suggested nearly 25 years ago by the demonstration of higher serum levels of C5b-9 in patients with aPL and stroke compared with non-APS related stroke (48). Others have reported hypocomplementemia (39) and higher levels of complement fragment Bb and C3a (40, 49) in patients with APS; however, the association with APS-related thrombotic events or serologic characteristics is inconsistent (39, 49). More recently, deposits of C1q, C4, C3, and C5b-9 were noted to co-localize with β2GPI and IgG in the affected artery wall of a patient with primary APS and arterial thrombosis who also had increased plasma levels of C5a and C5b-9 (50).

A minority (1%) of patients with aPL develop catastrophic APS (CAPS), manifesting as small vessel thrombosis in three or

**TABLE 1 |** Reports of Eculizumab therapy for patients with catastrophic APS or severe APS.

| | Patient | Prior therapies | Eculizumab dose/duration | Outcome |
|---|---|---|---|---|
| Shapira et al. (54) | 28/M with SLE and APS with a pulmonary embolism at age 12, and arterial ischemia leading to leg amputation, mesenteric ischemia and recurrent CAPS | Heparin, argatroban, fondaparinux, cyclophosphamide, steroids, intravenous immunoglobulin, lepirudin, bivalirudin, aspirin, and clopidogrel, plasma exchange | Eculizumab, 900 mg, then 1,200 q 2weeks for 1 year | Resolution of anemia, thrombocytopenia, and thrombotic events |
| Appenzeller et al. (55) | 30/F with ITP and primary APS developed CAPS after pregnancy. Complicated by myocardial infarction and renal failure | Hydroxychloroquine, heparin, steroids, rituximab, plasma exchange, immunoadsorption, hemodialysis | Eculizumab × 3 months, mycophenylate, steroids (homozygous for C3 mutation, c.1677C>T; p.C559C) | Resolution of MAHA and thrombocytopenia. Later had partial relapse, dialysis dependent |
| Muller-Calleja et al. (56) | 3 patients undergoing renal transplant, 2 with prior CAPS | Prednisone, rituximab, anticoagulation | Eculizumab, 900 mg weekly begun the day after transplant, then 1,200 q 2 weeks | Successful engraftment up to 4 years, continued treatment |
| Strakhan et al. (57) | 36/F with hypertension, acute renal failure, strokes, acute coronary syndrome, and MAHA | Plasma exchange, steroids | Eculizumab 900 mg/wk × 4 then 1,200 q 2 weeks | Gradual improvement of MAHA, continued dialysis |
| Wig et al. (58) | 47/M with APS, multifocal thrombi, and thrombocytopenia followed by renal and liver infarcts | Heparin, plasma exchange, intravenous immunoglobulin, steroids, argatroban, heparin | Eculizumab 900 mg × 2 weekly doses, then 1,200 mg every 7–10 days | Gradual improvement in all parameters, but remains dialysis dependent |
| Gustavsen et al. (59) | 22/F with arterial thrombosis and ischemic ulcerations during pregnancy | Warfarin, low molecular weight heparin, aspirin | Eculizumab 600 mg × 2 weekly doses, prior to Cesarean section | Improvement of ischemic pain, no further thrombosis, no adverse fetal effects |
| Marchetti et al. (60) | 33/F with factor V Leiden and triple positive APS developed TMA at 30 weeks of gestation | Rituximab, aspirin, heparin | Eculizumab 600 mg, Cesarean section at 32 weeks, repeat Eculizumab after surgery | Stabilization of thrombocytopenia, renal function and hematocrit |

more organs within the span of a week in the absence of small vessel inflammation on histopathologic examination (51); CAPS is fatal in over 40% of cases (52). Increased serum C5b-9 has been detected in CAPS with clinical improvement after treatment with eculizumab correlating with a reduction in serum C5b-9 and increase in C3 and C4 (53). While animal studies and anecdotal success of complement inhibitory therapy support a role for complement in aPL induced thrombosis (**Table 1**) (50, 54, 61), there is a paucity of controlled clinical research studies, and the mechanisms of complement activation in APS and its correlation with vascular events remains incompletely understood.

## MECHANISMS OF COMPLEMENT ACTIVATION IN APS

The aPL profile can predict risk of thrombosis and pregnancy morbidity. For example, LA was associated with a higher risk of thrombosis (OR 3.6, 95% CI 1.2–10.9) than anti-$\beta_2$GPI (OR 2.4, 95%CI 1.3–4.2) and anti-prothrombin (anti-PT) antibodies (OR 1.4, 95% CI 1.0–2.1) in the Leiden thrombophilia study (62). Retrospective and prospective studies have not shown a consistent association between thrombosis and aCL (3, 63). However, the differential ability of different aPL to activate complement has not been studied extensively. Since $\beta_2$GPI is the primary antigen in APS, the anti-$\beta_2$GPI antibody has been proposed as the more clinically significant and predictive aPL (62, 64, 65). Available studies highlight the role of $\beta_2$GPI as a complement regulator (66), though others suggest a role of anti-C1q antibodies (67).

The mechanisms of complement activation in APS are not fully understood. Some have suggested that immune complexes in APS bind to C1q and activate the classical complement pathway (68). However, aCL and anti-β2GPI are frequently of the IgG2 subclass (69, 70), which has relatively weak ability to activate complement compared with IgG1. Anti-C1q antibodies have been detected in patients with SLE, in whom they correlate with clinical manifestations particularly lupus nephritis (71, 72). In a mouse model, an anti-C1q monoclonal antibody enhanced complement activation by the classical pathway and caused renal injury (67). Oku et al. (73) have reported that antibodies against C1q were more prevalent in patients with primary APS (36%) than controls with other non-SLE autoimmune disorders, and concluded that these antibodies contribute to complement activation, since titers of anti-C1q correlated with levels of C4a. However, anti-C1q antibodies did not correlate

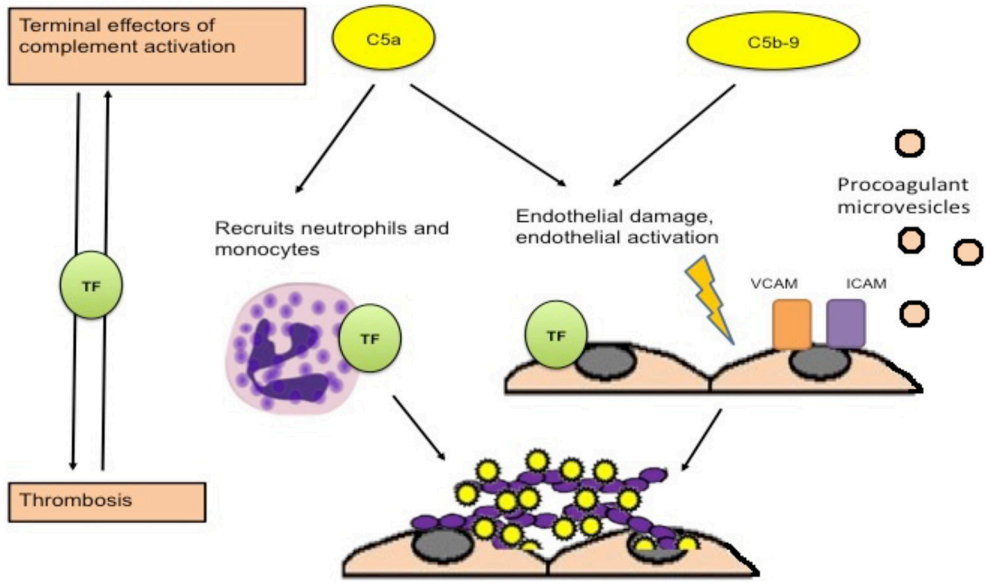


**FIGURE 2 |** Procoagulant effects of complement activation. Activation of complement leads to generation of C5a and C5b, which combines with other terminal complement components to form the membrane attack complex. C5a is an anaphylatoxin that recruits neutrophils and leads to expression of tissue factor on neutrophils, monocytes and endothelial cells, which is associated with procoagulant activity. Deposition of the membrane attack complex on the endothelium leads to endothelial injury and procoagulant changes including expression of adhesion molecules, secretion of von Willebrand factor, and release of procoagulant microvesicles [adapted from Ritis et al. (26)].

with thrombosis or pregnancy loss although a small subset of patients with recurrent thrombosis had a higher rate of anti-C1q antibodies (73). Finally, there is essentially no data on activation of complement by IgM anti-β2GPI antibodies.

Gropp et al. have reported a complement regulatory role of β2GPI, which inhibits complement activation by enhancing C3/C3b degradation (66). It has been proposed that when bound to a surface, β2GPI undergoes a conformational change from a circular form to an elongated form that can bind C3; in turn, C3 undergoes a conformational change to expose binding sites that make it susceptible to degradation by complement factor H (CFH) and factor I (66). In addition to its inhibitory effects on complement activation, CFH also has structural similarity to β2GPI and appears to share its property of inhibiting of contact pathway activation triggered by anionic phospholipids (74). Some APS patients, particularly those with recurrent thrombotic events, have autoantibodies against CFH suggesting a role of these antibodies in the predisposition to thrombosis (75, 76). A recent study reported low levels of CFH in patients with primary APS who also had low C3 suggesting complement activation (77).

## COMPLEMENT AND VASCULAR THROMBOSIS

Though the mechanisms of complement activation in APS are unclear, several mechanisms by which activated complement may contribute to thrombotic events have been suggested. Activation of complement leads to cleavage of C5, generating C5a and C5b (leading to membrane attack complex formation). Ritis et al. demonstrated that aPL-induced complement activation may lead to neutrophil expression of TF mediated through the C5a receptor, leading to expression of procoagulant activity (**Figure 2**) (26). C5a also induces TF expression on monocytes and endothelial cells (78, 79). In addition, deposition of C5b-9 on the endothelial surface leads to secretion of high molecular weight multimers of von Willebrand factor (80), expression of P selectin (81), and plasma membrane vesiculation that exposes a catalytic surface for the prothrombinase complex (82). Interestingly, a recent experiment by Müller-Calleja et al. indicated that a cofactor (β2GPI) -independent aPL induced exposure of procoagulant phosphatidylserine and activated TF on monocytes and induced thrombosis, and that C3 but not C5 was required (56). Complement activation can also contribute to depressed fibrinolysis, a recognized thrombogenic mechanism in APS (83–85). Events occurring during thrombosis and fibrinolysis can also activate complement leading to the generation of C5a and a functional membrane attack complex (86, 87), further amplifying activation of coagulation and thrombosis.

## THERAPEUTIC IMPLICATIONS

Though long-term anticoagulation with vitamin K antagonists and a combination of aspirin with low molecular weight heparin are the mainstay of therapy for thrombotic and obstetric APS, respectively, some patients develop recurrent aPL-related clinical events despite "adequate" therapies, indicating a need for other treatments (52). The expanding data supporting a role of complement in aPL associated complications makes

complement inhibition an attractive clinical target in APS. Moreover, complement targeted therapeutics are an active area of investigation, and the terminal complement inhibitor, eculizumab, is already widely used for the treatment of paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome. Although no complement inhibitors are approved for use in APS, eculizumab has been used successfully in patients with CAPS (54, 57, 61), to prevent recurrent CAPS in patients undergoing renal transplantation (88), and to prevent re-thrombosis in a patient with APS and recurrent arterial thrombosis (50). Recent reports of its successful use in CAPS (**Table 1**) are particularly encouraging since mortality in CAPS is as high as 40% with current treatment modalities (89). Pregnancy is considered a high-risk period for patients with aPL, with a high rate of fetal loss and pregnancy complications, and may serve as the "second hit" that leads to vascular complications. Preeclampsia and HELLP syndrome are also more common in women with aPL (55, 60), and may be complement mediated (90–92). Eculizumab crosses the placenta only minimally and does not affect the fetus (93). Hence, complement blockade may be an effective therapeutic modality for severe aPL related complications, including CAPS, during pregnancy (59, 94, 95).

Despite these encouraging data, there is still insufficient data to support the routine use of anti-complement therapy in APS, particularly patients without CAPS, and further mechanistic studies and randomized clinical trials are required. Ideally, complement-related biomarkers would be able to identify patients who are more likely to be refractory to standard therapy, and those who would benefit from complement inhibition as an adjunct to anticoagulation and antiplatelet therapy. However, standard measures of circulating complement cleavage products have not yet been shown to correlate with or predict the development of thrombosis.

## CONCLUSIONS

Recent experimental data indicate that complement activation plays a critical role in the pathogenesis of thrombosis and pregnancy complications in APS (25). However, the mechanisms by which aPL activate complement are not fully understood. Complement inhibition may provide a useful adjunctive therapy for patients with APS refractory to standard therapies, which is supported by reports of successful use of complement inhibition in patients with CAPS (54, 61). Mechanistic and clinical studies are needed to evaluate the efficacy of complement inhibition in APS and to develop biomarkers that can identify patients who might benefit from complement inhibition.

## AUTHOR CONTRIBUTIONS

SC wrote the primary manuscript with the assistance and editing by KM and RB.

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# Type I Interferon Signature in Primary Antiphospholipid Syndrome: Clinical and Laboratory Associations

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**Background:** Increased expression of type I interferon (IFN)-regulated genes has been described in blood and tissue cells from patients with systemic lupus erythematosus (SLE) and other rheumatic disorders. Only isolated studies have examined the type I IFN gene expression in antiphoshospholipid syndrome (APS), while efforts to evaluate associations with APS-related factors are scarce.

**Objective:** Our aim was to investigate the type I IFN signature in patients with primary APS (PAPS), SLE/APS, and SLE in comparison with healthy controls, and to evaluate associations with disease-related characteristics.

**Methods:** We measured the type I IFN score, derived from relative expressions of three IFN-inducible genes (MX-1, IFIT-1, and IFI-44) in peripheral blood mononuclear cells from 55 patients with PAPS, 34 with SLE/APS, 48 with SLE, and 28 controls. In patients with PAPS, we performed multivariate regression to examine associations of type I IFN score with their clinical, laboratory and treatment characteristics.

**Results:** Type I IFN score was increased in all patient groups vs. controls ($p = 0.028$, $p = 0.027$, $p = 0.028$ for PAPS, SLE/APS, and SLE, respectively). IFI-44 had the most pronounced expression. In patients with PAPS, multivariate linear regression revealed positive associations of type I IFN score with female gender (b-coefficient = 0.49; 95% CI 0.04, 0.94; $p = 0.034$) and IgG or IgM anti-β2GPI antibodies (b-coefficient = 0.53; 95% CI 0.10, 0.96; $p = 0.017$), and negative associations with age (b-coefficient = −0.02/year; 95% CI −0.04, −0.01; $p = 0.027$) and hydroxychloroquine use (b-coefficient = −0.51; 95% CI-0.96, −0.06; $p = 0.027$).

**Conclusion:** Type I IFN score is increased in PAPS and correlated positively with anti-β2GPI antibodies and negatively with hydroxychloroquine use.

**Keywords: antiphospholipid syndrome, systemic lupus erythematosus, type I Interferon signature, type I Interferon score, antiphospholipid antibodies, anti-b2-glycoprotein I antibodies, hydroxycloroquine**

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## INTRODUCTION

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by recurrent venous and/or arterial thrombotic events and/or obstetric complications and the presence of antiphospholipid antibodies (aPL) including anti-cardiolipin antibodies (aCL), anti-beta2-glycoprotein I antibodies (anti-β2GPI), and lupus anticoagulant (LA) (1). APS occurs either

as primary APS (PAPS) or secondary APS, when it is associated with other autoimmune diseases, mainly systemic lupus erythematosus (SLE/APS). Approximately 30–40% of SLE patients have positive aPL and almost one third of them develop APS (1, 2). In addition to well-characterized thrombotic events, other manifestations such as thrombocytopenia, livedo reticularis, valvular disease, APS nephropathy, or neurological disorders such as epilepsy and cognitive dysfunction may occur in APS, defined as non-criteria APS manifestations (3–5). Accordingly, in addition to thrombogenic mechanisms, aPL-mediated inflammatory processes have also been identified in APS, and emerging therapies associated with these inflammatory pathways include hydroxychloroquine, B-cell targeted therapy, complement inhibition, peptide therapy, mTOR inhibitors, and others (6).

Type I interferons (IFNs) are cytokines that have various effects on innate and adaptive immune cells and have been implicated in the pathogenesis of a number of systemic autoimmune diseases including SLE, Sjögren's syndrome, rheumatoid arthritis, systemic sclerosis and myositis (7–11). Gene expression profiling data from patients with SLE have shown high messenger RNA transcripts of genes regulated by type I IFN, also known as IFN signature, that correlated with clinical and laboratory indices of lupus activity in several studies (12, 13). These findings have spurred research evaluating type I IFN-blocking agents as therapeutic alternatives in SLE (14). Only a few studies have addressed the question of IFN-inducible gene expression in PAPS and SLE/APS (15–17). However, most studies had a small sample size and only one attempted to correlate the type I IFN signature to disease-related characteristics of patients with PAPS (17).

The aim of this study was to compare the type I IFN signature in peripheral blood mononuclear cells (PBMCs) from patients with PAPS, patients with SLE/APS, patients with SLE not fulfilling the classification criteria for APS, and healthy individuals. We also evaluated potential associations between type I IFN signature and several clinical, laboratory and treatment characteristics of patients with PAPS.

## MATERIALS AND METHODS

Consecutive adult patients with PAPS and age-matched patients with SLE/APS and SLE/non-APS followed in our department, were included in the study. Healthy individuals matched to PAPS patients by age distribution were recruited using brochures in the hospital and local community centers. Patients with APS (either PAPS or SLE/APS) fulfilled the updated Sapporo classification criteria (1), and patients with SLE met the updated ACR classification criteria for SLE (18). Candidates with active infection or hospitalization within the previous month, pregnancy, or history of malignancy, were excluded. The study protocol was approved by the local IRB ("Laikon Hospital Scientific Council") and all participants provided written informed consent.

At the study visit, all patients underwent thorough clinical and laboratory evaluation and their medical records were reviewed. For patients with APS, we recorded the details of prior thrombotic events (number, type, location) and/or obstetric complications establishing the diagnosis of APS, as well as the presence of non-criteria clinical manifestations as defined by the updated Sapporo classification criteria for APS (1). For patients with SLE, we recorded disease activity using the Systemic Lupus Erythematosus (SLE) Disease Activity Index-−2000 (SLEDAI-2K), as well as history of major SLE complications, including nephritis and CNS involvement. Medication use was reviewed and recorded including corticosteroids, hydroxychloroquine, immunosuppressives (cyclophosphamide, azathioprine, mycophenolate mofetil, methotrexate, leflunomide, and cyclosporine), antiplatelets, oral anticoagulants, and statins. Patients receiving biologic agents such as B cell depletion and adalimumab were excluded.

Blood samples were drawn from patients for immunologic tests including antinuclear antibodies, anti-dsDNA, anti-Sm, anti-Ro/SSA and anti-La/SSB antibodies, C3 and C4 levels, aCL and anti-β2GPI antibodies of IgG and IgM isotype using standard ELISA (1), and LA measured according to Scientific Standardization Subcommittee (SSC) on Lupus Anticoagulant/Phospholipid Antibodies guidelines (19). Additional 8–10 mL samples were collected from all participants for the isolation of PBMCs using Lymphoprep (Stem Cell Technologies) as the density gradient medium, according to the manufacturer's instructions.

Total RNA was extracted from PBMCs using Trizol reagent (Ambion, Life Sciences, USA) according to standard procedure. RNA concentration and quality was determined by spectrophotometry (Biospec Nano, Japan). Total RNA was transcribed into cDNA, which was then quantified by Quantitative Real-Time PCR (qRT-PCR) reaction as previously described (20). Three genes typically induced by type I IFN (10, 16, 20) were selected: myxovirus (influenza virus) resistance 1 (MX-1), interferon-induced protein with tetratricopeptide repeats 1 (IFIT-1) and interferon-induced protein 44 (IFI44). The levels of mRNA expression of type I IFN inducible-genes were determined based on the expression value of the glyceraldehyde phosphate dehydrogenase (GAPDH) reporter-housekeeping gene. Type I IFN score, was defined as the sum of relative expressions of MX-1, IFIT-1, and IFI-44 and calculated as previously described (20, 21). Type I IFN score was considered high if it exceeded the mean + 2*standard deviation (SD) value of the control group.

### Statistical Analysis

Between-group comparisons were tested using the Kruskal-Wallis and Mann-Whitney tests for continuous data, and Chi2 tests for nominal data. The Shidak-Holm method was used for correction of *p*-values for multiple comparisons. For patients with PAPS, we used the Mann-Whitney test to assess univariate associations of type I IFN score with nominal disease-related factors, and age as a binary variable, dichotomized on the median of PAPS group. We then applied linear regression to investigate multivariate associations of log-transformed type-I IFN score with clinical, laboratory and treatment characteristics of patients with PAPS. We applied

**TABLE 1 |** Baseline characteristics of study participant groups.

| | PAPS $n = 55$ | SLE/APS $n = 34$ | SLE $n = 48$ |
|---|---|---|---|
| Age (years, mean ± SD) | 44.6 ± 13.2 | 45.4 ± 11.5 | 47.4 ± 16.7 |
| Female gender [*n*, (%)] | 34 (62) | 28 (82) | 45 (94) |
| Disease duration (years, mean ± SD) | 7.6 ± 7.5 | 11.3 ± 9.3 | 9.6 ± 7.7 |
| Thrombotic events [*n*, (%)] | 48 (87) | 31 (91) | 2 (4) |
| Venous thrombosis [*n*, (%)] | 25 (46) | 19(56) | 1 (2) |
| Arterial thrombosis [*n*, (%)] | 33 (60) | 15 (44) | 1 (2) |
| Both arterial and venous events [*n*, (%)] | 10 (18) | 4(12) | 0 (0) |
| CVA [*n*, (%)] | 18 (33) | 12 (35) | 0 (0) |
| Recurrent thrombotic events [*n*, (%)] | 23 (42) | 13 (38) | 1(2) |
| Obstetric APS complications [*n*, (% among women)] | 14 (41) | 9(32) | 0 (0) |
| Non-criteria APS manifestations [*n*, (%)] | 18(33) | 16 (47) | - |
| Corticosteroid use [*n*, (%)] | 0 (0) | 15 (44) | 29 (60) |
| Immunosuppresives use [*n*, (%)] | 0 (0) | 18 (53) | 19 (40) |
| Hydroxychloroquine use [*n*, (%)] | 29 (53) | 25 (74) | 39 (81) |
| Antiplatelet use [*n*, (%)] | 28 (51) | 15 (44) | 19 (40) |
| Statin use [*n*, (%)] | 9 (16) | 10 (29) | 7 (15) |
| Oral anticoagulation [*n*, (%)] | 48 (87) | 32 (94) | 1 (2) |
| LA positivity [*n*, (%)] | 42 (76) | 24 (71) | 7 (15) |
| Anticardiolipin IgG positivity [*n*, (%)] | 26 (47) | 19 (56) | 10 (21) |
| Anticardiolipin IgM positivity[*n*, (%)] | 26 (47) | 17 (50) | 11 (23) |
| Anti-β2GPI IgG positivity [*n*, (%)] | 22 (40) | 11 (32) | 6 (13) |
| Anti-β2GPI IgM positivity [*n*, (%)] | 13 (24) | 10 (29) | 4 (8) |
| Double aPL positivity [*n*, (%)] | 35 (64) | 23 (68) | 9 (19) |
| Triple aPL positivity [*n*, (%)] | 22 (40) | 14 (41) | 5 (10) |
| Complement C3 levels (mean ± SD) | 105 ± 25 | 92 ± 19 | 87 ± 26 |
| Complement C4 levels (mean ± SD) | 20 ± 8 | 16 ± 8 | 16 ± 8 |
| Low complement levels [*n*, (%)] | 7 (13) | 8 (24) | 13 (27) |
| Antinuclear antibodies [*n*, (%)] | 19 (35) | 29 (85) | 39 (81) |
| SLEDAI (mean ± SD) | - | 2.61 ± 1.92 | 3 ± 4.48 |
| History of nephritis [*n*, (%)] | - | 6 (18) | 10 (21) |
| History of CNS involvement [*n*, (%)] | - | 8 (24) | 3 (6) |

*SD, Standard Deviation; HC, Healthy Controls; APS, Antiphospholipid Syndrome; PAPS, Primary APS; SLE, Systemic Lupus Erythematosus; SLE/APS, Systemic Lupus Erythematosus-associated APS; CVA, Cerebrovascular accident; SLEDAI, SLE Disease Activity Index; CNS, Central Nervous System.*

the stepwise backward algorithm ($p = 0.1$) to construct the final multivariate linear regression model, starting from an initial model containing the PAPS-related parameters that demonstrated univariate associations with the type I IFN score at the 0.25 level and adjusting for age and gender. Statistical analysis was performed using STATA version 12.0 (College Station™, TX, USA) and Graph Pad PRISM software (La Jolla™, CA, USA).

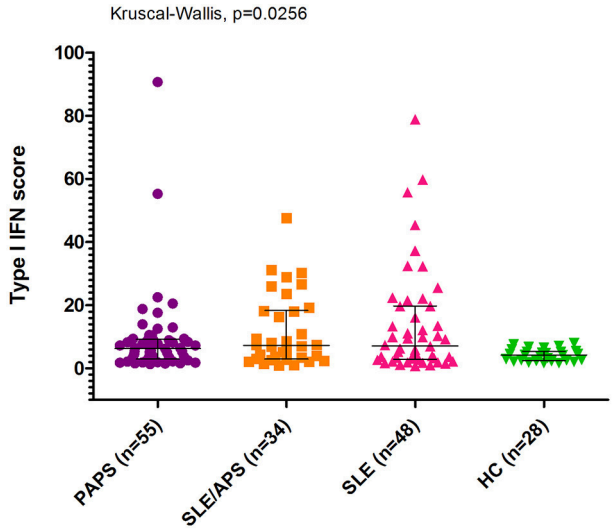


**FIGURE 1 |** Type I interferon score measurements with median and interquartile range in patients with PAPS, SLE/APS, and SLE vs. healthy controls. IFN, Interferon; HC, Healthy Controls; APS, Antiphospholipid Syndrome; PAPS, Primary APS; SLE, Systemic Lupus Erythematosus; SLE/APS, Systemic Lupus Erythematosus-associated APS.

## RESULTS

We included in the analysis 55 patients with PAPS, 34 age-matched patients with SLE/APS and 48 with SLE/non-APS, and 28 healthy controls. Patient characteristics are shown in **Table 1**. Female predominance was more pronounced in the SLE group, followed by patients with SLE/APS. The group of PAPS patients characterized by more arterial thrombotic events, recurrent venous or arterial thromboses, and obstetric APS events, but fewer non-criteria APS manifestations than the SLE/APS group. Among patients with PAPS, 35% had positive ANA in low titers, but none had SLE-specific antibody positivity such as anti-dsDNA or anti-Sm antibodies. None had anti-Ro/SSA or anti-La/SSB antibodies.

The median type I IFN score was highest in patients with SLE/APS (7.2, IQR: 3.0–18.1), followed by patients with SLE (7.1, IQR: 2.7–19.7), PAPS 6.3, IQR: 3.0–9.2), and controls (4.2, IQR: 2.5–5.4) (**Figure 1**). Mann-Whitney tests for comparison of the type I IFN score in each patient group vs. controls yielded unadjusted $p$-values of 0.014 for PAPS, 0.009 for SLE/APS, and 0.018 for SLE, and multiple-comparison adjusted $p$-values of 0.028, 0.027, and 0.028, respectively. Among the three genes comprising the type I IFN score, differences between patients and controls were most pronounced in the fold expression of IFI-44 (**Figure 2**). A high type I IFN signature was observed in 47.92% of patients with SLE, 44.12% of SLE/APS, and 38.18% of patients with PAPS (**Figure 3**).

Among patients with PAPS, the type I IFN score in univariate analysis was associated with anti-β2GPI antibodies of either IgG or IgM isotype, with a trend for an association with triple aPL positivity (**Table 2**). In multivariate regression, estimates

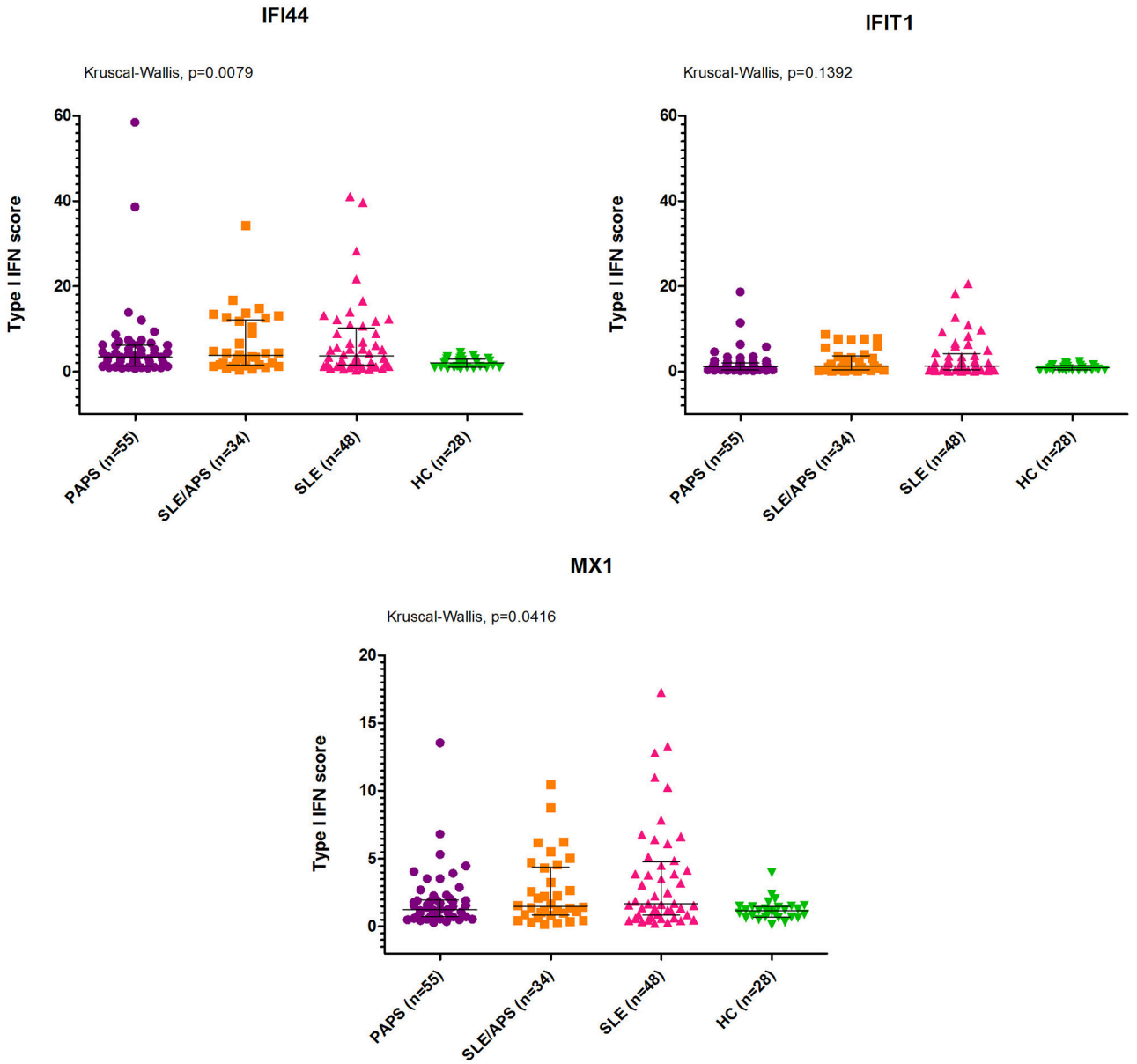


**FIGURE 2 |** IFI44, IFIT1, and MX1 mRNA expression levels with median and interquartile range in PAPS, SLE/APS, and SLE patients vs. healthy controls. HC, Healthy Controls; APS, Antiphospholipid Syndrome; PAPS, Primary APS; SLE, Systemic Lupus Erythematosus; SLE/APS, Systemic Lupus Erythematosus-associated APS.

derived from linear regression constructed by applying a stepwise backward algorithm ($p$ for removal = 0.1) to an initial model including age, gender, arterial events, aCL, antiβ2GPI, triple aPL, current use of hydroxycloroquine, and statins. The results of multivariate analysis (**Table 3**) showed that type I IFN score was significantly higher in females (b-coefficient = 0.49, 95% CI (0.04, 0.94), $p = 0.034$) and patients with medium-to-high IgG or IgM anti-β2GPI antibodies (b-coefficient = 0.53, 95% CI (0.10–0.96), $p = 0.017$), and lower with increasing age (b-coefficient = −0.02 per year, 95% CI: (−0.04, −0.01), $p = 0.027$) and hydroxychloroquine use (b-coefficient = −0.51, 95% CI (−0.96, −0.06), $p = 0.027$).

## DISCUSSION

In this study, we found that patients with PAPS had high type I IFN score relative to controls, and similar to that in SLE and SLE/APS patients. Patients with PAPS with medium-to-high anti-β2GPI antibody titers of either IgG or IgM isotype had higher IFN scores, whereas those treated with hydroxychloroquine had lower scores, after adjusting for age and gender and other APS-related factors.

The findings of high type I IFN score in patients with PAPS vs. controls, and in comparable levels to that in SLE and SLE/APS patients, indicate that inflammatory pathways

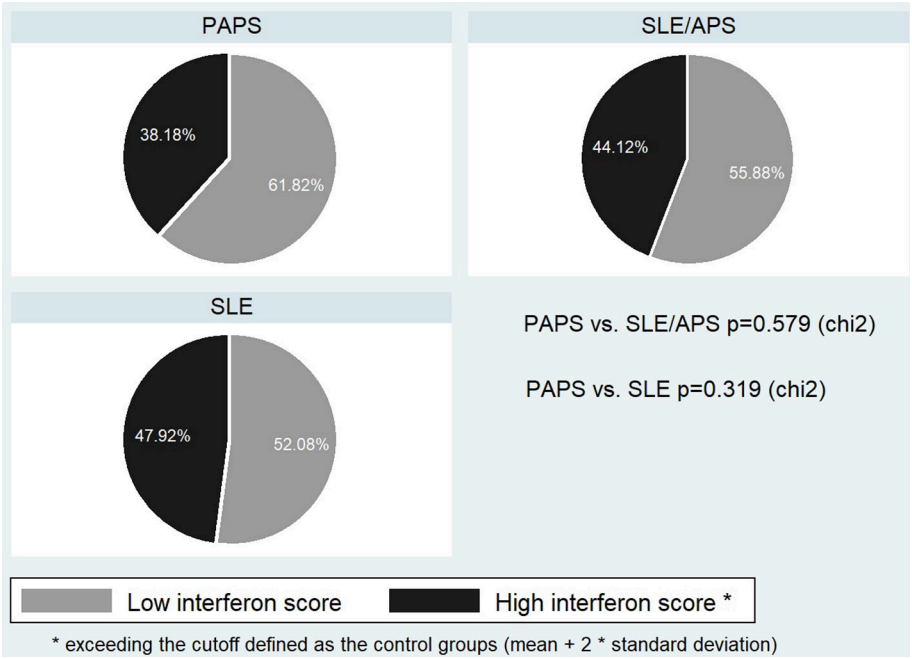


**FIGURE 3 |** Frequency of high type I IFN score in PAPS, SLE, and SLE/APS groups. APS, Antiphospholipid Syndrome; PAPS, Primary APS; SLE, Systemic Lupus Erythematosus; SLE/APS, Systemic Lupus Erythematosus-associated APS.

**TABLE 2 |** Univariate associations between type I interferon score and disease-related factors in patients with PAPS.

| Disease-related factor | Type I interferon score median, (interquartile range) | | *p*-value* |
|---|---|---|---|
| | Factor absent | Factor present | |
| Age >44 years# | 6.45 (3.63–9.38) | 5.13 (2.11–8.41) | 0.125 |
| Female gender | 4.99 (2.54–8.41) | 6.82 (3.56–9.38) | 0.239 |
| Arterial thrombotic events | 7.55 (2.98–12.97) | 5.30 (2.95–8.41) | 0.223 |
| Non-criteria APS manifestations | 6.45 (2.54–9.36) | 5.28 (3.56–8.38) | 0.733 |
| Recurrent thrombotic events | 6.44 (2.66–9.88) | 5.30 (3.56–8.41) | 0.682 |
| Obstetric APS complications | 6.27 (3.56–17.61) | 4.99 (2.37–8.41) | 0.277 |
| aCL positivity | 3.05 (1.82–9.18) | 6.45 (3.63–8.92) | 0.122 |
| Anti-β2GPI positivity | 4.99 (1.94–8.7) | 8.00 (4.30–9.37) | 0.045 |
| LA positivity | 7.21 (2.17–8.70) | 5.92 (3.55–9.38) | 0.663 |
| Triple aPL positivity | 4.99 (2.11–8.78) | 7.54 (5.03–9.38) | 0.057 |
| Hydroxychloroquine use | 8.00 (4.15–9.38) | 5.30 (2.95–8.38) | 0.231 |
| Antiplatelet use | 6.44 (2.54–8.78) | 5.92 (3.55–9.88) | 0.602 |
| Statin use | 6.83 (3.55–9.36) | 3.63 (1.94–5.30) | 0.101 |

**P-value derived from Mann-Whitney U.*
*#Cut off selected to coincide with the median age in the PAPS group.*
*Anti-β2GPI, anti-beta2-glycoprotein I antibody; aCL, anti-cardiolipin antibody; aPL, antiphospholipid antibody; APS, antiphospholipid syndrome; PAPS, primary APS; SLE/APS, Systemic Lupus Erythematosus-associated APS.*

involving type I IFN may be implicated in the pathophysiology of APS, independently of SLE co-existence. Our findings are congruent to those of previous studies on type I IFN signature in PAPS. Bernales and colleagues reported for the first time high type I IFN signature in a small series of 13 PAPS and 17 SLE patients compared to controls (15). Grenn and colleagues found elevated levels of the IFN-inducible genes IFIT-1, IFI44, and PRKR in PBMCs and sera from 42 PAPS patients, and in serum samples from an independent cohort of 26 patients with PAPS (16). Van den Hoogen and colleagues showed that type I IFN score was higher in 24 patients with PAPS than controls, but lower compared to 47 patients with SLE and 28 with SLE/APS (17). Recently, Knight and colleagues demonstrated a proinflammatory gene expression signature in PAPS using

**TABLE 3 |** Multivariate linear regression model of clinical and laboratory determinants of log-transformed type I interferon score in patients with PAPS.

| Parameter | β coefficient | 95% confidence interval | *P*-value |
|---|---|---|---|
| Age (per year) | −0.02 | −0.04, −0.02 | 0.027 |
| Female gender | 0.49 | 0.04, 0.94 | 0.034 |
| Hydroxychloroquine use | −0.51 | −0.95, −0.06 | 0.027 |
| Anti-β2GPI positivity | 0.53 | 0.10, 0.96 | 0.017 |

*Anti-β2GPI, anti-beta2-glycoprotein I antibody; PAPS, primary APS.*

RNA sequencing, mainly driven by up-regulation of type I IFN-inducible genes and more specifically by high transcription of IFIT-1 and MX-1 (22), which was also highly expressed in our patients with PAPS.

In our PAPS patients, anti-β2GPI antibodies were significantly correlated with type IFN scores. Our findings are in accordance with those of Grenn and colleagues, showing a significant correlation between anti-β2GPI positivity and elevated levels of IFIT-1 and IFI44 in PBMCs of PAPS patients (16). Basic research findings also support that aPL can induce IFNα production in cell cultures from pDCs from patients with APS (23). Furthermore, antibodies to β2GPI, especially to domain I of β2GPI, emerge in current research as one of the main pathogenic autoantibody subsets in APS associated with several phenotypes in the APS spectrum (24).

We also found that hydroxychloroquine use is associated with significantly lower type I IFN scores in patients with PAPS, confirming the results by van den Hoogen and colleagues (17). Hydroxychloroquine can attenuate the IFN signature via modulating Toll-like receptor (TLR) signaling and blocking neutrophil extracellular traps activity (22, 25). Hydroxychloroquine in patients with PAPS is the object of rigorous investigation, and promising evidence currently exists for its role in aPL production (26), and prevention of thrombotic (6, 27, 28) and obstetric event recurrences (29–31).

Although anticoagulation is the mainstay of treatment for APS, several patients experience recurrent thrombotic events and pregnancy morbidity despite the use of conventional treatments. In addition, anticoagulation is largely ineffective for non-criteria APS-related manifestations (5), which may be better addressed by alternative treatments targeting inflammatory pathways. Grenn and colleagues recently demonstrated that the impaired ability of endothelial progenitors to differentiate into endothelial cells was reversed by a type I IFN receptor-neutralizing antibody (16). Further research into IFN-inducible gene expression (including both type I and II IFN signature) and inflammatory dysregulation in APS could pave the way for new emerging therapies in APS potentially including interferon-blocking agents.

Our study has certain limitations. Due to the rarity of APS, the sample size was not adequate to support multivariate models which would include all individual APS-related parameters potentially affecting the type I IFN score, and power to detect weaker associations may be limited. Nevertheless, our results are derived from a cohort of well-characterized and closely monitored patients, and this is the first study to show a multivariate association between high IFN signature and anti-β2GPI antibody positivity in patients with APS, while adjusting for age, gender, and other significant disease-related factors.

In conclusion, patients with PAPS are characterized by a high type I interferon signature, which seems to be more pronounced in those with anti-β2GPI positivity, and limited in those receiving hydroxychloroquine treatment. Our findings highlight the need for further investigation of IFN pathways in PAPS, aiming to elucidate their role in the pathogenesis of thrombotic and non-thrombotic complications and investigate their potential as therapeutic targets for selected patients.

## DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of local IRB ("Laikon Hospital Scientific Council") with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Laikon Hospital Scientific Council.

## AUTHOR CONTRIBUTIONS

MT conceived the original idea, supervised and interpreted the findings of this work, and the manuscript writing. EP performed the experiments and wrote the manuscript. EK contributed to sample preparation, performed the statistical analysis, and helped to draft the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Blood Cell-Bound C4d as a Marker of Complement Activation in Patients With the Antiphospholipid Syndrome

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Antiphospholipid syndrome (APS) is a chronic and disabling condition characterized by recurrent thrombosis and miscarriages mediated by antibodies against phospholipid-binding proteins (aPL), such as beta$_2$glycoprotein I ($\beta_2$GPI). Complement is involved in APS animal models and complement deposits have been documented in placenta and thrombotic vessels despite normal serum levels. Analysis of circulating blood cells coated with C4d displays higher sensitivity than the conventional assays that measure soluble native complement components and their unstable activation products in systemic lupus erythematosus (SLE). As C4d-coated blood cell count has been reported to be more sensitive than serum levels of complement components and their activation products in systemic lupus erythematosus (SLE) patients, we decided to evaluate the percentage of C4d positive B lymphocytes (BC4d), erythrocytes (EC4d), and platelets (PC4d) in primary APS patients and asymptomatic aPL positive carriers as marker of complement activation in APS. We assessed by flow cytometry the percentages of BC4d, EC4d, and PC4d in primary APS (PAPS; n. 23), 8 asymptomatic aPL positive carriers, 11 APS-associated SLE (SAPS), 17 aPL positive SLE, 16 aPL negative SLE, 8 aPL negative patients with previous thrombosis, 11 immune thrombocytopenia (ITP) patients, and 26 healthy subjects. In addition, we used an *in vitro* model to evaluate the ability of a monoclonal anti-$\beta_2$GPI antibody (MBB2) to bind to normal resting or activated platelets and fix complement. EC4d and PC4d percentages were significantly higher in PAPS and aPL carriers as well as aPL positive SLE and SAPS than in aPL negative controls. The highest values were found in PAPS and in SAPS. The EC4d and PC4d percentages were significantly correlated with serum C3/C4 and anti-$\beta_2$GPI/anti-cardiolipin IgG. *In vitro* studies showed that MBB2 bound to activated platelets only and induced C4d deposition. The detection of the activation product C4d on circulating erythrocytes and platelets supports the role of complement activation in APS. Complement may represent a new therapeutic target for better treatment and prevention of disability of APS patients.

**Keywords: anti-phospholipid syndrome, beta2–glycoprotein I, complement, C4d, platelets, erythrocytes**

## INTRODUCTION

Antiphospholipid syndrome (APS) is a chronic autoimmune disease characterized by recurrent thrombotic events and pregnancy morbidity, in the presence of antibodies targeting anti-phospholipid binding proteins (aPL) (1). APS can be found in patients with no evidence of associated diseases (primary APS, PAPS) or with other autoimmune disorders, mainly systemic lupus erythematosus (SLE) (SAPS) (1).

The major aPL antigenic target is $\beta_2$-glycoprotein I ($\beta_2$GPI), a phospholipid binding plasma protein of 50 kDa (2). Upon binding to anionic phospholipids or to membrane receptors of different cell types, $\beta_2$GPI changes conformation, exposing an immunodominant cryptic epitope located at domain I (D1), which is recognized by aPL. There is sound evidence from *in vitro* and *in vivo* models that $\beta_2$GPI-dependent aPL play a pathogenic role both in thrombosis and pregnancy complications (3, 4). Moreover, epidemiological data support a strong diagnostic/prognostic value of anti-D1 antibodies in APS patients (5).

Complement activation was initially suggested to be involved in APS animal models since the induction of fetal loss or thrombosis by passive infusion of aPL IgG was prevented by treatment with inhibitors of complement activation or the use of animals deficient in complement components (6–10). Moreover, a human monoclonal antibody against $\beta_2$GPI D1 lacking the complement-fixing portion of the molecule (MBB2ΔCH2), unlike the complement-fixing parent molecule (MBB2) that reacts with the same epitope (11) fails to exhibit pathogenic effect. In contrast, low C3 and C4 serum levels were described in some APS patients only and few studies reported high levels of complement activation products (fragment Bb and anaphylatoxins C4a, C3a, and C5a) with no association with the vascular manifestations of the syndrome (12–15). On the other hand, we recently reported deposition of C1q, C4, C3, and C9 on the endothelium of the vessel wall close to the thrombotic occlusion in a PAPS patient who underwent bypass surgery to treat an arterial thrombotic occlusion. Notably, complement components co-localized with $\beta_2$GPI and IgG, suggesting that aPL caused complement activation and contributed to the pathogenesis of the thrombotic event (16).

Measurement of serum levels of complement activation products has been reported to be more sensitive than that of native complement components in SLE (17). In particular, the number of C4d-coated B lymphocytes, erythrocytes and platelets in circulating blood of SLE patients with active disease was higher than in controls (17–24).

The number of C4d-bound platelets was associated with lupus disease activity and complement consumption but contrasting results regarding the association with arterial or venous events and aPL were reported (25, 26). This finding is in contrast with the ability of aPL to activate complement and promote binding of complement split products to fixed platelets *in vitro* (25, 27, 28).

We have investigated the percentage of C4d positive circulating blood cells in PAPS and report a higher number of C4d positive erythrocytes and platelets in aPL positive patients than in controls supporting the hypothesis that complement is activated *in vivo*. A mechanistic model of complement deposition on platelets in the presence of a human monoclonal antibody specific for D1 of $\beta_2$GPI was investigated as well.

## MATERIALS AND METHODS

### Patients and Controls

We recruited the following patients: 23 PAPS; 11 SAPS, 17 aPL positive SLE, and 16 aPL negative SLE. As controls we included: 11 patients with primary immune thrombocytopenia (ITP), 8 aPL negative patients with previous thrombotic episodes, 8 persistently positive aPL healthy subjects (aPL positive carriers), and 26 normal healthy subjects (NHS). SLE patients were classified according to the 1997 American College of Rheumatology updated criteria for SLE and the new 2012 SLICC SLE criteria (29, 30). Disease activity in SLE patients was assessed by the SELENA version of SLEDAI index in SLE (31). APS patients fulfilled the revised Sapporo criteria (32). The characterization of vascular and obstetric APS was carried out as previously reported (33). Primary ITP was diagnosed according to the International Working Group criteria (34).

All ITP patients were in stable clinical remission. Anti-phospholipid negative patients with thrombosis had previous documented episodes of myocardial infarction, stroke, transient ischemic attack, pulmonary embolism, or deep vein thrombosis.

When entering the protocol, all subjects underwent a detailed clinical evaluation and comprehensive medical history registration. Recorded variables included sex, age, disease duration and therapy (**Supplementary Table 1**). To avoid any modification due to the acute phase reactants, samples have been collected at least six months after the thrombotic event (35). The protocol was approved by the local Institutional Review Board (IRB) (Comitato Etico Milano area 2–426_2014) and all subjects gave written informed consent.

### Blood Samples

Venous blood samples were collected from an antecubital vein using a 21-gauge butterfly needle with minimal stasis. The first 3 mL were collected into K2E EDTA tubes (Becton Dickinson vacutainer, North Ryde, NSW, Australia) and analyzed by coulter hematology analyser for blood cell count (DxH-800, Beckman Coulter, Milano, Italy). Additional blood samples included: 3 mL in K2E EDTA for measurement of C4d deposition on B lymphocytes, red blood cells and platelets by flow cytometry; 3 mL in CTAD vacutainer for lupus anticoagulant (LAC) determination; 5 mL in vacutainer without anticoagulant for serum collection. Five mL of blood were collected into desirudin (Revasc, 25 μg/mL) and used for experiments of C4d deposition on *in vitro* stimulated and resting platelets.

### Serum Complement Determination

Serum concentrations of complement components C3 and C4 were determined by an immunoturbidimetric method (Roche/Hitachi cobas c 701/702): C3 and C4 normal ranges indicated by the manufacturer were 55–180 and 20–50 mg/dL, respectively.

## Detection of aPL

Serum anticardiolipin (anti-CL) and anti-$\beta_2$GPI IgG/IgM autoantibodies were detected as previously described (36). LAC was measured according to international ISTH guidelines (37).

## Detection of C4d Bound to Cells by Flow Cytometry

The percentage of C4d bound to B lymphocytes (BC4d), erythrocytes (EC4d), and platelets (PC4d) was measured by flow cytometry after subtraction of background signals. All analyses were performed using a FACS Calibur cytometer and Cell Quest software (BD Biosciences, San Jose, CA). In all experiments, control procedures to establish proper calibration, compensation, and linearity were performed.

## C4d Bound to B-Lymphocytes

Erythrocytes lysis was performed by addition of ammonium chloride–based reagent (BD Pharm Lyse; BD Biosciences, San Jose, CA) to EDTA-whole blood (300 μL), left 10 min at 4°C and centrifuged at 800 g at 4°C for 5 min. Cell pellet was suspended in 1 mL of Dulbecco's phosphate buffered saline (DPBS, Sigma-Aldrich, St. Louis, MO) supplemented with 1% heat-inactivated fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO) (DPBS/1% FCS) and stained at 2–8°C with 10 μg/mL purified mouse monoclonal antibodies against human C4d (mouse anti-human C4d; Quidel, San Diego, CA) or 10 μg/mL mouse anti-human isotype control IgG1κ (MOPC-21; BD Biosciences, San Jose, CA) for 45 min. Samples were then washed with DPBS/1%FCS and centrifuged at 800 g at 4°C for 5 min. Pellets were suspended in a DPBS/1%FCS solution containing fluorescein isothiocyanate (FITC) conjugated goat anti-mouse antibody (Cappel, MP biomedicals, Santa Ana, CA) (10 μg/mL) and R-phycoerythrin (PE)-conjugated monoclonal antibody against human CD-19 (a 95-Kd type I transmembrane glycoprotein expressed on B cells) (BD Biosciences, San Jose, CA), and stained at 2–8°C in the dark for 45 min. Samples were washed again and suspended in 250 μL cold DPBS/1%FCS. 5,000 events in the lymphocytes gate (based on their size) were acquired at high flow rate. A single fluorochrome dot plot strategy was used to identify B-lymphocytes CD19 positive (SSC vs. CD19-PE) and the percentage of C4d was assessed on B-lymphocytes gate (SSC vs. FITC).

## C4d Bound to Erythrocytes

EDTA-whole blood (50 μL) was suspended in 1,5 mL DPBS/1%FCS and centrifuged for 5 min at 800 g at 4°C. The pellet was suspended in 500 μL DPBS/1%FCS; 10 μL of the suspension was subsequently stained with anti-C4d monoclonal antibody or mouse anti-human isotype control IgG1κ at 2–8°C for 45 min. Samples were then washed and cell surface C4d was detected by addition of FITC-conjugated goat anti-mouse antibody at 2–8°C in the dark for 45 min. Samples were washed again and suspended in 250 μL of cold DPBS/1%FCS. 5,000 events in the erythrocytes gate were acquired with a high flow rate. A single fluorochrome dot plot strategy (FSC vs. FITC) was used for quantification of percentage of C4d on erythrocytes. Details regarding the erythrocyte gating strategy are shown in **Supplementary Figure 1**.

## C4d Bound to Resting Platelets

EDTA-whole blood samples (50 μL) were diluted with DPBS/1%FCS (1:2) and stained with anti-C4d monoclonal antibody or mouse anti-human isotype control IgG1κ at 2–8°C for 45 min, followed by staining with FITC-conjugated goat anti-mouse antibody at 2–8°C in the dark for 45 min. A PE–conjugated monoclonal antibody against human CD42b (a 145 kD glycoprotein known as GPIbα) was used to identify platelets. 5,000 events in the platelet gate were acquired at low flow rate. A single fluorochrome dot plot strategy was used to identify CD42b positive platelets (SSC vs. CD42b-PE) and the percentage of C4d was measured (SSC vs. FITC). Details regarding the platelet gating strategy are shown in **Supplementary Figure 2**.

## MBB2 Antibody

The human monoclonal antibody against D1 of $\beta_2$GPI (MBB2) has been produced and characterized as previously published (11).

## *In vitro* Model of C4d Binding to Normal Platelets

Hirudin-anticoagulated whole blood from healthy blood donors was diluted in home-made PBS (1:15) and incubated with or without 500 μg/mL of MBB2 and/or thrombin receptor activating peptide (TRAP, Sigma-Aldrich, Germany) (20 μM) at 37°C for 20 min. Samples were then diluted with PBS (1:10) and stained with 10 μg/mL mouse anti-human C4d or 10 μg/mL mouse anti-human isotype control IgG1κ at RT for 20 min. Then, FITC-conjugated goat anti-human antibody (Cappel, MP biomedicals, Santa Ana, CA) and/or APC-conjugated goat anti-mouse antibody (ThermoFisher scientific Massachusetts, USA), and/or anti-CD42b-PE, and/or anti-CD62p-Pe/Cy7 (Biolegend, San Diego, CA) were added to the samples and incubated at RT in the dark for 20 min. After incubation, samples were diluted in 300 μL PBS and immediately analyzed by flow cytometry. All analyses were performed using a FACS Verse cytometer and FACS Suite software (BD Biosciences, San Jose, CA). 5,000 events in the platelet gate were acquired with a medium flow rate. Dot plot strategy was used for analysis: first of all, platelets were identified with CD42b-PE vs. SSC, then C4d-APC or MBB2-FITC or CD62p-Pe/Cy7 vs. SSC were used to determine the percentage of C4d and MBB2 platelet bound and of CD62p expression.

## Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, CA, USA). All results were expressed as median with interquartile range. The normality of data was evaluated by the D'Agostino-Pearson test. Comparisons between groups were performed using Kruskal-Wallis or Friedman tests as appropriate followed by Dunn's *post-hoc* test. Comparison between two groups were performed using Wilcoxon *t*-test. All tests were two tailed. Correlations between variables were expressed as Spearman's correlation coefficient. $p < 0.05$ was chosen as the cut-off level for statistical significance.

# RESULTS

## aPL Profile of Patients and Controls

Normal healthy subjects and pathological controls (ITP, SLE, and patients with previous thrombosis) were negative in all aPL assays. Among the 17 aPL positive SLE patients, three displayed single, 7 double and 7 triple positivity. Four out of 11 SAPS patients were single-positive, 1 double-positive and 6 triple-positive for aPL; 4/8 aPL positive carriers were double- and 4/8 triple-positive; of the 23 PAPS patients, 1 was single-, 5 double-, and 17 triple-positive.

## C3 and C4 Serum Levels

aPL positive carriers and PAPS patients displayed lower serum levels of C3 and C4 than normal healthy subjects, albeit their values fell in the normal range (**Table 1**).

## Measurement of Cell-Bound C4d

Significantly higher percentages of both EC4d and PC4d were found in aPL positive SLE, SAPS and PAPS patients compared to NHS, ITP patients, and aPL negative thrombotic patients; moreover, higher percentages of EC4d and PC4d were detected also in aPL negative SLE and asymptomatic healthy aPL positive carriers (**Figures 1A,B**). Patients with SLE with or without aPL or full blown APS displayed higher BC4d percentages than PAPS or aPL positive carriers. On the contrary, the percentages of BC4d in aPL positive carriers and PAPS were comparable with the controls (**Supplementary Figure 3**).

Cell-bound C4d percentages were comparable both in thrombotic and obstetric APS patients, suggesting that they were not associated with the main APS clinical manifestations (**Supplementary Figure 4**); moreover, we did not find any correlation also with non-classification criteria (e.g., thrombocytopenia; **Supplementary Figures 5–7**) (32).

## Correlations Between the Percentage of PC4d and EC4d With Other Laboratory Parameters

C4 Serum levels were found to be inversely correlated with the percentage of PC4d ($r = -0.4682$, $p < 0.0001$; **Figure 2A**) and EC4d ($r = -0.5163$, $p < 0.0001$; **Figure 2B**).

There was a positive correlation of both anti-$\beta_2$GPI IgG ($r = 0.2486$, $p = 0.0157$) and anti-CL IgG ($r = 0.4537$, $p < 0.0001$) titers with PC4d (**Figures 3A,B**). The correlation between PC4d and anti-CL IgM was mild, but statistically significant ($r = 0.2472$, $p = 0.0163$; **Supplementary Figure 8B**). In contrast, there was no correlation between PC4d and anti-$\beta_2$GPI IgM titers ($r = 0.09486$, $p = 0.3631$) (**Supplementary Figure 8A**). EC4d percentages positively correlated with anti-$\beta_2$GPI IgG ($r = 0.3503$, $p = 0.0005$) and anti-CL IgG ($r = 0.4805$, $p < 0.0001$) (**Supplementary Figures 9A,B**). There was also a positive correlation between EC4d and both anti-$\beta_2$GPI IgM ($r = 0.2672$, $p = 0.0092$) and anti-CL IgM ($r = 0.4177$, $p < 0.0001$) (**Supplementary Figures 9C,D**).

## *In vitro* Studies: MBB2 and C4d Deposition on Resting and Activated Platelets

Since $\beta_2$GPI-dependent aPL may recognize $\beta_2$GPI adhered on activated platelets (3), we set up an *in vitro* model to investigate whether the bound antibody was responsible for cell membrane deposition of C4d. To this end, hirudin-anticoagulated whole blood samples from NHS ($n = 11$) were incubated with TRAP, a platelet agonist. In the absence of exogenous stimuli, the expression of CD62p on platelets was negligible, while it dramatically increased after addition of TRAP. These results indicate that circulating NHS platelets were not activated, offering a suitable *in vitro* model to investigate the possible role of platelet activation in $\beta_2$GPI adhesion to cell membranes and antibody binding (**Figure 4A**). Blood samples, which provided the source of resting platelets, autologous $\beta_2$GPI and complement, were incubated with MBB2. The binding of MBB2 to resting platelets was negligible (0.4%) but it increased up to 18 times (7.5%; $p = 0.002$) following platelet stimulation by TRAP (**Figure 4B**). This finding suggests that pathogenic aPL may recognize autologous $\beta_2$GPI bound only to activated platelets. We then investigated whether the bound antibody was able to activate complement and to induce C4d deposition on the platelet membranes. The percentages of PC4d increased significantly when the blood samples were simultaneously incubated with TRAP and MBB2, suggesting that MBB2 reacted with adhered $\beta_2$GPI and activated complement, leading to C4d deposition on activated platelets (**Figure 4C**).

# DISCUSSION

Complement has been shown to be activated in several systemic autoimmune diseases and plays a key role in SLE and in SLE-related disorders (38). Although there is ample evidence that thrombosis and fetal loss are complement dependent in experimental models of APS, complement activation in APS patients is still matter of debate (15). Our results show for the first time increased percentages of C4d complement activation product that is stably deposited on cell membranes of platelets and erythrocytes in PAPS.

High percentages of platelet C4d were reported to be specific for SLE (17–24). We confirmed this finding in aPL negative SLE patients included in this study as a pathological control group. The percentages of C4d bound to circulating blood cells, in particular to platelets, display higher sensitivity than the conventional assays that measure soluble complement components and their unstable activation products. Consequently, platelet C4d percentages have been suggested as useful and sensitive tool for mirroring complement activation in SLE patients (17).

The finding of increased percentages of PC4d and EC4d in PAPS and in aPL positive carriers but not in healthy controls and patients with ITP strongly suggests that the presence of aPL is specifically associated with *in vivo* complement activation. Comparable percentages were found in aPL negative and aPL positive SLE patients with no APS clinical manifestations supporting the hypothesis that SLE and the presence of aPL may

**TABLE 1 |** Serum levels of C3 and C4.

| | NHS ($n = 26$) | ITP ($n = 11$) | aPL neg thrombosis ($n = 8$) | aPL neg SLE ($n = 16$) | aPL pos SLE ($n = 17$) | SAPS ($n = 11$) | aPL pos carriers ($n = 8$) | PAPS ($n = 23$) |
|---|---|---|---|---|---|---|---|---|
| C3 (mg/dL) (normal range: 55–180 mg/dL) | 129 ± 9 | 124 ± 11 | 159 ± 11 | 92 ± 6 | 76 ± 5 | 73 ± 7 | 97 ± 11 | 89 ± 4 |
| C4 (mg/dL) (normal range: 20–50 mg/dL) | 31 ± 3 | 25 ± 3 | 34 ± 4 | 19 ± 3 | 10 ± 1 | 15 ± 4 | 20 ± 2 | 20 ± 2 |

*NHS, normal healthy subjects; ITP, primary immune thrombocytopenia; aPL, anti-phospholipid antibodies; SLE, systemic lupus erythematosus; SAPS, secondary antiphospholipid syndrome; PAPS, primary antiphospholipid syndrome.*
*Values are reported as mean ± SEM.*

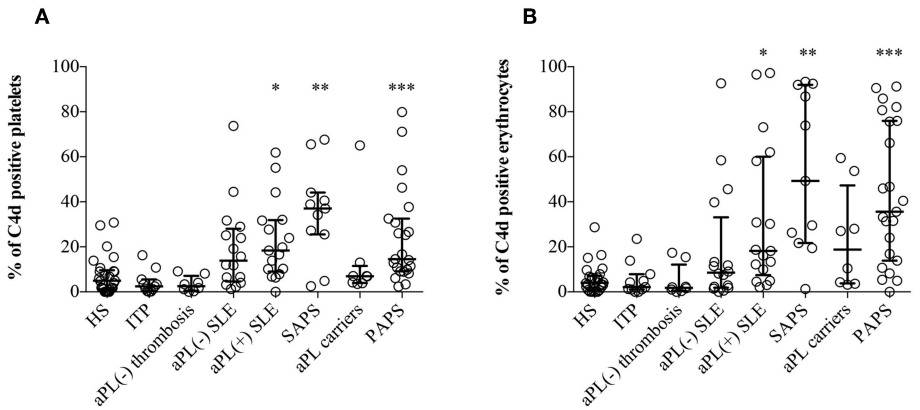


**FIGURE 1 |** C4d deposition on platelets and erythrocytes. Flow cytometry was performed on EDTA-whole blood samples ($n = 120$). C4d-positive cells were detected by purified anti-human C4d and FITC conjugated goat anti-mouse antibody on platelets **(A)** and on erythrocytes **(B)**. Results are expressed as percentage, median with interquartile range, and analyzed by Kruskal-Wallis test and Dunn's multiple comparison *post-hoc* test. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NHS.

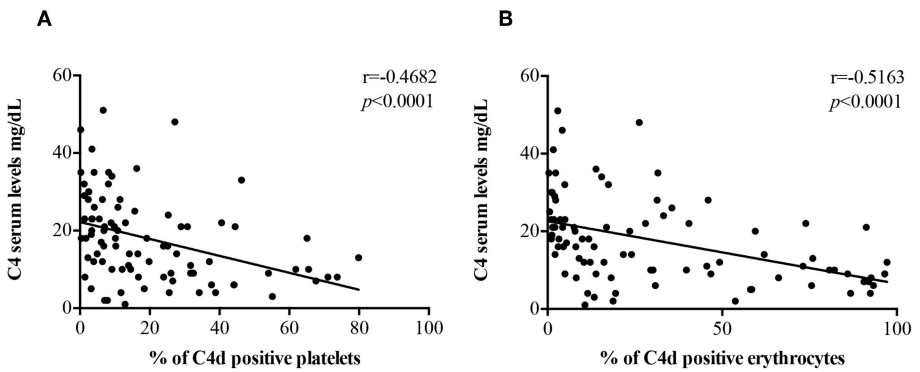


**FIGURE 2 |** Correlations between C4 serum levels and EC4d and PC4d percentages. C4 serum levels (mg/dL) negatively correlated with the percentages of PC4d **(A)** and EC4d **(B)**.

be independently linked to complement activation. On the other hand, full blown APS was associated with the highest EC4d and PC4d percentages both in PAPS and in SLE-associated APS. Since the majority of these patients had a history of vascular thrombotic events, we investigated whether a previous thrombosis may affect the percentages of C4d positive cells in the absence of aPL. This possibility was ruled out by the observation that the control group of aPL negative thrombotic patients had normal percentages of C4d positive cells further supporting the specificity of this parameter for *in vivo* complement activation in APS.

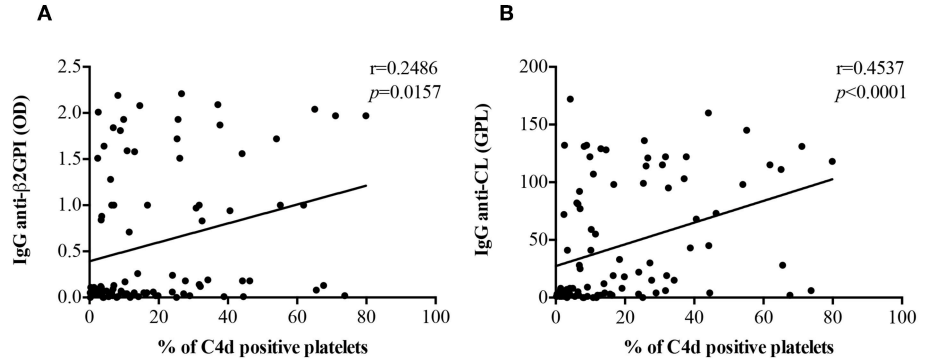


**FIGURE 3 |** Correlations between anti-β2GPI and anti-CL IgG titers PC4d. PC4d titers positively correlated with both IgG anti-$\beta_2$GPI **(A)** and IgG $\beta_2$GPI-dependent anti-CL titers **(B)**.

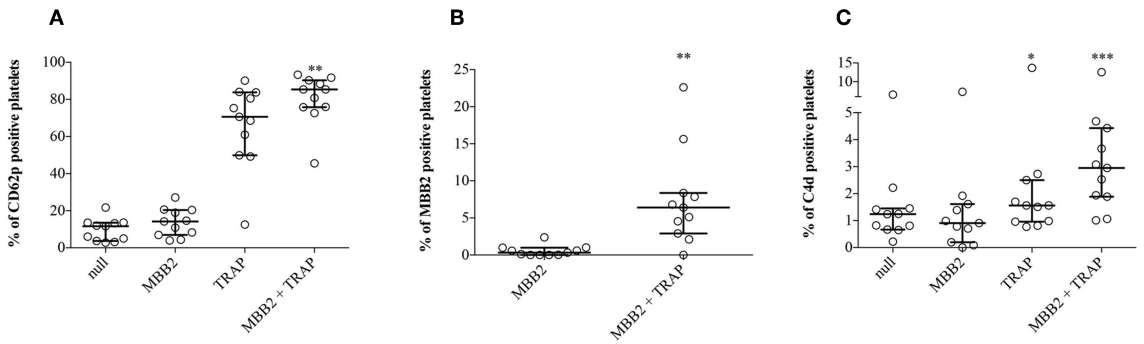


**FIGURE 4 |** Effects of MBB2 and TRAP, alone and in combination, on the expression of CD62p on platelets. Percentages of CD62p-, MBB2-, and C4d-positive platelets were measured in hirudin-anticoagulated blood from 11 NHS after its incubation with or without MBB2 and/or TRAP at 37°C for 20 min. Data were analyzed by Friedman test and Dunn's multiple comparisons *post-hoc* test **(A,C)** and by Wilcoxon matched paired test **(B)**. Results are expressed as percentage, median with interquartile range. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$ vs. MBB2.

Although the serum levels of C3 and C4 in PAPS and in aPL positive carriers were reduced in comparison with healthy controls and patients with ITP or previous thrombosis without aPL, they were still within the normal range. Moreover, C4d positive cells significantly correlated with both serum C3 and C4 values and titers of anti-$\beta_2$GPI and $\beta_2$GPI-dependent anti-CL IgG. These findings are consistent with the hypothesis that the presence of aPL is associated with complement activation in APS.

Few recent studies reported reduced complement levels and increased levels of activation products in some APS patients albeit without clear association with the clinical manifestations of the syndrome (12–14). The high percentages of both EC4d and PC4d in our study further support the involvement of complement in APS. The lack of association with the clinical manifestations of the syndrome was confirmed in our study in spite of the use of a more sensitive and reliable laboratory tool for detection of complement activation.

Complement activation in APS patients should be associated with complement deposition in damaged tissues. There is sound evidence that this is the case for placenta both in animals and in patients (15). A role of complement for the aPL-mediated placenta damage has been suggested in animals while it is still matter of research in humans (15). On the other hand, although less studies looked at vessel complement deposition, the available data are suggestive for a role of complement in endothelial perturbation that may be pivotal for the thrombophilic state (16, 39).

Both circulating and tissue immune complexes are known to play a critical role in activating the complement cascade in SLE (38). Their deposition on the membrane of circulating blood cells explains the increased percentages of C4d positive cells; however additional pathways have been suggested to mediate the binding of complement components to activated platelets without the need of immune complex formation (40). In contrast, the mechanism(s) responsible for complement activation in APS has not been clarified yet. Although circulating anti-$\beta_2$GPI/$\beta_2$GPI complexes can be found in a limited proportion of patients (41, 42), they are undetectable in most of them suggesting that tissue bound complexes (e.g., on vessel walls) (16, 39) and/or additional mechanisms play a role in complement activation.

Beta$_2$GPI is the main target for diagnostic and pathogenic aPL and it is general accepted that binds to activated platelets

through several candidate membrane receptors or just because of the electrostatic interaction between the cationic PL-binding site of $\beta_2$GPI and the anionic phospholipid (i.e., phosphatidylserine) that is exposed in the outer cell membrane leaflet upon cell activation (3). Once bound, the adhered $\beta_2$GPI exposes the immunodominant epitope on D1 that is recognized by pathogenic aPL that in turn may activate complement (3). With this in mind, we set up an *in vitro* model in which normal platelets were incubated with anti-D1 monoclonal antibody in the presence or absence of a platelet agonist (i.e., TRAP) and autologous serum as a source of $\beta_2$GPI and complement. Our *in vitro* results support only in part the role of immune complexes for complement deposition on the platelet cell membrane, because only a small percentage of activated platelets actually displayed membrane C4d. Additional mechanisms that do not require immune complex formation at the platelet surface may play a role as also suggested in SLE patients (40). Moreover, we can speculate that abnormalities in PAPS or SLE platelets themselves may be responsible for the increased complement deposition on their surface.

In conclusion the detection of complement activation products on circulating erythrocytes and platelets using a highly sensitive and specific assay further supports the view that APS is a complement-mediated disorder. Increased EC4d and PC4d percentages are associated with the active inflammatory disease in SLE. It is difficult to translate this finding to APS which is a non-acute inflammatory disorder. We failed to find an association with both the classification and non-classification criteria, including thrombocytopenia. However, we believe that this sensitive tool to evaluate complement activation may offer more information in monitoring the dynamics of the aPL-mediated pathogenic pathways during pregnancy rather than in patients far from the acute thrombotic events.

## ETHICS STATEMENT

This study was carried out in accordance with the standard of the good clinical practice with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the ethical committee "Milano Area 2 426_2014."

## AUTHOR CONTRIBUTIONS

PL, MS, and MG wrote the first draft of the manuscript. PL and MS performed the *ex-vivo* and *in-vitro* studies and interpreted data. MG, GP, and WB evaluated and recruited the patients. FP performed statistical analysis. DC detected aPL. EAF contributed to developing the *in-vitro* system. MB, MC, FT, and PM contributed to conception and design of the study. All authors contributed to manuscript revision, read and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00773/full#supplementary-material

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REVIEW
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# New Biomarkers for Atherothrombosis in Antiphospholipid Syndrome: Genomics and Epigenetics Approaches

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Antiphospholipid Syndrome (APS) is an autoimmune disorder, characterized by pregnancy morbidity and/or a hyper coagulable state involving the venous or the arterial vasculature and associated with antiphospholipid antibodies (aPL), including anti-cardiolipin antibodies (aCL), anti-beta2-glycoprotein I (anti-ß2GPI), and Lupus anticoagulant (LA). In recent years there have been many advances in the understanding of the molecular basis of vascular involvement in APS. APS is of multifactorial origin and develops in genetically predisposed individuals. The susceptibility is determined by major histocompatibility complex (MHC). Different HLA-DR and HLA-DQ alleles have been reported in association with APS. Moreover, MHC II alleles may determine the autoantibody profile and, as such, the clinical phenotype of this disease. Besides, polymorphisms in genes related to the vascular system are considered relevant factors predisposing to clinical manifestations. Antiphospholipid antibodies (aPL) induce genomic and epigenetic alterations that support a pro- thrombotic state. Thus, a specific gene profile has been identified in monocytes from APS patients -related to aPL titres *in vivo* and promoted *in vitro* by aPL- explaining their cardiovascular involvement. Regarding epigenetic approaches, we previously recognized two miRNAs (miR-19b/miR-20a) as potential modulators of tissue factor, the main receptor involved in thrombosis development in APS. aPLs can further promote changes in the expression of miRNA biogenesis proteins in leukocytes of APS patients, which are translated into an altered miRNA profile and, consequently, in the altered expression of their protein targets related to thrombosis and atherosclerosis. MicroRNAs are further released into the circulation, acting as intercellular communicators. Accordingly, a specific signature of circulating miRNAs has been recently identified in APS patients as potential biomarkers of clinical features. Genomics and epigenetic biomarkers might also serve as indices for disease progression, clinical pharmacology, or safety, so that they might be used to individually predict disease outcome and guide therapeutic decisions. In that way, in the setting

of a clinical trial, novel and specific microRNA–mRNA regulatory networks in APS, modified by effect of Ubiquinol treatment, have been identified. In this review, current and previous studies analyzing genomic/epigenetic changes related to the clinical profile of APS patients, and their modulation by effect of specific therapies, are discussed.

**Keywords: Antiphospholipid Syndrome, cardiovascular disease, genomics, microRNAs, therapy**

## INTRODUCTION

Antiphospholipid Syndrome (APS) is an autoimmune disorder, clinically characterized by pregnancy morbidity and/or a hypercoagulable state involving the venous or the arterial vasculature and associated with antiphospholipid antibodies (aPLs), including anti-cardiolipin antibodies (aCL), anti-beta2-glycoprotein I (anti-ß2GPI), and Lupus anticoagulant (LA).

Patients with APS have enlarged incidence of vascular damage, involving thrombosis, accelerated atherosclerosis, stroke or myocardial infarction, among others (1, 2). Numerous mechanisms have been postulated to contribute to the development of thrombosis in APS patients, comprising synergic effects of autoantibodies with pro-thrombotic molecules, adhesion receptors, inflammatory mediators, oxidative stress, netosis, and a plethora of intracellular signaling molecules.

Thus, a number of studies have established that aPLs provoke a pro-atherothrombotic status through the induced expression of both pro-thrombotic and pro-inflammatory molecules such as tissue factor (TF), vascular endothelial growth factor (VEGF) and its receptor Flt-1, as well as by inducing oxidative stress and mitochondrial dysfunction in monocytes and neutrophils, along with increased neutrophil extracellular traps (NETs) formation (3–5). Moreover, it has been demonstrated that endothelial cells (EC) express significantly higher amounts of adhesion molecules (ICAM-1, VCAM-1, and E-selectin) and other inflammatory mediators when incubated with aPL antibodies and ß2GPI *in vitro* (6, 7). Likewise, the incubation of ECs with antibodies reacting with ß2GP1 induce their activation, accompanied by the upregulation of TF, (8) adhesion molecules and IL-6, along with the alteration of the prostaglandin metabolism.

Genetic predisposition to APS and aPLs has been stated by different reports. Animal models and human studies have highlighted HLA associations with the disease and the occurrence of aPLs in APS patients. Specifically, different HL-DR and HLA-DQ alleles have been associated with APS. In addition, major histocompatibility complex (MHC) genes seems to influence not only autoantibody production but also disease expression itself (9).

Genetic polymorphisms have also been linked to thrombosis in APS patients, including variants of coagulation factors, anti-thrombotic and fibrinolytic molecules [i.e., FXIII, tissue factor pathway inhibitor (TFPI), type-I plasminogen activator inhibitor (PAI-1)] inflammatory mediators [i.e., tumor necrosis factor alpha (TNFα)], parameters related to platelet activity (i.e., platelet FC receptor IIa, platelet glycoproteins GP Ia/IIa and GP IIb/IIIa), endothelial factors (i.e., thrombomodulin), etc. (9). Besides, the Fcγ receptor as well as a β2-GPI-domain V polymorphism have been demonstrated to be relevant factors predisposing to APS (10, 11).

More recently, microarrays studies allowed the identification of APS and systemic lupus erythematosus (SLE) specific gene signatures explaining the pro-atherosclerotic, pro-thrombotic and inflammatory states in these autoimmune diseases (12). However, the modulation of gene expression has left significant gaps in our understanding of the development and progression of these co-morbidities in APS and SLE. Epigenetics, defined by the changes or modifications in DNA that influence phenotype without altering the genotype, present a new and entirely different mechanism of gene regulation. Several interrelated epigenetic and post-transcriptional regulatory mechanisms altered in numerous autoimmune and cardiovascular diseases are DNA methylation changes, histone modifications and microRNA activity, all of which act by altering gene and protein expression levels (13).

While extensive epigenomic studies have identified specific DNA methylation changes and histone modifications -linked to the development, the disease activity and even the organ involvement- in a closely related disease to primary APS, such as SLE, to date no studies have been developed to analyze those epigenetic alterations in APS patients and their contribution to cardiovascular disease.

Conversely, microRNAs, which markedly affect immune system and have an important role in the pathogenesis of numerous autoimmune and inflammatory conditions, have been demonstrated to act as main regulators of a number of gene targets involved in clinical features of APS, such as immune response, atherosclerosis and thrombosis (14).

This paper reviews genomic and epigenetic approaches (mainly focused on the role of microRNAs) used to deep into the mechanisms associated with vascular involvement in primary APS.

## PRO-THROMBOTIC AND ATHEROGENIC CHANGES INDUCED BY ANTIPHOSPHOLIPID ANTIBODIES ON IMMUNE AND VASCULAR CELLS

Subjects positive for LA, higher titers of anti-CL, and anti-ß2GPI antibodies (known as "triple positives"), have the highest risks for thrombosis (15). Moreover, various studies have demonstrated that triple-positive aPL patients usually have high titers of antibodies to the major ß2GPI epitope on domain I, which confer LA activity, associated with the highest risk for thrombosis (16).

Multiple molecules, essential in the hemostatic system, and acting as key players in thrombosis and atherosclerosis development, are altered in APS immune and vascular cells by effect of aPLs, including TF, the VEGF/Flt1 axis, several toll-like receptors (TLRs), annexins, protein disulfide isomerase, etc.

Thus, the expression of TF, the major initiator of the blood coagulation, was firstly described by our group to be significantly elevated in monocytes from APS patients with a previous history of thrombosis and related to the presence of high titres of aPLs (17). Thereafter, the molecular mechanisms underlying the aPL-induced increased expression of TF in monocytes were delineated, suggesting that aPLs induce TF expression in monocytes from APS patients by activating, simultaneously and independently, the phosphorylation of MEK-1/ERK proteins, and the p38 MAP kinase-dependent nuclear translocation and activation of NF-kappaB/Rel proteins (18). Parallel studies performed in ECs also concluded that p38MAPK phosphorylation and NFkB activation mediated the aPL-induced TF expression and function, along with the upregulation of IL-6, IL-8 and the inducible nitric oxide synthase (iNOS) (19).

Signaling TF activities are mainly mediated by the protease activator receptors (PARs), major mediators of thrombosis, hemostasis and inflammatory processes. Thus, it has been shown that TF complexes with coagulation factors VIIa or/and Xa induce PAR1 and PAR2 signaling. Some years ago, we provided evidence of increased expression of PAR-1 and PAR-2 in monocytes from APS patients with previous history of thrombosis (20). That study also demonstrated a correlation between PAR-2 levels and IgG aPL titers, as well as a parallel behavior of TF and PAR-2 expression, so that PAR-2 inhibition prevented the IgG aPL-induced TF expression. Overall, this study suggested that TF/PAR2 axis is directly involved in the pathogenesis of the thrombotic complications associated with APS.

Previous reports indicated a close relationship between TF and VEGF, protein involved in normal vascular development, as well as in pathologies related to inflammation and cardiovascular disease (21). Prior studies reported increased plasma levels of VEGF in APS patients (22). Later, it was shown that monocytes from APS patients expressed increased levels of both, VEGF and its receptor Flt1. Besides, these molecules were produced by monocytes when treated with aPLs, in a process involving the p38 MAPK signaling pathway (23). Thus, VEGF can be considered a regulatory factor in aPL-mediated monocyte activation and TF expression, contributing to the proinflammatory–prothrombotic status of APS.

Proteomic studies have demonstrated that aPLs are also responsible for the altered protein profile of APS monocytes, involving deregulated expression of annexin A1 (AnxA1), annexin A2 (AnxA2), ubiquitin-like protein Nedd8, Rho A protein, protein disulfide isomerase (PDI), and Heat shock protein-60 (Hsp60). These proteins were associated with a hypercoagulable state, as well as with autoimmune-related responses (24).

Toll-like receptors (TLR)-2 and−4 are membrane receptors known by their roles in the activation of immune and endothelial cells, pathogen recognition and production of cytokines. The TLR pathway is activated in APS patients, in which peripheral mononuclear cells show a significant increase in the gene expression of TLR2 and TLR4 that mediate aPL-induced vascular abnormalities (25). *In vivo* studies in mice and *in vitro* experiments in human monocytes and an endothelial cell line have shown that TLR2 and TLR4 mediate the inflammatory activation of monocytes and endothelial cells induced by aPLs (26, 27), suggesting that these receptors might be considered a therapeutic target to prevent the thrombotic effects of aPLs in APS.

aPLs can also activate platelets, inducing the expression of the fibrinogen receptor glycoprotein IIb/IIIa (GlIb/IIIa) as well as thromboxane B2 (TXB2), promoting its aggregation and thus contributing to the development of thrombosis (9).

Pierangely and coworkers further described other potentially significant antigenic targets for aPLs, which included prothrombin, tissue plasminogen activator (tPA), phosphatidylserine (PS), plasmin, annexin 2, activated protein (APC), thrombin, antithrombin III (AT-III), and annexin V (9). Finally, aPLs are associated to the development of atherosclerosis, so that *in vivo*, it has been demonstrated a direct correlation between serum levels of aCL and anti-ß2GPI antibodies and the occurrence of coronary syndrome, myocardial infarction and stroke (28). A recent study demonstrated a strong association among higher aPL-IgG titers and development of thrombotic events, as well as with the presence of early atherosclerosis, as demonstrated by increased intimae-media thickness (IMT) (5). *In vitro*, several studies confirmed the involvement of aPLs in the formation of the atheroma plaque, through the activation of endothelial cells and leukocytes, and the induction of foam cell generation, by facilitating the adsorption of oxidized low-density lipoproteins by monocytes (3).

## ROLE OF OXIDATIVE STRESS IN THE DEVELOPMENT OF ATHEROTHROMBOSIS IN APS

Various studies have proven that oxidative stress is involved in the pathophysiology of APS. Thus, an increased oxidative status has been demonstrated in plasma of APS patients, more significantly associated to the triple positivity for aPLs, so that those patients showed higher plasma levels of prostaglandin 2 and 8-isoprostane compared to healthy donors (29).

In a recent study (5) we showed an increased production of reactive oxygen species (ROS) by monocytes and neutrophils, accompanied by significant losses in mitochondrial membrane potential, which disturbed the redox status, and were related to both, the autoimmune condition and the inflammatory and pro-atherothrombotic status of APS patients. Accordingly, *in vitro* studies demonstrated that the binding of aPL-IgG to the monocytes provoked a redox- sensitive signaling pathway that controlled the prothrombotic phenotype. Other studies demonstrated that the generation of superoxide induced by aPLs in plasmacytoid dendritic cells and monocytes upregulate the expression of the TLRs −7 and −8 (30).

All that data underlies the relevant role of autoimmunity in the induction of an oxidative status in these patients, which further acts as an underlying mechanism promoting cardiovascular disease.

## NETOSIS IN APS PATIENTS: A NEW MECHANISM OF THROMBOSIS STIMULATED BY ANTIPHOSPHOLIPID ANTIBODIES

A number of studies have recognized that NETosis generation is associated with autoimmunity, deep vein thrombosis, tissue damage and atherosclerosis (31). In the setting of APS, Knight and co-workers have established a key role for neutrophil extracellular traps (NETs) in the development of thrombosis, so that they revealed, *in vivo*, high circulating levels of NETs in the plasma of APS patients, related to the occurrence of thrombotic events. Moreover, they demonstrated through *in vitro* studies that aPLs, especially those targeting ß2GPI, activate neutrophils to release NETs, thus predisposing to thrombosis (32, 33). These results open a new point of debate about the potential role of NETs as therapeutic targets.

## GENETIC RISK FACTORS OF ATHERO-THROMBOSIS IN APS

APS is strongly associated with genetic abnormalities (**Table 1**). Family and population studies have indicated that genetic factors play a key role in the etiopathogenesis of this disorder, suggesting the existence of a genetic predisposition to this disease, either when it presents as a primary disorder or within the context of SLE (34). This predisposition can be explained by the influence of genes at the major histocompatibility complex (MHC) locus and outside the MHC. Thus, certain human leukocyte antigen (HLA) alleles (HLA-DR and HLA-DQ) are strongly linked to the presence of aPL autoantibodies (35, 51, 52). The HLA allele most frequently associated with APS are HLA-DRB1*04 (DR4), DRB1*07 (DR7), DRB1*1302 (DR6), DRw53, DQA1*0102, DQA1*0201, DQA1*0301, DQB1*0302 (DQ8), and DQB1*0604/5/6/7/9 (34).

Several of these HLA alleles determine the susceptibility to produce aPL (LA, aCL, and anti-β2GPI) antibodies to prothrombin, annexin V, phosphatidylethanolamine-phosphatidylserine, independently of the clinical context, primary APS or SLE. In fact, the same associations have been found among aPLs and the HLA system in primary APS and in APS secondary to SLE (35, 51, 52). In addition, the pattern of HLA associations is also influenced by the different ethnic groups (36, 53, 54).

Other genes, outside the MHC, contribute to the development of the disease. Thus, genetic variations of various components in the hemostatic system, favoring blood coagulation, may modulate the clinical manifestation of thrombosis (37):

1. A single nucleotide variation (SNV) of the type 1 plasminogen activator inhibitor (PAI-1) gen has been related to high levels of this inhibitor in plasma in APS patients. This is a common 4G/5G single nucleotide insertion/deletion variation in the promoter region 675 pb upstream from the start of transcription (675 insertion/deletion 4G/5G). Individuals with the 4G allele have higher plasma levels of PAI-1 than those with the 5G allele. High levels of PAI-1 have been found associated with high titers of aPLs in one third of the patients. In turn, 4G/5G genotype was associated with the occurrence of both types of thromboses, arterial and venous, especially with venous thromboembolism in patients with APS (38, 39, 55).
2. The protein C pathway is a key modulator of the blood coagulation, having an anticoagulant activity. In APS, there is evidence of acquired resistance to activated protein C (PC) induced by aPLs. Endothelial protein C receptor (EPCR) might have a procoagulant action by inhibiting the activation of PC. Elevated levels of soluble EPCR have been found in SLE, which suggests its role on the development of thrombotic manifestations. Several haplotypes of EPCR gene, PROCP, have been described according to the SNVs (H1-H4). Thus, T6333C, A6936G, G6147A, and all in common would correspond to H1, H3, H4 and H2, respectively. The PROCP T6333C and A6936G variations (H1 and H3) have been linked with EPCR levels and thrombosis. In addition, H1 haplotype has been associated with reduced sEPCR levels and increased levels of activated PC, which would have a protective effect against thrombosis (40). In this regard, a recent study showed a lower prevalence of the PROCR H1 haplotype in APS patients with arterial thrombosis (40).

**TABLE 1 |** Genetic risk factors associated with athero-thrombosis in APS.

| Gene | Genetic factor | Association with APS | References |
|---|---|---|---|
| MHC | HLA-DR and HLA-DQ alleles | Presence of autoantibodies aPL | (32–35) |
| PAI-1 | 675 insertion/deletion 4G/5G | Occurrence of thrombosis | (36–38) |
| EPCR | T6333C | Lower prevalence in APS with arterial thrombosis | (39) |
| Prothrombin | G20210A | Not clear relationship with thrombotic events | (40–42) |
| TFPI | T33C C399T | Venous thrombosis | (43) |
| TNFa | G238A | Arterial thrombosis | (44) |
| GPIa | C807T | Arterial thrombosis | (45) |
| GPIb-alpha | Kozak TC polymorphism | | (46) |
| p-selectin | G1902A | Thrombotic events | (47) |
| PSGL-1 | Tandem repeats (VNTR) | Thrombosis | (48) |
| B2-Glycoprotein | G796T G1004C | Production of autoantibodies | (49); (50) |
| TLR4 | A896G C1196T | Lower prevalence in APS patients with thrombosis | (24) |

*MHC, major histocompatibility complex; HLA, human leukocyte antigen; PAI-1, type 1 plasminogen activator inhibitor 1; EPCR, endothelial protein C receptor; TFPI, tissue factor pathway inhibitor; TNFα, tumor necrosis factor alpha; GPIa, glycoprotein Ia; PSGL-1, p-selectin glycoprotein ligand 1; TLR4, toll like receptor 4.*

3. The proteolytic cleavage of prothrombin leads to the generation of thrombin, the end- product of the coagulation cascade. The alterations involving prothrombin lead to multiple imbalances in hemostasis, since this factor has procoagulant, anticoagulant and antifibrinolytic activities. The prothrombin G20210A SNV is a common gene mutation where guanine is changed by adenine at position 20210, and it has been associated with an increased risk of venous thrombosis in the general population (41). The frequency of the G20210A mutation of prothrombin (F2) gene is has been observed significantly increased in APS patients with previous thromboses (42, 56). Thus, this mutation could be considered an important genetic risk factor for clinical manifestations in APS. However, other studies performed in aPL positive patients (SLE) did not find association among this SNV and the occurrence of thrombotic events (43).
4. Tissue factor pathway inhibitor (TFPI) (the natural inhibitor of TF) can regulate the blood coagulation through the inhibition of the tissue factor-activated factor VII complex. Decreased TFPI plasma levels have been related to deep vein thrombosis in APS (44). Several TFPI SNVs have been studied in APS patients with or without vein thromboembolism. Lincz et al., observed a significant relationship among both, the T33C and C399T variations (single nucleotide variations located in the intron region of TFPI gene) and venous thrombosis in APS patients (45). Therefore, TFPI SNV is another genetic risk factor for the development of thrombosis in APS.
5. Tumor necrosis factor alpha (TNFα) is a cytokine with a well-recognized role in inflammation. Bertolaccini et al., reported high levels of this proinflammatory molecule in patients with APS. In addition, they showed an increased frequency of the G238A SNV of TNFα gene, strongly related to arterial thrombosis, suggesting the importance of this genetic marker for the development of thrombosis (46).
6. Glycoprotein (GP) Ib and GP Ia/IIa bind to von Willebrand factor (vWF) and collagen, respectively, to mediate the adhesion of platelets to the vascular wall. The presence of SNVs in these genes might increase the platelet adhesion and aggregation, predisposing to the development of arterial and/or venous thrombosis. Thus, the frequency of platelet GPIa C807T SNV has been shown higher in APS patients having thrombosis compared to those without thrombosis or controls. In addition, the frequency of the T/C polymorphism in the kozak sequence of GPIb-alpha has been found increased in APS patients with arterial thrombosis compared to APS patients with venous thrombosis, or APS patients without thrombosis. That data suggest that these two polymorphisms may be responsible for the thrombosis occurrence in APS patients (47, 57).
7. P-Selectin is a cell adhesion molecule that mediates the attachment and rolling of leukocytes on activated endothelial cells and the recruitment of leukocytes to the thrombi. Plasma levels of p-selectin are increased in APS patients (48). A study of 40 APS patients showed an increased prevalence of a single nucleotide variation associated with the coding region of p-selectin, G1902A, compared to healthy controls. Among APS patients, those who had thrombotic events presented more significantly augmented prevalence of this genotype compared to APS without thrombosis (49). Moreover, the interaction of p-selectin on activated platelets or endothelial cells with the p-selectin glycoprotein ligand (PSGL-1) on monocytes is considered relevant in processes such as inflammation and thrombosis, since it can activate TF expression. PSGL-1 presents several tandem repeats (VNTR) polymorphisms. This genotype frequency is elevated in APS patients, especially in those with thrombosis (50). These studies pointed out to selectin-PSGL1 system genetic variations as determinant of thrombotic predisposition in patients with APS.
8. Two single nucleotide variations in β2-Glycoprotein gene have been found increased in APS: G796T and G1004C, more known by the changes in aminoacidic sequence; Val247Leu and Trp316Ser, respectively (58, 59). The presence of these SNVs may lead to a conformational change in β2GPI that affects the exposure of potential epitopes, which in turn may favor thrombosis. Previous studies suggested that the presence of Val247Leu genotype is related to the production of anti- β2GPI antibodies. However, the relationship with either arterial or venous thrombosis is not clear (59). In the same way, the presence of Trp316Ser SNV may increase the risk of APS, but there is no relationship with thrombosis or antibodies production (58). Thus, further studies might be required to evaluate the pathogenic link between these SNVs and thrombosis in APS.
9. Pierangeli et al. evaluated the prevalence of two SNVs in Toll-like receptor 4 (TLR4) gene in 100 APS patients: A896G and C1196T. Both of the TLR4 SNVs confer an alteration to the extracellular domain of the TLR4 receptor, which may affect the binding of ligands. The frequency of these two SNVs was significantly reduced in APS patients with thrombosis compared to healthy donors (26). These two TLR4 SNVs are supposed to be protective against thrombosis. Thus, decreased prevalence of these polymorphisms in APS patients might suggest a higher susceptibility to an aPL-mediated procoagulant endothelium activation (26).

Considering all these genetic alterations associated with APS (at the MHC locus and outside the MHC), it is becoming increasingly clear that interactions between more than one genetic abnormality could determine whether an individual will develop the disease or suffer from the different clinical manifestations.

## GENE EXPRESSION SIGNATURES ASSOCIATED TO CVD IN APS

As detailed in the first part of this review, in the last years a number of studies have identified several genes involved in thrombosis, inflammation and endothelial dysfunction altered in APS patients (i.e., TF, PAR1, PAR2, VEGF, Flt1, IL8, TLR2, TLR4, etc.), most of them showing increased expression in cells integrating the immune and vascular systems, including

monocytes, platelets, neutrophils and endothelial cells, favoring the thrombin generation, and leading to a procoagulant activity (17, 20, 23, 25–27).

Patsouras et al. (60) further reported a significant increase of gene expression of the platelet factor (CXCL4) and its variant, CXCL4L1, in platelets of APS patients compared to the control group and to SLE patients. CXCL4 and CXCL4L1 are chemokines produced by the platelets during the aggregation. They are involved in a variety of biological processes, including inflammation, blood coagulation, and angiogenesis. They have also pro-coagulant effects and anti-angiogenesis activity, inducing angiostasis by inhibiting endothelial cell proliferation and chemotaxis. That study showed that APS patients having high levels of CXCL4/CXCL4L1 in plasma were characterized more often by IgG aCL, double antibody or triple antibody positivity, and presented more than 3 thrombotic events, thus underlying the relevance of the increased expression of these molecules in the occurrence of thrombosis.

Recent advances in gene expression analysis allowed to perform broad-based gene expression profiling. Thus, a study by Hamid C and coworkers (61), using Affymetrix Human Genome U133A-2.0 arrays, revealed a complex gene expression response in HUVECs to *in vitro* treatment to anti- β2GPI antibodies, involving multiple chemokines, pro-inflammatory cytokines, pro-thrombotic and pro-adhesive genes. Moreover, that study showed that some of these newly identified anti-β2GPI antibody-regulated genes could contribute to the vasculopathy associated with this disease.

Recently, Ripoll VM and colleagues analyzed the *in vitro* effects of aPLs from thrombotic or obstetric APS patients in monocytes, in order to identify different molecular pathways related to the pathogenesis of the APS subtypes (62). Thus, genes related to cell response to stress, MAPK signaling modulation and cell interactions were induced by IgGs isolated from patients with vascular thrombosis. In contrast, genes associated with cell adhesion, extracellular matrix and embryonic and skeletal development were modulated by IgGs purified from patients with pregnancy morbidity, suggesting that the IgGs from the different clinical subtypes of APS induce disease-specific genome profiles in monocytes, associated with different physiological mechanisms.

Using microarray technology, our group identified, *in vivo,* shared and differential genetic patterns related to atherosclerosis and cardiovascular disease in APS, APS plus SLE, and SLE patients. Thus, the gene expression analysis of monocytes led to the segregation of APS, APS plus SLE and SLE, with specific profiles associated with the pro-atherosclerotic, pro-thrombotic and inflammatory alterations. The specific features of APS monocytes comprised genes involved in mitochondria biogenesis and function, oxidative stress and antioxidant defense, processes directly related to the development of thrombosis in APS (12). In addition, those alterations were related to the levels of aPLs, a fact that was demonstrated with *in vitro* studies, where treatment of healthy monocytes with aPLs modulated the expression of genes involved in such processes including CCL2, IFIT1, PPAR gamma, SLC25A27, ARHGEF5, and IL11RA (12).

Finally, a very recent systematic review, by using bioinformatic analyses, identified a number of genetic risk factors in thrombotic APS (37). They found that sixteen genes (CXCL4L1, P-Selectin, TLR2, TLR4, PAI-1, β2GPI, GP1a, GP1BA, PAR1, PAR2, TFPI, TF, VEGFA, FLT1, TNF, and Prothrombin) contribute significantly, while six (PLSCR1, PTPN22, ACAPMTS13, F13A1, ACE, and F5) were not associated with thrombosis in primary APS. These genes affected mostly the immune system and blood coagulation pathways. Moreover, the authors suggested that these genes, expressed in 32 different organs, may pose higher risk of developing thrombosis anywhere in the body of primary APS patients.

Overall data suggest that a complex network of genetic factors (involving altered gene expression mainly induced by aPL, multiple alleles and polymorphisms) including inflammatory mediators, oxidative stress, prothrombotic molecules, leukocyte activators, and adhesion receptors are responsible for the APS pathophysiology (**Figure 1**).

## EPIGENETIC MECHANISMS UNDERLYING THE PATHOPHYSIOLOGY OF APS AND THE DEVELOPMENT OF ATHEROTHROMBOSIS

Since genome-wide profiling does not give a sufficient resolution to explain the complex pathological features of patients with autoimmune disorders, epigenetic modifications are engaged additional regulators in immune responses. Epigenetic mechanisms, known for their ability to regulate gene transcription and genomic stability, are key players for maintaining normal cell growth, development, and differentiation (13). The term "epigenetics" can be defined as the heritable alterations in gene expression, related to environmental factors, without changes in the sequence of bases in the DNA.

Epigenetic modifications can be broadly categorized into: (1) Histone modifications -including methylation, acetylation, phosphorylation ubiquitination, ADP ribosylation, and sumoylation- (2) DNA methylation and emerging RNA methylation; and (3) Non-coding RNAs mechanisms, such as microRNAs (63). Distinct from genetic mutations, epigenetic alternations are reversible, and vulnerable to nutritional and environmental factors, and thus more manageable for modification and/or drug targeting (64).

In the last years, several new findings about epigenetic modifications of gene expression have been reported in different autoimmune disorders. These modifications designate changes in the expression of DNA that result from methylation, posttranslational modifications of histone proteins, i.e., acetylation/deacetylation, methylation, and microRNAs. Remarkably, these modifications seem to act jointly (65).

### Histone Modifications

Histone modification refers to the post-transcriptional modification of a specific amino acid in the polypeptide side-chain of a histone protruding from the nucleosome.

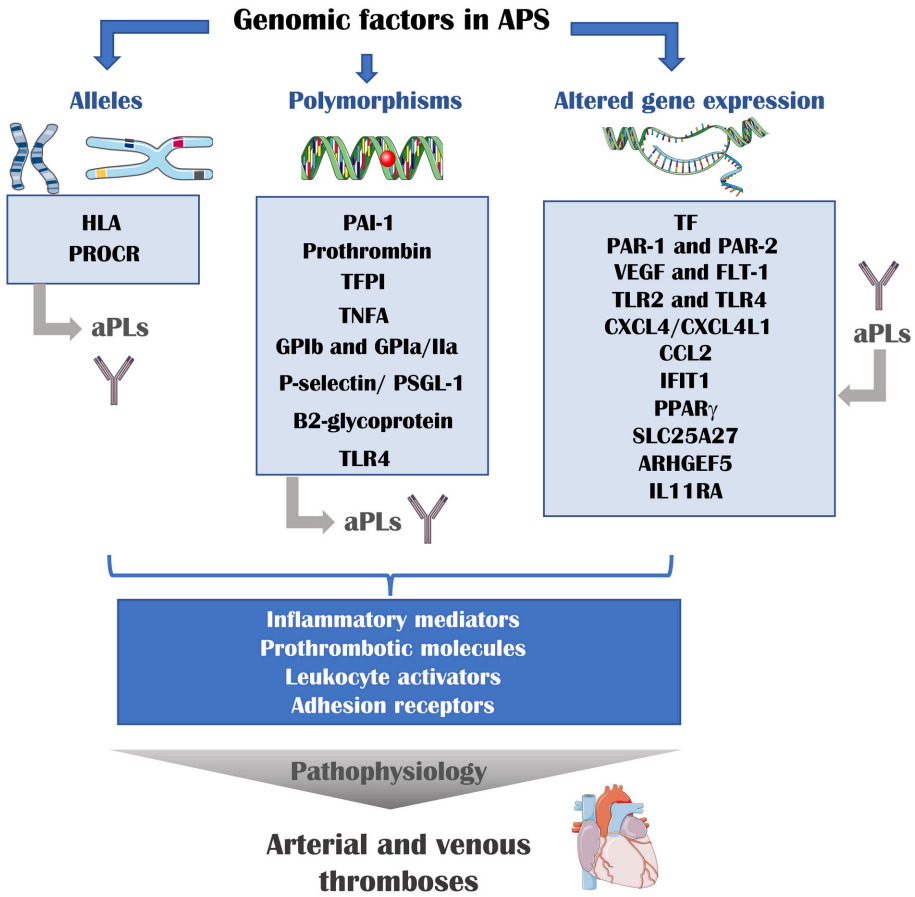


**FIGURE 1 |** Genomic risk factors of athero-thrombosis in APS. Multiple genomic factors are involved in the pathophysiology of APS. The presence of determined alleles or polymorphisms is associated with the presence of aPLs and thrombotic events. In addition, aPLs can modulate the expression of several genes. These genes encode for inflammatory mediators, prothrombotic molecules, leukocyte activator and adhesion receptors, proteins that are directly involved in the development of thrombosis. HLA, human leukocyte antigen; PROCR, endothelial protein C receptor gene; PAI-1, plasminogen activator inhibitor 1; TFPI, tissue factor pathway inhibitor; TNFA, tumor necrosis factor A; GP, glycoprotein; TLR, toll-like receptor; TF, tissue factor; PAR, protease activator receptor; VEGF, vascular endothelial growth factor; Flt-1, VEGF receptor 1; CXCL4, platelet factor 4; CXCL4L1, platelet factor variant 1; CCL2, C-C motif chemokine ligand 2; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; PPAR gamma, peroxisome proliferator-activated receptor gamma; SLC25A27, Solute Carrier Family 25 Member 27; ARHGEF5, Rho guanine nucleotide exchange factor 5; IL11RA, interleukin 11 receptor subunit alpha.

Histone modification affects local chromatin conformation altering its accessibility, and thus influencing gene transcription activity. Histone modifications are coordinated in cellular processes such as the cell cycle, development, and differentiation (66).

They include acetylation, methylation, ubiquitination, and sumoylation, each with different function and biological significance. To date, histone acetylation and histone methylation are the best studied processes (67).

Acetylation of histones induces the relaxing of compacted chromatin and grants the access of transcription factors to gene promoter regions, while deacetylation of terminal lysine residues silences the transcription. In this process, a number of identified histone acetyl transferases (HAT, including PCAF, Tip60 and p300/CBP) add the acetyl group, and the histone deacetylases (HDAC, including HDAC1 and sirtuins) remove it. Thus, HATs and HDACs coordinately regulate the acetylation status of proteins, so that changes in HAT/HDAC activity influence the cellular gene transcription in response to extracellular stimuli.

Likewise, the methylation of lysine or arginine residues by histone methyltransferases (HMTs) is reversed by demethylating enzymes (i.e., lysine-specific demethylase 1 and JmjC domain-containing histone demethylase). This reversible property of histone modifications provides great potential for the modulation of these epigenetic mechanisms (68).

SLE is the paradigm of disease on which epigenetic abnormalities and their patterns of inheritance are exceptionally complex. Global H3 and H4 hypoacetylation and hypermethylation characterize CD4+ T cells from active SLE patients compared with CD4+ T cells from patients with inactive SLE and healthy individuals, indicating that histone H3 and H4 acetylation inversely correlates with disease severity (69). Furthermore, there are a number of clusters comprising aberrantly expressed genes in SLE (including those codifying

for a set of chemokines) strongly associated with altered H4 acetylation (70).

To date, no studies have been developed to analyze histone modifications in primary APS patients.

## DNA Methylation Alterations

DNA methylation is a relatively stable and heritable epigenetic mark present in eukaryotic organisms. It refers to the addition of a methyl group to the 50 carbon in the pyrimidine ring of a cytosine residue, usually occurring in the context of cytosine-guanine dinucleotides (CpG) in mammal DNA. Although CpG dinucleotides are present throughout the whole DNA sequences and represent only approximately 4% of the human genome, research interest in DNA methylation has been mainly focused on those CpG sites located within the 50 upstream promoter regions of genes. The methylated status of CpG sites in a promoter region blocks the accessibility to transcriptional activators and thus inhibits the gene transcription, serving as a repressive "lock," while an unmethylated state at the promoter permits transcription.

The methylation process is catalytically mediated by DNA methyltransferases (DNMTs), which mainly include DNMT1, DNMT3a, and DNMT3b. DNMT1 acts as a maintenance methyltransferase enzyme that recognizes and copies the preexisting methylation profiles of a DNA strand to the new strand during DNA replication in the S phase of the cell cycle, whereas DNMT3a and DNMT3b induce *de novo* methylation (71).

Differential DNA methylation is a known epigenetic feature of SLE that not only differentiates SLE from healthy individuals, but also correlates with various organ-specific manifestations, and may play a dynamic role in disease activity via mediation of T helper cell response, among other pathophysiologic mechanisms (72). Candidate gene studies have identified several pathways in which aberrant gene expression promoted by DNA demethylation is closely related to the development of SLE (73–76). Epigenomics has further suggested that specific DNA methylation changes in lupus CD4+ T cells are correlated with different clinical phenotypes of SLE such as skin lesions only, skin and renal involvement only, and skin and renal involvement with polyarticular disease, while there are also common methylation changes found in all groups of SLE compared to controls (77).

One of most relevant advances in this field has been the identification of IFN-induced protein 44-like (IFI44L) promoter methylation in peripheral blood cells as a biomarker with high sensitivity and specificity for diagnosis of SLE superior to that of other available tests (78). Besides, the IFI44L promoter methylation level may also be a potential biomarker of disease activity of SLE, because this gene promoter exhibit significantly increased methylation level in lupus patients in remission stage than in active stage. Moreover, the IFI44L promoter methylation also shows significantly lower level in SLE patients with renal damage than those without renal damage (78).

Likewise, genome-wide studies of DNA methylation have identified many differential methylation loci in T and B lymphocytes from SLE patients compared to those from healthy individuals, with methylation status of several genes correlating with disease activity (i.e., RAB22A, STX1B2, LGALS3BP, DNASE1L1, and PREX1), (79).

Previous studies further demonstrated epigenetic aberrancies in SLE neutrophils, with demethylation in a number of interferon-regulated genes (80). Thereafter, a proinflammatory transcriptional signature in APS neutrophils was demonstrated, suggesting a role of neutrophils in the pathogenesis of APS (81).

Although the DNA methylation profile of APS remains to be characterized, a very recent study examined the genome-wide DNA methylation signatures in neutrophils from individual with primary APS vs. healthy individuals and compared the differential methylation profile of primary APS to that of SLE (82).

Seventeen hypomethylated and 25 hypermethylated CpG sites were identified in relation to healthy donors. Remarkable hypomethylated genes included ETS1, a genetic risk locus for SLE, and PTPN2, a genetic risk locus for other autoimmune diseases. Gene ontology analysis of the hypomethylated genes revealed enrichment of genes involved in pregnancy. None of the differentially methylated sites in primary APS were differentially methylated in SLE neutrophils, and there was no demethylation of interferon signature genes in primary APS as is seen in SLE. Furthermore, no other differentially methylated genes or gene regions were shared between the two disease methylation profiles.

The most notable finding of this study was the association between differential methylation in APS and genetic regions regulating pregnancy, a defining feature of APS. Thus, gene ontology analysis revealed enrichment of hypomethylated genes known to be associated with human pregnancy, namely ETS1, EMP2, and OXT. Hypomethylation of ETS1 and EMP2 in primary APS neutrophils may indicate dysregulation of trophoblast differentiation and migration which could contribute to increased fetal morbidity. DPPA3, also hypomethylated in primary APS, plays an important role in embryogenesis in murine and bovine models, though its role in humans is less clear. Functional studies of how DNA methylation affects these genes and their associated cellular functions may elucidate the mechanisms by which APS causes pregnancy morbidity and potentially guide future treatment strategies.

As in the case of histone modifications, the DNA methylation profile of APS remains to be characterized in leukocyte subsets of APS patients, as well as their involvement in the development of atherothrombosis.

## Cellular miRNAs as Biomarkers of Disease in APS

Current genome-wide studies have shown that the human genome is extensively transcribed and produces many thousands of regulatory non-protein-coding RNAs (ncRNAs), including miRNAs, small interfering RNAs, and various classes of long ncRNAs. It is now clear that these RNAs fulfill critical roles as transcriptional and post-transcriptional regulators and as guides of chromatin-modifying complexes. Among them, miRNAs are small ncRNAs ubiquitously expressed, with a profound influence in the regulation of almost every cellular process investigated,

and whose expression changes are observed in numerous human pathologies (83).

MicroRNAs (miRNAs) control posttranscriptional expression of genes by destabilizing target transcripts or by inhibiting protein translation (84). In humans, more than 2,500 miRNAs been described so far, and the number is still increasing. They act as potential modulators of the transcription of more than 20,000 genes encoding human proteins. The first step in the canonical pathway of miRNA biogenesis is the transcription of miRNA genes by RNA polymerase II/III. The pri-miRNA is cleaved by the microprocessor complex Drosha and DGCR8 to generate the pre-miRNA. Exportin 5 protein facilitates the exportation of the pre- miRNA to the cytoplasm, where it is again processed by the RNase Dicer. This enzyme produces a miRNA duplex of 5 p and 3 p strands of 22 base pairs approximately. Finally, one strand is loaded into the proteins Argonaute (AGO), generating the complex miRISC (miRNA-induced silencing complex). In recent years, non-canonical pathways for miRNA biogenesis are further emerging, including those that are independent of Drosha or Dicer (85, 86).

miRNAs have been demonstrated to control a wide range of physiological functions such as embryogenesis, cellular differentiation, proliferation, cytokine production and apoptosis. Furthermore, their altered levels have been associated to a number of pathophysiological processes such as cancer, cardiovascular disease, viral infections, neurodegenerative diseases and immune-related diseases, among others (87).

The first study that intended to characterize the role of miRNAs in the pathogenesis of APS was published in 2011 (88) (**Table 2**). That study identified two miRNAs as main regulators of the expression of TF, as mentioned above, a key procoagulant molecule involved in the development of thrombotic complications in APS. Thus, the expression levels of miR-19b and miR-20a by RT- PCR in monocytes from APS and SLE patients were found significantly reduced when compared with healthy donors and negatively correlated with the increased expression of TF in the cell surface of monocytes from both APS and SLE patients. This study suggested for the first time the potential role of those miRNAs in the pathogenesis of thrombosis in APS patients. However, the mechanism by which both, miR-19b and miR-20a are reduced in monocytes from APS and SLE patients remains to be clarified.

In a later study, we identified and characterized a number of miRNAs related to the cardiovascular disease present in APS and SLE patients (89) recognized as the main regulators of targets involved in clinical features of APS such as atherosclerosis, thrombosis, immune response and oxidative stress: miR-124a-3p, miR-125a-5p, miR-125b-5p, miR-146a-5p, miR-155- 5p, and miR-222-3p. The levels of these miRNAs were reduced in neutrophils purified from both, APS and SLE patients in relation to the control group. Moreover, the expression of molecules related to the miRNA biogenesis such as Dicer, Drosha, Exportin-5, Argonaute-1 and Argonaute-2, were decreased in both groups of patients. In monocytes isolated from APS and SLE patients, a reduction in the levels of miR-124a and miR-125a was found, while miR-155 and miR-146a appeared increased. Furthermore, the altered levels of both, miRNAs and their biogenesis proteins, correlated with markers of thrombosis, inflammation and oxidative stress, and were associated to the presence of thrombotic events, as well as with an increased Carotid Intimae Media Thickness (CIMT). The *in vitro* treatment of monocyte and neutrophils, isolated from healthy donors, with aPL-IgG purified from the serum of APS patients promoted an alteration of the levels of selected miRNAs, along with those of their biogenesis proteins. The pathogenic role of aPLs in miRNA expression was also supported by the fact that SLE patients positive for aPLs displayed a specific dysregulation of miRNAs in relation to those without such autoantibodies. In addition, a parallel cohort of 20 non-autoimmune (aPL negative) patients with previous thrombotic events showed differential miRNAs

**TABLE 2 |** microRNAs differentially expressed in Antiphospholipid Syndrome patients.

| microRNA | Alteration | Source | Technique | References |
|---|---|---|---|---|
| miR-19b, miR-20a | Reduced | Monocytes | RT-PCR | (88) |
| miR-124a-3p, miR-125a-5p, miR125b-5p, miR-146a-5p, miR-155-5p, miR-222-3p | Reduced | Neutrophils | RT-PCR | (89) |
| miR-124a-3p, miR-125a-5p | Reduced | Monocytes | RT-PCR | |
| miR-155-5p, miR-146a-5p | Increased | Monocytes | RT-PCR | |
| miR-146a-3p | Increased | Exosomes (plasma) | RT-PCR | (90) |
| miR-299-3p, miR-579, miR-494, miR-221-3p, miR-4516, miR-145-5p, miR-146b-5p, miR-371a-3p, miR-18a-5p, miR-26a-5p, miR-199a-5p, miR-376c, miR-126-3p, miR-7f-5p, miR-30b-5p, miR-106a-5p | Reduced | Monocytes | Nanostring | (91) |
| miR-29a-3p, miR-451a miR-150-5p | Increased | Monocytes | Nanostring | |
| miR-19b/miR-34a, miR-19b/miR-15a, miR-19b/miR-124, miR-19b/miR-145, miR-20a/miR-145, miR-20a/miR-374a, miR-20a/miR-210, miR-20a/miR-133b, miR-206/miR-34a | Increased | Plasma | RT-PCR | (92) |
| miR-124/miR-296 | Reduced | Plasma | PCR-Array | |
| miR-125b, miR-99a, miR-99b, miR-127, miR-181a, miR-590-3p, miR-744, miR-27a, miR-30a-5p, miR-126, miR-30e-3p, miR-335, miR-27b, miR-20a, miR-29a, miR-942, let-7c, let-7f, let-7g, let-7e, let-7a | Reduced | Plasmacytoid dendritic cells | Open-Array | (93) |

alteration than thrombotic APS patients (aPL positive). Taken together, these results established that: (1) Specific miRNAs might be considered potential biomarkers of immune activation and atherothrombotic development in APS. (2) Antiphospholipid antibodies are involved in the deregulated expression of both, miRNAs related to cardiovascular disease, and biogenesis proteins in leukocytes from APS and SLE patients.

In a very recent study (93), van den Hoogen L et al. analyzed the expression profiles of miRNAs and mRNAs in plasmacytoid dendritic cells (pDCs), the major producers of IFN-gamma in APS and SLE. A global reduced expression of miRNAs in all groups of patients was found, further related to their activation status, whereas the miRNA profiles among patients with SLE, SLE + APS and primary APS did not show strong differences. The global miRNA downregulation seemed not to be due to alterations in the miRNA machinery, since the miRNA biogenesis proteins did not show altered expression. However, pDC miRNA expression was related to the type I IFN signature in pDCs, so that miRNA expression was strongly reduced in patients with an IFN-high signature. Three miRNAs (miR-361-5p, miR128-3p, and miR-181-2-3p) were expressed at lower levels in IFN-high patients and found downregulated in pDCs activated by TLR7 agonist R837 (imiquimod). Pathway enrichment analysis revealed that the genes upregulated and predicted as target of these three miRNAs were involved in pDC activation and apoptosis. These finding suggested that aberrances in miRNA expression may have a key role in regulating pDC activity and the immunopathology of SLE and APS (**Figure 2**).

## Circulating miRNAs as Biomarkers of Disease in APS

Publications involving circulating miRNAs as diagnostic and prognostic biomarkers in many diseases have grown exponentially over the past decade. miRNAs are present in almost all human body fluids (including blood, plasma, serum, saliva, urine, seminal fluid, and pleural effusion) as a consequence of either, necrotic or apoptotic cell death, or an active release. Growing evidence highlights the role of miRNAs in cell-to-cell communication, having the capacity of regulating gene expression outside of the cell of origin. Circulating miRNAs are encapsulated in exosomes and/or bound to proteins and lipoproteins, and thus protected from endogenous RNAses, which make them stable and suitable for non-invasive analysis in patient samples (94). It has been shown that the circulating profile of miRNAs might have potential as biomarker of diagnosis, therapeutic response and prognosis in a wide range of cardiovascular pathologies and autoimmune diseases such as systemic sclerosis, rheumatoid arthritis, and SLE. With this in mind, we recently analyzed the circulating miRNA signature of APS and their potential role as biomarkers of disease and atherothrombotic status in a cohort of 90 patients (92) (**Table 1**). MicroRNA expression profiling identified 39 miRNAs differentially expressed in APS, including 19 increased and 20 reduced. Bioinformatic tools allowed to identify a set of them that showed potential mRNA targets involved in the physiopathology of APS. Eleven miRNAs were validated in the whole cohort of patients, including miRNAs 34a-5p, 15a-5p, 133b-3p, 145a-5p, 124-3p, 20a-5p, 19b-3p, 210-3p, 206, 296-5p, and 374a-5p. The signature generated by these miRNAs allowed to identify APS patients with a marked accuracy (AUC of 0.81), was found associated to the presence of both, fetal loss and type of thrombosis, and correlated with parameters related to inflammation and thrombosis (TF, PAI-1, VEGF-A, VEGF-R1, and MCP-1). In line with these findings, hard clustering analysis differentiated 3 clusters of APS patients representing different thrombotic risk groups, and significant differences between groups for several miRNA ratios were found. Among them, the ratios generated by the miR-124, miR-19b, and miR-296 were associated with an increased CIMT, thus recognizing APS patients with early atherosclerosis. These results showed the potential role of circulating miRNAs as biomarkers of atherothrombotic in APS.

We could also prove that the plasma miRNA signature remained stable over time after the analysis of samples from the same patients 3 months after the first sample collection. In addition, the specificity of the miRNA signature in APS was also confirmed in relation to both, SLE-aPL negative patients and thrombotic non-autoimmune patients.

A significant correlation between the circulating miRNA signature in APS and the titers of aPL was also noticed. Moreover, *in vitro* treatment of healthy monocytes and EC with IgG-aPL purified from the serum of APS patients, promoted a deregulated secretion of miRNAs and target proteins into the culture supernatant. These results reinforced the pathological effects of these autoantibodies, which also modulate the circulating miRNA profile found in APS patients related to their atherothrombotic status.

Similarly, a study conducted by Wu et al. (95), revealed that the treatment of ECs with anti β2GPI antibodies isolated from APS patients promoted the secretion of extracellular vesicles (EVs), whose miRNA content was different from that secreted after treatment with a non- immune-IgG. The treatment with anti-β2GPI antibodies induced the expression of miR-100, miR-1185-1, miR-10b, miR-576, miR-1251, miR-26a and miR-32, while the levels of miR-126, miR-543, miR-365b, and miR-339 were found down-regulated. The EVs secreted after aPLs exposition, were enriched in IL1β and inflammasome components which, in turn, were able to activate unstimulated endothelial cells. Thus, that study suggested that alterations in miRNA profile may contribute to the ability of EVs, derived from endothelial cells treated with aPLs, to activate unstimulated endothelial cells in an autocrine or paracrine manner.

Interestingly, circulating miRNAs might also have a potential role as biomarkers in obstetric APS. Gysler et al. (90) identified that patients with aPLs and adverse pregnancy outcomes expressed significantly higher levels of circulating miR-146a-3p compared with "healthy pregnant." Moreover, the *in vitro* treatment of a trophoblast cell line with aPLs from APS patients significantly increased cellular and exosome expression of miRs associated to TLR signaling, including miR-146a-5p, miR-146a-3p, miR-155, and miR-210. It was also established that the upregulation of miR-146a-3p contributed to the pathogenesis of obstetric APS, driving the cells to secrete interleukin (IL)-8 by activating the RNA sensor, TLR8 (**Figure 2**).

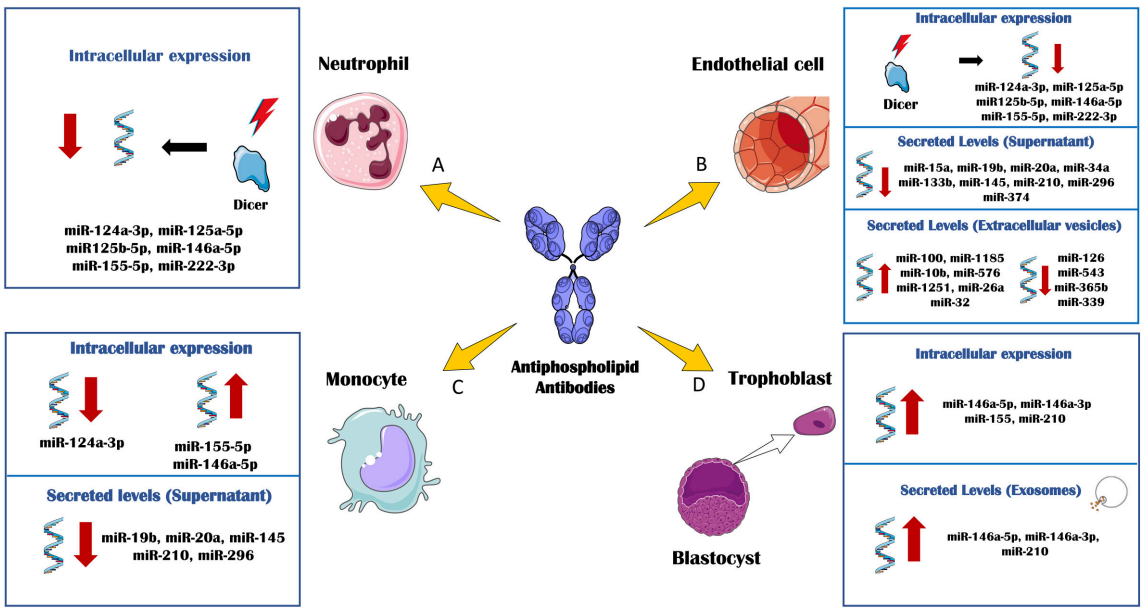


**FIGURE 2 |** *In vitro* effects of antiphospholipid antibodies (aPL) through miRNA biology. The *in vitro* treatment of several immune and vascular cells with aPL, has allowed delineating the regulation of cellular and extracellular levels of miRNAs associated to the APS pathology. **(A)** aPL- IgG treatment of neutrophils purified from healthy donors promoted the down-regulation of DICER and other related miRNA biogenesis proteins. Accordingly, the intracellular levels of several miRNAs, including miR-124a-3p, miR-125a-5p, miR125b-5p, miR-146a-5p, miR-155-5p, and miR-222-3p were also reduced. **(B)** Human umbilical vein endothelial cells (HUVEC) cultured in the presence of aPL-IgGs, showed a downregulation of DICER and miRNA biogenesis proteins along with the intracellular levels of miR-124a-3p, miR-125a-5p, miR125b-5p, miR-146a-5p, miR- 155-5p, and miR-222-3p. aPLs also induced effect through the circulating miRNA profile. The secreted levels of miR-15a, miR-19b, miR-20a, miR-34a, miR-133b, miR-145, miR-210, miR-296, and miR-374 were found reduced in the supernatant of HUVECs treated with aPL-IgGs compared to IgG control. The treatment of HUVECs with ß2GPI antibodies promoted the secretion of extracellular vesicles whose miRNA profile was increased in miR-100, miR-1185, miR-10b, miR- 576, miR-1251, miR-26a and miR-32, and reduced in miR-126, miR-543, miR-365b and miR-339, in relation to the IgG-control treatment. **(C)** aPL-IgGs treatment on monocytes isolated from healthy donors, promoted the reduction of the intracellular levels of miR-124a-3p while the levels of miR-155-5p and miR-146a-5p were up-regulated. The secreted levels of miR-19b, miR- 20a, miR-145, miR-210 and miR-296 were reduced in the supernatant of healthy monocytes treated with aPL-IgGs purified from APS patients compared to the control-IgGs treatment. **(D)** The culture of human first trimester trophoblast cell line, HTR8, in the presence of b2-GPI antibodies elevated the intracellular levels of miR-146a-5p, miR-146a-3p, miR-155 and miR-210. In parallel, secreted exosomes derived of the ß2GPI antibodies treatment on these cells, showed augmented levels of miR-146a-5p, miR-146a-3p, miR-210.

## Specific miRNAs Display Common Roles in Autoimmune and Cardiovascular Diseases

Several miRNAs displaying an altered expression in APS have been previously shown to play critical roles in the development of other autoimmune and inflammatory disorders, as well as in the pathogenesis of several cardiovascular diseases. Thus, miR-146 and miR-155 are key modulators of both innate and adaptive immune responses. Both miRNAs modulate the expression of several TLR4 effectors, such as IRAK1, IRAK2, TRAF6, IRF3, IRF5 (miR-146a), SHIP1, SOCS1, TNFα, and PU.1 (miR-155). Deregulated miR-146a and miR-155 expression have been associated with several chronic inflammatory disorders, such as SLE, RA, periodontitis, nephropathy and atherosclerosis (96). Other miRNAs such as miR-124a and miR-125 have been also previously reported as modulators of targets involved in the inflammatory chemokine pathway such as MCP1 (miR-124) or RANTES (miR-125). Furthermore, their expression was also found altered in other systemic autoimmune diseases, including SLE and RA (97, 98). The miRNA cluster 17–92, which includes miR-19 and miR-20, has been linked to different cardiovascular pathologies. Through the modulation of key proteins like MAPK, ERK, PTEN, PI3K, AKT, this cluster has been associated with mechanisms leading to coronary heart disease, myocardial infarction, and cardiac aging (99).

Concerning circulating miRNAs deregulated in APS patients, miR-133 and miR-145 have also shown high potential as biomarkers for both, diagnosis and prognosis for survival in patients with coronary artery disease, atherosclerosis, and acute coronary syndrome. They regulate targets related to angiogenesis, endothelial function, apoptosis and differentiation of both vascular smooth muscle cells and cardiac myocytes (100).

Altogether, miRNAs have a versatile range of abilities to manipulate posttranscriptional mechanisms leading to regulate coding genes engaged in different but interrelated diseases, including autoimmune and cardiovascular disorders, thus showing that they could play a regulatory role in the common pathways shared by these diseases. Therefore, the analysis of the miRNA expression profile could be a useful tool to identify biomarkers able to early recognize patients prone to develop more severe complications. At the same time, the identification of specific altered miRNAs might allow the identification of specific pathogenic pathways and suggest new treatment strategies.

## NOVEL THERAPEUTIC OPTIONS FOR MANAGING THROMBOSIS IN PATIENTS WITH ANTIPHOSPHOLIPID SYNDROME

As more insight is gained about the pathophysiology of APS and the involvement of receptors, intracellular pathways and genetic and epigenetic alterations, new treatment modalities have been proposed, on which the patient's cellular and molecular profiles are becoming more and more relevant.

## PHARMACOGENOMIC STUDIES DEVELOPED IN APS PATIENTS WITH CV RISK

In autoimmune diseases, such as APS, pharma genomics has led to several DNA-based tests to improve drug selection, adjust dosing, and diminish the risk of toxicity.

It has been shown that the cytochrome P450 (CYP) 2CP inactivates warfarin -the most common oral anticoagulant drug for APS-, but there is a percentage of population carrying variants in the CYP2CP gene that confer low enzyme activity (called *2 or *3 variants). These patients require significantly lower doses of warfarin, and even have risk of life-threatening bleeding when take standard doses (101). In this regard, a study by Kondrat'eva et al. (102) evaluated in 88 APS patients the estimated frequency of thrombotic and hemorrhagic complications during moderately intensive therapy with warfarin. They found that mutant cytochrome P450 gene variants (CYP2C9*2 and 3) were present in 38.5% of the patients. The number of nasal and gingival hemorrhages was increased in patients with CYP2C9*3. That study concluded that the determination of CYP2C9 genotype in APS patients before warfarin use might allow to avoid excessive hypo coagulation and related hemorrhages.

On the other hand, a number of genes have been reported to exhibit polymorphisms that influence the response to antithrombotic/anti-inflammatory drugs. For example, common single nucleotide polymorphisms (SNPs) in the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR, statins) gene (a A-T substitution at position 74726928 and a T-G substitution at position 74739571) were related to differential response to pravastatin treatment (103), so that individuals with a single copy of the minor allele of these SNPs showed reduced to 22% the overall efficacy for modifying total cholesterol concentration.

Other pleiotropic genes whose changes have been demonstrated with statins are the angiotensin-converting enzyme (ACE) gene, the b-fibrinogen (FGB) gene, the glycoprotein IIIa (Gilia) gene, the stromelysin-1 (MMP3) gene, the CD36 gene, and the estrogen receptor a (ESR1) gene (104).

That overall data suggest that genetic diagnostic tests might help to identify patients at high risk for adverse drug reactions, allowing to personalized dosage modifications and thus making medications safer.

## GENES AND MIRNAS AS POTENTIAL BIOMARKERS FOR THROMBOSIS MONITORING IN APS

A recent study (5) demonstrated that aPLs can disrupt the mitochondrial function of monocytes and neutrophils, leading to the generation of various intracellular ROS and the subsequent expression of TF and other proinflammatory cytokines. The inhibition of intracellular ROS in monocytes with the use of Coenzyme Q10 (CoQ10, an antioxidant with anti-inflammatory and anti-thrombotic properties), *in vitro*, prevented the upregulation of TF and VEGF/Flt-1 induced by IgG-aPL.

Previous studies have proven that changes in mitochondrial morphology and function may affect several features of cardiovascular biology. Moreover, inhibiting mitochondrial fission has been reported to be cardioprotective (105). Accordingly, we showed that CoQ10, *in vitro*, prevented mitochondrial dysfunction (involving both fission and altered mitochondrial membrane potential), oxidative stress, and the suppressed the expression of prothrombotic markers relevant to the pathophysiology of APS.

Subsequently, we analyzed the potential *in vivo* beneficial effects of $CoQ_{10}$ supplementation in the prevention of athero-thrombosis in APS patients, by developing a clinical trial (Clinical Trials.gov: NCT02218476). To develop this study (91), thirty-six APS patients were randomized to receive the reduced form of coenzyme Q10 (ubiquinol or Qred; 200 mg/d) or placebo for 1 month. The results showed that Qred modulated the overexpression of prothrombotic and proinflammatory mediators along with the improvement on endothelial function, and the reversion in the expression and/or activity of thrombosis-related protein kinases, peroxides levels, mitochondrial function, and NETosis process.

In parallel, the effect of $Q_{red}$ treatment in the miRNA profile of monocyte from APS patients was evaluated, along with their relationship with the changes observed on the inflammatory and prothrombotic profile. By using Nano string array technology and RT-PCR validation analysis, we identified for the first time in APS, the altered mRNA and miRNA signatures of monocytes related to atherosclerosis. Monocytes gene profiling showed differential expression of 29 atherosclerosis-related genes. In parallel, 21 miRNAs were found differentially expressed in relation to healthy donors, including 18 reduced and 3 increased, whose functional classification indicated preponderance in processes such as inflammatory response, reproductive system disease, and connective tissue disorders. $Q_{red}$ treatment for 1 month promoted a significant reversion of the expression levels of 23 mRNAs and 16 miRNAs. The interaction network miRNA- mRNA demonstrated that the presence of several $Q_{red}$-upregulated microRNAs seemed to control simultaneously the expression of various $Q_{red}$-downregulated genes. Thus, we have identified novel and specific miRNA–mRNA regulatory networks, related to CVD in patients with APS and modified by effect of Qred. Altogether, these results might open the scenario for the search of specific miRNAs as novel biomarkers of response to treatment and monitor of disease in APS.

## CONCLUSIONS

In recent years, there have been many advances in the understanding of the molecular basis for vascular involvement in APS, but many areas need to be further investigated, in particular the association between altered genetic/epigenetic profiles, autoantibodies and clinical manifestations, and the effectiveness of new therapeutic strategies.

It would be interesting to apply next generation sequencing technologies like RNA-Seq along with GWAS to screen both, the gene profile and the whole transcriptome of large cohorts of primary APS patients, in order to reveal the mutations/polymorphisms, post-transcriptional modifications, and changes in the gene expression as compared to healthy controls, and their relationship with the risk of thrombosis. Additionally, epigenomic studies (DNA methylation, histones modifications and miRNA profiles) on patients with primary APS would help to identify and better characterize the regulatory mechanisms that influence the abnormal expression and activities of the genes contributing to inflammation, thrombosis and organ damage in primary APS.

To date, a vast number of genetic and epigenetic biomarkers have been identified and probed to be specifically associated to the main clinical features of APS patients. Although no study has delineated which biomarkers could be considered as the most clearly associated with the highest risk of thrombosis, emerging studies are evaluating, by using bioinformatic analyses and based on a significant number of previous works, the genetic risk factors that most significantly contribute to the development of thrombosis in primary APS. As described in a precedent section, a recent study allowed to find 16 genes as the most clearly involved, including a number of them that mostly regulate the immune system and the blood coagulation pathways (CXCL4L1, P-Selectin, TLR2, TLR4, PAI-1, β2GPI, GP1a, GP1BA, PAR1, PAR2, TFPI, TF, VEGFA, FLT1, TNF, and prothrombin). In parallel, several studies have defined a number of miRNAs (mir-19b, miR20a, miR-124a-3p, miR-125a-5p, miR-125b-5p, miR-146a-5p, miR-155- 5p, and miR-222-3p), altered in their expression in key cells involved in the development of thrombosis (i.e., monocytes and neutrophils) or found deregulated in the plasma of thrombotic APS patients (miRNAs 34a-5p, 15a-5p, 133b-3p, 145a-5p, 124-3p, 20a-5p, 19b-3p, 210-3p, 206, 296-5p, and 374a-5p). Interestingly, those miRNAs were demonstrated to regulate the expression of the above defined as "*the highest prothrombotic genes*." Furthermore, all of them were deregulated by effect of antiphospholipid antibodies, of which it has been shown that their persistent and redundant presence (i.e., triple positivity) along with their titres, directly influence the highest risk of thrombosis.

Thus, although further wider studies are required to definitively demonstrate that coordinated deregulation in APS patients, and even when other molecular actors such as polymorphisms might influence these alterations, these achievements in our understanding of the disease have opened the door to the possibility of new model targeted therapeutic options for the prevention of thrombotic events in APS.

## AUTHOR CONTRIBUTIONS

CL-P and CP-S provided initial planning and wrote sections of the manuscript, edited the text, and gave final approval. NB, MA, AP-T, and EC participated in the planning and writing of sections of the manuscript, edited the text, and gave final approval.

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# The IgA Isotype of Anti-β2 Glycoprotein I Antibodies Recognizes Epitopes in Domains 3, 4, and 5 That Are Located in a Lateral Zone of the Molecule (L-Shaped)

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**Background:** Antiphospholipid syndrome (APS) is characterized by thrombosis and/or pregnancy morbidity with presence of anti-phospholipid antibodies (aPL). The APS classification criteria only consider the aPL of IgG/IgM isotype, however testing of aPL of IgA isotype is recommended when APS is suspected and consensus aPL are negative. IgA anti-βeta-2 glycoprotein-I (B2GP1) has been clearly related with occurrence of thrombotic events. Antibodies anti-B2GP1 of IgG/M isotypes recognize an epitope in Domain 1 (R39-G43), the epitopes that recognize IgA anti-B2GP1 antibodies are not well-identified.

**Aim:** To determine the zones of B2GP1 recognized by antibodies of IgA isotype from patients with APS symptomatology and positive for IgA anti-B2GP1.

**Methods:** IgA antibodies to Domain-1(D1) and Domain-4/5(D4/5) of B2GP1 (ELISA) and epitope mapping on oligopeptide arrays of B2GP1 were evaluated in sera from a group of 93 patients with at least one thrombotic and with isolated positivity for IgA anti-B2GP1 antibodies (negative for other aPL).

**Results:** A total of 47 patients (50.5%) were positive for anti-D4/5 and 23(25%) were positive for anti-D1. When peptide arrays were analyzed, three zones of B2GP1 reactivity were identified for more than 50% of patients. The center of these zones corresponds to amino acids 140(D3), 204(D4), and 264(D5). The peptides recognized on D3 and D4 contain amino acid sequences sharing high homology with proteins of microorganism that were previously related with a possible APS infectious etiology. In the three-dimensional structure of B2GP1, the three peptides, as the R39-G43 epitope, are located on the right side of the molecule (L-shape). The left side (J-shape) does not bind the antibodies.

**Conclusions:** Patients with thrombotic APS clinical-criteria, and isolated IgA anti-B2GP1 positivity appear to preferentially bind, not to the D1 or D4/5 domains of B2GP1, but rather to three sites in D3, D4, and D5. The sites on D3 and D4 were

previously described as the target identified by human monoclonal antibodies derived from patients that were capable of inducing APS in animal models. The localization of these epitopes opens a new route to explore to increase understanding of the pathophysiology of the APS and to propose new alternatives and therapeutic targets.

**Keywords: antiphospholipid antibodies, antiphospholipid syndrome, kidney transplant, graft thrombosis, epitope mapping, peptide arrays, B2GP1**

## INTRODUCTION

Antiphospholipid syndrome (APS) is an autoimmune vascular disorder characterized by recurrent thrombosis and gestational morbidity in carriers of antiphospholipid autoantibodies (aPL) (1).The most common form of the disease is the Primary antiphospholipid syndrome (P-APS) (2). There are two other forms of APS, one is that associated with systemic autoimmune diseases (SAD-APS), mainly systemic lupus erythematosus (SLE) and the other catastrophic APS (3, 4).

The main antigenic target for aPL is Beta 2 Glycoprotein I (B2GP1), a plasma protein that can circulate freely in the blood or that is bound to lipoproteins or phospholipids, like the cardiolipin (5).

B2GP1 is composed of 5 short consensus repeat domains ("sushi" domains). There are several different conformations for B2GP1, the most important of which are the circular plasma conformation and an open conformation which resembles a fish-hook or "*hockey* stick" (6, 7).

The classification criteria of APS established by an International consensus statement in 2006 are based on the simultaneous presence of at least one clinical and one laboratory criterion. The laboratory criteria include positivity of any lupus anticoagulant, anticardiolipin antibodies (aCL) and/or of anti-B2GP1, persistently positive and at medium to high titer. Only antibodies of IgM and IgG Isotypes were included in classification criteria. IgA anti-BGP1 antibody positivity was not included due to lack of sufficient supporting evidence at that time (1).

The question of whether IgA anti-$B_2$GPI may have diagnostic value for APS was subsequently addressed by the "non-criteria" antiphospholipid task force during the 13th International Congress on Antiphospholipid Antibodies held in April 2010 in Galveston, Texas. The task force concluded that the IgA anti-β2GP1 antibodies should be tested in patients with clinical signs and symptoms of APS, particularly when other antiphospholipid tests are negative (8). Importantly, IgA anti-β2GP1 antibodies are included as serologic markers of SLE in the revised classification criteria for SLE (9).

Over the past several years interest in the significance of anti-B2GP1 antibodies of the IgA isotype has been growing. IgA anti-B2GP1 has been shown to be strongly associated with DVT and stroke (10) and the thrombogenic effects of IgA anti-β2GP1 antibodies *in vitro* and *in vivo* animal models has been shown (11). Along this line our group shown that the presence of IgA anti-B2GP1 was strongly associated with thrombosis in various situations including: thrombosis in hemodialyzed patient (12), graft-thrombosis after kidney transplantation (13), and thrombosis and mortality after heart transplantation (14).

It has been described that anti-B2GP1 antibodies of IgG/M isotypes recognize epitopes on all 5 domains of B2GP1 (15, 16) with a conformational epitope in Domain 1 (R39-G43) being the immunodominant target (17). Although it is known that 50% of IgA anti-B2GP1 positive patients have specificity to D4/5, the epitopes that are targeted by IgA anti-B2GP1 antibodies are not well-identified.

The aim of the study is to define the zones of B2GP1 recognized by anti-B2GP1 antibodies of IgA isotype and to determine if these epitopes are similar or different from those described for IgG and IgM anti-B2GP1 isotypes.

## METHODS

### Patients and Serum Samples

The sera of patients were obtained from the APS Serum Collection of the Hospital 12 de Octubre Immunology Department (period 2011–2014). Initially it was planned to study 100 patients with thrombotic antecedents: 50 with Stage 4 of Chronic kidney disease (CKD) on the waiting list for renal transplant and 50 with normal renal function (NKF), plus 25 asymptomatic carriers. For reasons of peptide array availability, the number of sera studied was reduced to 93 patients with thrombotic antecedents and 18 asymptomatic controls. The selection criteria for patient group were that they had at least one thrombotic event consistent with clinical APS classification criteria and that they were only positive for IgA anti-β2GP1 (negative for other aPL). All patients were IgA anti-B2GP1 "true positive" (positivity confirmed in at least two samples separated by more than 6 months). Fifty patients were selected from among those who suffered CKD and 43 were selected from among the NKF patients. Nine of the 93 patients (all in the NKF group) had concomitant autoimmune diseases (patient selection and disposition, **Supplementary Figure 1**).

### CKD Subgroup

Patients in the CKD group had vascular thrombosis during their time on dialysis or in early post-transplant period. Twenty patients were selected because they had thrombotic episodes during the time period when they were undergoing dialysis (regardless of whether they also had thrombosis in the post-transplant). The other 30 were selected because they had thrombosis in the first 3 months of the post-transplant period

**Abbreviations:** OR, odds ratio; anti-B2GP1, anti-βeta-2 glycoprotein-I antibodies; aCL, anti-cardiolipin antibodies; CIC, circulating immune-complexes; B2-CIC, circulating immune-complexes of IgG or IgM bounded to Beta-2 glycoprotein-I.

(regardless of whether they also had thrombosis in the pre-transplant period). Fourteen of these patients lost their graft due to graft-thrombosis in the first 3 months after transplant. All the sera were obtained at the time of the dialysis (before the transplant).

Many of these patients also had other thrombotic events in addition to those used to select them, so that the total number of thrombotic events is greater than the number of patients (**Table 1**).

In addition, a reference group of 18 carriers of IgA anti-β2GP1 (negative for other aPL) without any history of vascular events or gestational morbidity and with preserved renal function was analyzed. Of these, 66% were women. Mean age was 61 ± 4 years and 6 (33%) suffered from systemic autoimmune diseases.

## Ethical Issues

The study was approved by the Hospital 12 de Octubre Ethics Committee for Clinical Research (Reference Numbers CEIC PI13/405, CEIC 14/354, and CEIC 15/008).

The patients were not asked to sign an informed consent because the legislation does not require it for observational studies without intervention in which genetic material is not used. To assure the anonymity of the data, including both sera (blood drawn) and the related clinical data, a blind code was assigned to each patient.

## Antiphospholipid Antibodies

Anti-B2GP1 and anticardiolipin autoantibodies of IgA isotype were quantified by enzyme-linked immunosorbent assays (ELISA) using IgA anti-B2GP1 and IgA anticardiolipin QUANTA Lite® (INOVA Diagnostics Inc., San Diego, CA, USA). Antibody levels higher than 20 Units were considered positive (99th percentile of a healthy population in our hospital, $N = 321$) (18). Levels of anti-cardiolipin and anti-B2GP1 of IgG and IgM isotypes were evaluated using BioPLex 2200 multiplex immunoassay system (Bio-Rad, Hercules CA, USA). Antibody levels higher than 18-GPL/mL (aCL IgG), 18 MPL/mL (aCL IgM), and 18 U/mL (anti-B2GP1, IgG/IgM) were considered positive.

The autoantibodies anti-Domain 1 (D1) and Anti-Domain 4/5 (D4/5) of B2GP1 were evaluated with ELISA tests from Inova as previously described (19), changing the secondary antibody to peroxidase-conjugated anti-human IgA (Inova). The control sera available in the kit were used as reference to convert the OD values to UA.

The 99th percentile values of anti-D1 and anti-D4/5 were obtained by using a healthy population in our hospital (blood donors, $N = 168$). Anti-D1 levels higher than 23.8 U and Anti-D4/5 higher than 22 U were considered positive.

We have no data about the presence of lupus anticoagulant (LA) in most patients in the study. LA is not routinely evaluated in patients with a first thrombotic event in our hospital. Following the protocols established in the hospital, LA is only evaluated in patients with recurrent thrombosis and in situations that the hematologist considers appropriate due to the clinical characteristics of each patient.

## Oligopeptide Microarrays

Peptide arrays (CelluSpots) containing 79 peptides of 15-aa derived from the amino acid sequence of B2GP1 (NCBI Reference Sequence: NP_000033.2, Table-fig 2) were purchased from Intavis AG, Cologne, Germany.

The design of the peptides was done with the objective that the antibodies of the patients could recognize any linear B2GP1 peptide with a length of up to 11 amino acids. Peptide 1 contains the first 15 aa. Peptides (from 2 to 79) were "bled" 4 positions in order to maintain an overlap of 11 aa with the previous one. Number, sequence and position of each peptide are described in **Table 1**.

To identify IgA-binding epitopes on B2GP1, the peptide arrays were first blocked for 3 h on a horizontal shaker at room temperature (RT) with blocking buffer: TBS-T (Tris-buffered saline, 50 mM, pH 7.4, Tween, 2.5%) with 5% skimmed milk powder. After washing for 10 min with TBS-T, patient sera were placed in a dilution of 1:100 in blocking buffer according to previous optimizations of patient serum and secondary antibody concentrations. Serum incubation was performed overnight at 4°C on a horizontal shaker. The slides were washed 3× for 10 min with TBST on a horizontal shaker at room temperature (RT) and then anti-human-IgA conjugated to alkaline phosphatase (Mabtech AB, Nacka Strand, Sweden) was placed at a dilution of 1:5,000 in blocking buffer and incubated for 2 h at RT.

After washing 5× for 10 min., detection was performed using NBT/BCIP Color Substrate Solution (0.4 mg/ml NBT; 0.19 mg/ml BCIP; 50 mM $MgSO_4$100 mM Tris buffer, pH 9.5;) was prepared from NBT/BCIP Ready-to-Use Tablets (Sigma-Aldrich, St.Louis, Mo, USA), 30 min at room temperature.

Substrate solution was removed and slides were washed 3× for 10 min. with TBST. The slides were rinsed to eliminate possible remains of salt by a rapid immersion in distilled water and dried at RT.

## Microarray Data Analysis

Images (gray scale, 16 bits) from slides were read-out using an image scanner and quantitation of the signals were quantified by image analysis. The image segmentation for the identification of the spots was made by the "fixed circle" method. The alkaline phosphatase activity of the spots was determined by image analysis using UTHSCSA ImageTool, version 3.0 (University of Texas Health Science Center, San Antonio, TX, USA). Signals from peptides were quantified measuring the Integrated Optical Density (IOD) of the area corresponding to the spot of each peptide.

A positive signal was defined as a positive IOD value after subtraction of background: the mean IOD of the five negative spots (without any peptide) plus two-fold standard deviation derived from 15 random peptides on each microarray slide. This involves a probability of >95% of positivity for a positive signal (20).

Peptides of high antigenicity were defined as those that were recognized by 50% or more of the patients. When several contiguous peptides were recognized by more than 50% of the patients, only the peptide that had the greatest

**TABLE 1 |** Description of the 79 peptides used in the peptide-array.

| Peptide number | Sequence | Position | Domain | Peptide number | Sequence | Position | Domain |
|---|---|---|---|---|---|---|---|
| 1 | GRTCPKPDDLPFSTV | 1–15 | 1 | 41 | MFGNDTITCTTHGNW | 161–175 | 3 |
| 2 | PKPDDLPFSTVVPLK | 5–19 | 1 | 42 | DTITCTTHGNWTKLP | 165–179 | 3 |
| 3 | DLPFSTVVPLKTFYE | 9–23 | 1 | 43 | CTTHGNWTKLPECRE | 169–183 | 3 |
| 4 | STVVPLKTFYEPGEE | 13–27 | 1 | 44 | GNWTKLPECREVKCP | 173–187 | 3–4 |
| 5 | PLKTFYEPGEEITYS | 17–31 | 1 | 45 | KLPECREVKCPFPSR | 177–191 | 3–4 |
| 6 | FYEPGEEITYSCKPG | 21–35 | 1 | 46 | CREVKCPFPSRPDNG | 181–195 | 3–4 |
| 7 | GEEITYSCKPGYVSR | 25–39 | 1 | 47 | KCPFPSRPDNGFVNY | 185–199 | 4 |
| 8 | TYSCKPGYVSRGGMR | 29–43 | 1 | 48 | PSRPDNGFVNYPAKP | 189–203 | 4 |
| 9 | KPGYVSRGGMRKFIC | 33–47 | 1 | 49 | DNGFVNYPAKPTLYY | 193–207 | 4 |
| 10 | VSRGGMRKFICPLTG | 37–51 | 1 | 50 | VNYPAKPTLYYKDKA | 197–211 | 4 |
| 11 | GMRKFICPLTGLWPI | 41–55 | 1 | 51 | AKPTLYYKDKATFGC | 201–215 | 4 |
| 12 | FICPLTGLWPINTLK | 45–59 | 1 | 52 | LYYKDKATFGCHDGY | 205–219 | 4 |
| 13 | LTGLWPINTLKCTPR | 49–63 | 1–2 | 53 | DKATFGCHDGYSLDG | 209–223 | 4 |
| 14 | WPINTLKCTPRVCPF | 53–67 | 1–2 | 54 | FGCHDGYSLDGPEEI | 213–227 | 4 |
| 15 | TLKCTPRVCPFAGIL | 57–71 | 1–2 | 55 | DGYSLDGPEEIECTK | 217–231 | 4 |
| 16 | TPRVCPFAGILENGA | 61–75 | 1–2 | 56 | LDGPEEIECTKLGNW | 221–235 | 4 |
| 17 | CPFAGILENGAVRYT | 65–79 | 2 | 57 | EEIECTKLGNWSAMP | 225–239 | 4 |
| 18 | GILENGAVRYTTFEY | 69–83 | 2 | 58 | CTKLGNWSAMPSCKA | 229–243 | 4 |
| 19 | NGAVRYTTFEYPNTI | 73–87 | 2 | 59 | GNWSAMPSCKASCKV | 233–247 | 4–5 |
| 20 | RYTTFEYPNTISFSC | 77–91 | 2 | 60 | AMPSCKASCKVPVKK | 237–251 | 4–5 |
| 21 | FEYPNTISFSCNTGF | 81–95 | 2 | 61 | CKASCKVPVKKATVV | 241–255 | 4–5 |
| 22 | NTISFSCNTGFYLNG | 85–99 | 2 | 62 | CKVPVKKATVVYQGE | 245–259 | 5 |
| 23 | FSCNTGFYLNGADSA | 89–103 | 2 | 63 | VKKATVVYQGERVKI | 249–263 | 5 |
| 24 | TGFYLNGADSAKCTE | 93–107 | 2 | 64 | TVVYQGERVKIQEKF | 253–267 | 5 |
| 25 | LNGADSAKCTEEGKW | 97–111 | 2 | 65 | QGERVKIQEKFKNGM | 257–271 | 5 |
| 26 | DSAKCTEEGKWSPEL | 101–115 | 2 | 66 | VKIQEKFKNGMLHGD | 261–275 | 5 |
| 27 | CTEEGKWSPELPVCA | 105–119 | 2 | 67 | EKFKNGMLHGDKVSF | 265–279 | 5 |
| 28 | GKWSPELPVCAPIIC | 109–123 | 2–3 | 68 | NGMLHGDKVSFFCKN | 269–283 | 5 |
| 29 | PELPVCAPIICPPPS | 113–127 | 2–3 | 69 | HGDKVSFFCKNKEKK | 273–287 | 5 |
| 30 | VCAPIICPPPSIPTF | 117–131 | 2–3 | 70 | VSFFCKNKEKKCSYT | 277–291 | 5 |
| 31 | IICPPPSIPTFATLR | 121–135 | 3 | 71 | CKNKEKKCSYTEDAQ | 281–295 | 5 |
| 32 | PPSIPTFATLRVYKP | 125–139 | 3 | 72 | EKKCSYTEDAQCIDG | 285–299 | 5 |
| 33 | PTFATLRVYKPSAGN | 129–143 | 3 | 73 | SYTEDAQCIDGTIEV | 289–303 | 5 |
| 34 | TLRVYKPSAGNNSLY | 133–147 | 3 | 74 | DAQCIDGTIEVPKCF | 293–307 | 5 |
| 35 | YKPSAGNNSLYRDTA | 137–151 | 3 | 75 | IDGTIEVPKCFKEHS | 297–311 | 5 |
| 36 | AGNNSLYRDTAVFEC | 141–155 | 3 | 76 | IEVPKCFKEHSSLAF | 301–315 | 5 |
| 37 | SLYRDTAVFECLPQH | 145–159 | 3 | 77 | KCFKEHSSLAFWKTD | 305–319 | 5 |
| 38 | DTAVFECLPQHAMFG | 149–163 | 3 | 78 | EHSSLAFWKTDASDV | 309–323 | 5 |
| 39 | FECLPQHAMFGNDTI | 153–167 | 3 | 79 | SLAFWKTDASDVKPC | 312–326 | 5 |
| 40 | PQHAMFGNDTITCTT | 157–171 | 3 | | | | |

*The text of the sequence is marked in one color for each domain. Domain 1, green; Domain 2, blue; Domain 3, red; Domain 4, black; Domain 5, purple.*

number of recognitions by the patients was considered to have high antigenicity.

## Visualization of 3D Structure of B2GP1

Jmol (21), an open-source Java viewer for chemical structures in 3D (http://www.jmol.org), was used to visualize and analyze the tertiary structure of B2GP1. The data of crystal structure of human B2GP1 were obtained from Protein Data Bank (doi: 10.2210/pdb1C1Z/pdb).

## Statistical Analysis

Association between qualitative variables was determined with the Pearson $\chi 2$ test (or Fisher exact test, when appropriate). Mann-Whitney test was used for comparisons in scaled variables with 2 categories. Pearson's correlation coefficient was used to evaluate the association between two continuous variables; the size of Correlation Coefficient was interpreted following the Hinkle's Rule of Thumb (22). Data were processed using Medcalc for Windows version 17.9 (Medcalc Software, Ostend, Belgium). Probabilities $<0.05$ were considered significant.

**TABLE 2 |** Description of APS events in the 93 patients studied.

| APS event | Global | Chronic kidney disease patients (N50) | Normal kidney function ($N = 43$) | *P*-value |
|---|---|---|---|---|
| Deep venous thrombosis | 58 (32.3%) | 31 (62%) | 27 (62.8%) | 0.938 |
| Arterial thrombosis | 15 (16.1%) | 9 (18%) | 6 (14%) | 0.597 |
| Pulmonary embolism | 16 (17.2%) | 1 (2%) | 15 (34.9%) | <0.001 |
| Stroke | 6 (6.5%) | 1 (2%) | 5 (11.6%) | 0.092 |
| Myocardial infarction | 11 (11.8%) | 5 (10%) | 6 (14%) | 0.556 |
| Graft Thrombosis (kidney transplanted patients) | 14 (15%) | 14 (28%) | – | – |
| Vascular access thrombosis (dialyzed patients) | 22 (23.7%) | 22 (44%) | – | – |

# RESULTS

The mean levels of IgA anti-B2GP1 autoantibodies in the 93 patients were 86.7 ± 6.4 U/mL (mean ± standard error). The median was 60.8. Interquartile range (IQR) was 40.1–121.

The values of IgA anti-B2GP1 in the transplanted patients were slightly higher (Median 83.2; IQR: 52–136) than in the patients with renal function (median 49.4; IQR: 33.1–92.3; $p = 0.009$). The mean age of the patients was 57.8 ± 1.7 years (median 59.5; IQR: 43–72). The sex ratio was very balanced: 53% women.

No significant differences were observed in the sex ratio ($p = 0.715$), age ($p = 0.428$) between CKD and NKF groups, except a greater prevalence of pulmonary embolism in NKF patients. Graft loss due to thrombosis and thrombotic events in dialysis vascular access obviously only occurred in the CKD group (**Table 2**).

## Antibodies Against D1 and D4/5

A total of 47 patients (50.5%) were positive for Anti-D4/5 and 23 (25%) were positive for Anti-D1. No correlation was observed in antibody levels of IgA anti-B2GP1 and IgA anti-D1: $R = 0.183$ (95% CI: −0.022 to 0.373; $p = 0.079$; **Figure 1A**).

The levels of IgA anti-β2GP1 correlated significantly, although moderately, with the levels of Anti-D4/5: $R = 0.582$ (95% CI: 0.429–0.702; $p < 0.001$; **Figure 1B**).

When we closely examine the anti-B2GP1 vs. anti-D4/5 correlation graph **Figure 1B**, we can observe that there are two patterns of behavior in the patients' serum. In the first one—in 66 patients (clear area), there was a strong correlation ($R = 0.81$; 95% CI: 0.701–0.881; $p < 0.001$) and in the second pattern (subgroup of 27 patients, 29%) there was a practically null correlation ($R = 0.08$; shaded area in **Figure 1B**) To clarify, there was either a strong correlation in the patients or no correlation at all.

There were no significant differences in the proportion of anti-D1 positive among CKD and NKF patients (18 vs. 32%: $p = 0.101$). The proportion of patients positive for IgA anti-D4/5 was significantly lower in the CKD group (32 vs. 74%; $p < 0.001$).

Considering the 93 patients with thrombosis, no significant association was observed between the presence of the various types of thrombosis and the positivity of the IgA Anti-D1 or IgA Anti-D4/5 antibodies (**Table 3**).

Furthermore, no significant differences were observed in patients with CKD with the presence of anti-D1 ($P = 0.957$) or anti-D4/5 and the occurrence of thrombotic events or loss of the graft by thrombosis (**Table 4**).

## Analysis of Peptide Arrays

Of the 93 sera analyzed, 5 sera did not recognize any peptide in the peptide arrays (evaluated twice). The remaining sera recognized several peptides, although no common pattern was identified. The average number of peptides recognized by the sera of patients was 24.5 (28.6 in anti-β2GP1 asymptomatic carriers).

We assessed the degree of antigenicity of each peptide by the number of sera that recognized it (**Figure 2**). Highly antigenicity peptides were considered to be those that were recognized by more than 50% of the sera of patients with APS clinical signs. Three zones of the molecule were identified where there were peptides with high antigenicity: Zone 1 in domain 3, formed by the peptides 33–35 (P33-P35) and centered in P34 (aa140). Zone 2 in the domain 4 (P46-P52). As peptide P48 is the most recognized, it was considered to be the center of this zone, although the midpoint corresponds to aa 204. Zone 3 in domain 5 (P62-P67) in which peptide P64 was the most recognized of said zone (midpoint: aa264) (**Figure 2**).

Furthermore, a fourth zone located between domains 1 and 2 was also found: a peptide (P14) was recognized by 46% of the sera, very slightly below the cutoff of 50%, so that it was considered an antigenic-like zone to make comparisons. This was named zone 4. The three zones described were also recognized by the two groups of patients although region 4 was recognized better by the NKF group (not shown).

Likewise, these zones were also recognized by the sera of the patients who were negative for the ELISA anti-D4/5 (**Figure 3A**). Of the 88 patients in whom peptides were recognized in domains 4 or 5, only 45 (51%) were positive in the anti-D4/5 ELISA test. Furthermore, only 12 (46%) of the 43 patients in whom some peptide were recognized in domain 1, only 12 were positive in the anti-D1 ELISA test.

The 18 asymptomatic IgA anti- β2GP1 carriers showed a polyclonal response, however the three zones described above can be clearly identified (**Figure 3B**).

## Localization of High Antigenicity Peptides in the Tertiary Structure of B2GP1

The tertiary structure of B2GP1 acquires a "fishhook" shape when open. If we observe the molecule from the right, it has an L shape

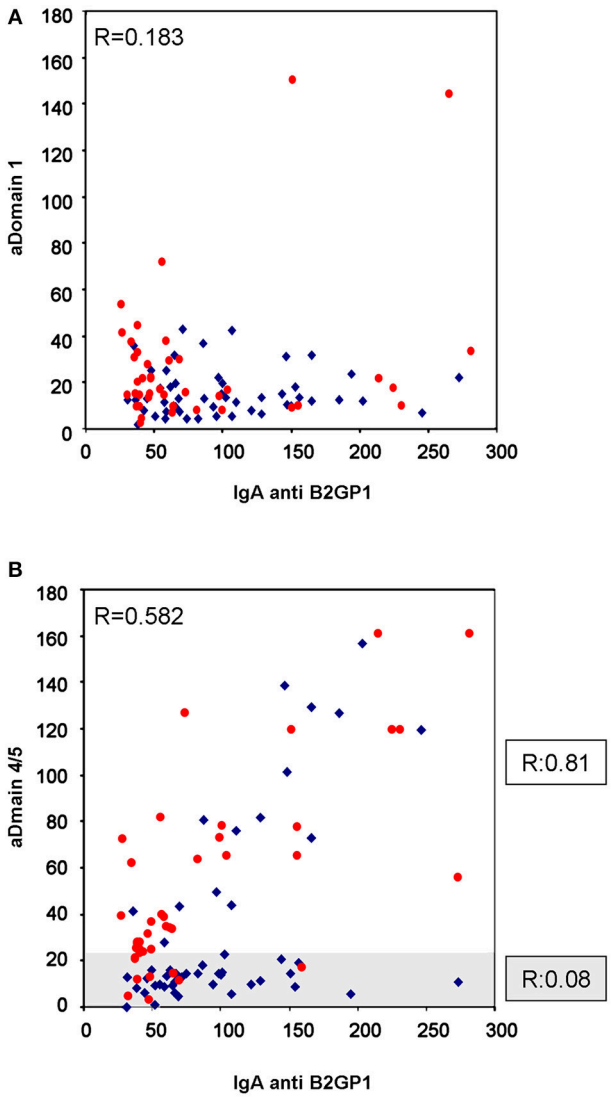


**FIGURE 1 |** Correlation of IgA levels of anti-B2GP1 vs. anti-D1 and anti-D4/5. **(A)** Correlation of the levels of IgA anti-D1 and IgA anti-B2GP1 in the 93 patients with thrombotic events. No correlation should be found ($R = 0.183$; $p = 0.079$). **(B)** Correlation of the levels of IgA anti-D4/5 and IgA anti-B2GP1 in the same patients. In the overall study, moderate correlation was found ($R = 0.582$; $p < 0.001$). The graph has been dividend into two zones: the shaded zone includes the patients in whom no correlation is observed ($R = 0.08$). When only the patients of the non-shaded area are analyzed, a high correlation is observed ($R = 0.81$; $p < 0.001$). The patients with renal failure are in blue and those with normal kidney function in red.

and is called Face L. If we look at it from the other side, it has a J shape and is called Face J.

Once the two faces are defined, if we mark the highly antigenicity peptides with colors, we can only see them on the L face, but not on the J Face (**Figure 4A**). Our three peptides (P34, P48, and P64) are shown in orange. The epitope on Domain 1 focused on the residues R39-G43 (23) is also marked, this time in red. However, when we turn the molecule and then observe face J, the peptides can no longer be visualized. Only one part of P48 can be observed, that which is located in the "elbow" of the molecule, part of it being visualized on Face J (**Figure 4B**). The intermediate steps of the rotation in **Figure 4C** from the layout in L to the layout in J can be visualized in this figure and in the **Supplementary Video 1**.

When we mark all the amino acids that correspond to the four zones (all the peptides of each zone) on the same model 3D of B2GP1, we can verify again that it is on face L where most of the amino acids recognized by the sera of the patients are located (**Figure 5A**). On face J (**Figure 5B**) only part of the amino acids corresponding to the angle of the molecule (zone 2). Zones 1, 3, and 4 can only be visualized from face L (see **Supplementary Video 2**).

## DISCUSSION

In this work, we have demonstrated for the first time that the antibodies of isotype IgA from patients with thrombotic clinical signs consistent with APS who are only positive for the IgA against B2GP1 (negative for other isotypes) have affinity to specific zones of the B2GP1 molecule.

The group of patients studied include patients with normal kidney function and patients with end-stage kidney failure that represent the two clinical situations of the medical practice in which IgA anti-B2GP1 associated to thrombotic disease can have affinity to specific zones of the Target molecule be more frequently observed. This difference may be because the patients included are receiving very complete medical control, especially during the pre-transplant period during which they come to dialysis 3 times a week. This tight control facilitates the detection of the thrombosis in very early phases and results in immediate treatment before patients can evolve into pulmonary embolism.

### Antibodies Against ELISA Domains 1 and 4/5

The domain of B2GP1 identified as immunodominant in the response of anti-B2GP1 autoantibodies (IgG/IgM isotypes) was D1 (24). However, after having analyzed our Elisa-assay results and protein mapping analysis, we cannot consider that D1 behaves as immunodominant for anti-B2GP1 autoantibodies of IgA isotype. The determination of IgA anti-D1 antibodies does not seem to provide diagnostic advantages since they are less sensitive than the determination of the IgA antibodies against the complete molecule, as already described for the anti-B2GP1 IgG class (25).

Half of the patients were positive for the IgA anti-D4/5 antibodies, a level similar to that previously described by other authors in patients positive for the antibodies against the complete protein (11, 25). The correlation between the levels of the IgA anti-B2GP1 and IgA anti-D4/5 antibodies is strong, but only in 71% of the patients; it being almost null in the remaining 29% that do not recognized the D4/5.

These findings suggest that there should be other zones of the molecule involved in the recognition by the IgA of the patients

**TABLE 3 |** Clinical characteristics of patients with thrombotic events depending on the positivity of the presence of IgA autoantibodies to D1 or D4/5 of B2GP1.

| | aD1 negative | a D1-positive | | aD4/5 negative | aD4/5-positive | |
|---|---|---|---|---|---|---|
| **Characteristic** | **Number/mean** | **Number/mean** | ***P*-value** | **Number/mean** | **Number/mean** | ***P*-value** |
| Age (years) | 57.1 ± 2.0 | 60.2 ± 3.3 | 0.434 | 56.8 ± 2.3 | 58.9 ± 2.5 | 0.540 |
| Sex (men) | 34 (46.6%) | 9 (45%) | 0.9 | 21 (46.7%) | 22 (45.8%) | 0.936 |
| Deep venous thrombosis | 45 (61.6%) | 13 (65%) | 0.784 | 29 (64.4%) | 29 (60.4%) | 0.689 |
| Arterial thrombosis | 12 (16.4%) | 3 (15%) | 0.877 | 8 (17.8%) | 7 (14.6%) | 0.676 |
| Pulmonary embolism | 12 (16.4%) | 4 (20%) | 0.709 | 7 (15.6%) | 9 (18.8%) | 0.683 |
| Stroke | 3 (4.1%) | 3 (15%) | 0.079 | 2 (4.4%) | 4 (8.3%) | 0.446 |
| Myocardial infarction | 8 (11%) | 3 (15%) | 0.62 | 5 (11.1%) | 6 (12.5%) | 0.836 |
| Graft loss by thrombosis | 9 (12.3%) | 5 (25%) | 0.160 | 9 (20%) | 5 (10.4%) | 0.197 |
| Vascular access thrombosis | 20 (27.4%) | 2 (10%) | 0.105 | 14 (31.1%) | 8 (16.7%) | 0.101 |

*No significant differences were observed.*

**TABLE 4 |** Clinical characteristics of patients with thrombotic events associated with renal failure as a function of anti-D1 or D4/5 IgA autoantibodies positivity.

| | aD1 negative | a D1-positive | | aD4/5 negative | aD4/5-positive | |
|---|---|---|---|---|---|---|
| **Characteristic** | **Number/mean** | **Number/mean** | ***p*-value** | **Number/mean** | **Number/mean** | ***p*-value** |
| Age (years) | 56.8 ± 2.4 | 55.9 ± 5.3 | 0.868 | 55.4 ± 2.7 | 59.3 ± 3.3 | 0.411 |
| Sex (men) | 18 (43.9%) | 6 (66.7%) | 0.216 | 17 (50%) | 7 (43.8%) | 0.680 |
| Deep venous thrombosis | 24 (58.5%) | 7 (77.8%) | 0.282 | 21 (61.8%) | 10 (62.5%) | 0.960 |
| Arterial thrombosis | 7 (17.1%) | 2 (22.2%) | 0.716 | 7 (20.6%) | 2 (12.5%) | 0.487 |
| Pulmonary embolism | 1 (2.4%) | 0 (0%) | 0.636 | 1 (2.9%) | 0 (0%) | 0.488 |
| Stroke | 1 (2.4%) | 0 (0%) | 0.636 | 1 (2.9%) | 0 (0%) | 0.488 |
| Myocardial infarction | 4 (9.8%) | 1 (11.1%) | 0.902 | 4 (11.8%) | 1 (6.3%) | 0.544 |
| Graft loss by thrombosis | 9 (22%) | 5 (55.6%) | 0.042 | 9 (26.5%) | 5 (31.3%) | 0.726 |
| Vascular access thrombosis | 19 (46.3%) | 3 (33.3%) | 0.478 | 14 (41.2%) | 8 (50%) | 0.558 |

*No significant differences were observed.*

and that detection of the class IgA and the anti-D1 and anti-D/45 antibodies cannot be used as the only substitute of the complete protein.

## Analysis of the Linear Epitopes

The recognition of the different peptides located in the peptide arrays by the IgA of the patients is wide-ranging and heterogeneous. Although there are some zones of the molecules that are more frequently recognized by part of the groups of patients, in the practice, each patient is different. These data suggest that the response of antibodies of the IgA isotype against the B2GP1 in our patients is polyclonal and directed against multiple zones of the molecule.

As it was not possible to localize profiles of patients, we decided to analyze the grade of recognition of each peptide by the sera of the patients. This strategy made it possible to identify both peptides that are highly recognized as zones in which there are peptides with a high grade of recognition.

It has been previously described that in addition to the classic cryptic epitope G40-R43 (26), there are other areas of B2GP1 molecule recognized by aPL (IgG and IgM isotypes) correlating with an increased risk for thrombosis. Blank et al., using pathogenic human monoclonal IgM antibodies anti-B2GPI (that can induce experimental APS) and a hexapeptide phage display library, identified three hexapeptides that react specifically with these antibodies (27).

These antibodies, called ILA-1 (28), ILA-3 (28), and H-3 (29), recognized the following peptides: Peptide ILA-1: 58-LKTPRV-63 (first and second domains of B2GP1). Peptide ILA-3: 208-KDKATF-213 (fourth domain of the B2GPI) and Peptide H-3: 133-TLRVYK-138 (this corresponds to the third domain) (27).

The Zone 2 of our mapping, the most frequently recognized by the sera of the patients, contains the sequence of the peptide ILA-3 in the P50-P53 peptides. An interesting fact in regards to future research is that the currently commercially available recombinant proteins formed by the 4/5 domains of B2GP1 and recommended for use in the ELISA technique do not contain the whole domain 4. In fact, they are truncated forms that lack the first 33 amino acids of domain 4. Thus, these proteins lack zone 2 and are not adequate to detect antibodies against this zone.

Zone 1 contains the hexapeptide recognized by H-3. The peptide 34, that having the highest grade of recognitions of the Zone 1, begins precisely with the six H3 amino acids.

The peptides corresponding to the cryptic epitope of the domain 1 (30) are only partially recognized. Very few sera react with the peptides that contain the motif R39-R43 (P8-P10).

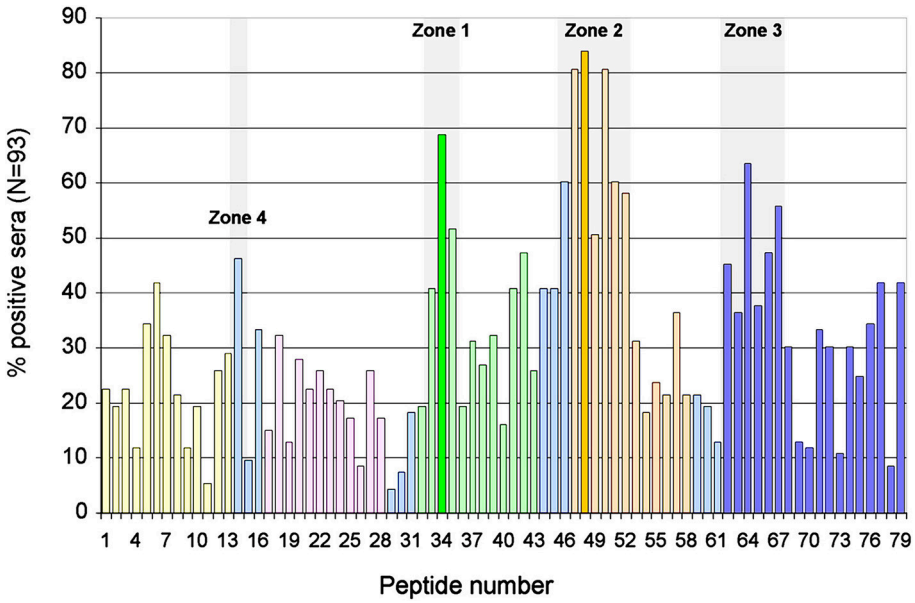


**FIGURE 2 |** Identification of immunodominant regions of B2GP1. Description of the degree of antigenicity of the 79 peptides of B2GP1 evaluated in the 93 patients with thrombotic events. On the Y axis the percentage of sera that are positive for each of the peptides is indicated. Three peptides with high antigenicity corresponding to numbers 34 (P34; aa: 133–147), 48 (P48; aa 189–203), and 64 (P68; aa 253–267) were detected. Likewise, three areas of accumulation of peptides with positivity (gray overlap) located around these peptides can be observed: ZONE 1 (peptides 33–35) zone 2 (peptides 46–52), and zone 3 (peptides 62–67). The peptides corresponding to each domain are marked in: Domain 1, Yellow; Domain 2, Pink; Domain 3, Green; Domain 4, Orange; Domain 5, Dark Blue; Interlinking regions corresponding to two domains, cyan.

However, there is a moderate grade of recognition (46%) of the interlinking region between D1 and D2 that corresponds to the peptide containing the sequence of the exapeptide recognized by ILA-1 (P14). The different grade of recognition of the two parts of the epitope located in D1 could be explained because we are using linear peptides to identify antibodies that originally recognized a conformational epitope. With our current strategy, we only can detect antibodies that have high affinity for the linear part of the epitope and cannot detect the binding when dealing with the antibodies that recognize the conformational part.

Notably, the peptides recognized by ILA-1, ILA-3, and H3 are present in various microbial proteins. This fact suggests that molecular mimicry could be involved in the pathogenic production of these antibodies (31). The context in which the antigenic presentation of the microbial molecules is produced would direct that the response of the antibodies against them would be mediated by IgA if presented to the mucosal-associated immune system, or by IgM/IgG if presented in other organs and tissues of the immune system.

It is significant that the cryptic epitope D1, the sequences of amino acids recognized by the monoclonal antibodies ILA-1, ILA-3, and H3 and the antigenic zones described in this work cannot be visualized in face J and, nevertheless, all of them can be identified when we observed face L. This fact suggests that the two faces of the molecule probably have different behaviors from both functional and antigenic points of view.

The induction and stabilization of the "fishhook" conformation occurs after the binding of domain 5 to anionic structures (32). In the same way, the stabilization of the circular conformation of β2GP1 is due to the interaction between D1 and D5 of β2GP1 (6).

The epitopes suitable for binding antibodies (such as R39-R43) can only be exposed when the B2GP1 is arranged in the "fishhook" configuration. These epitopes would not be accessible in the circular and S forms (5, 33).

We do not know the localization of the zones we have described in the B2GP1 molecule when it acquires the circular form. As of the writing of this manuscript (September 2018), we have not found any data concerning the tridimensional structure of the circular conformation of B2GP1 in the Protein Data Bank (PDB) that could help us answer this question (34).

It could be hypothesized that the epitopes of Face L would all be cryptic and their accessibility would depend on the shape of the molecule, so that, as occurs with D1, they would lose their antigenicity when B2GP1 acquires the circular form. When the molecule takes on the circular form, the amino acids located in "Face L" could remain in the inside the ring after the union of domains 1 and 5, thus remaining inaccessible.

If, in contrast, they stay on the exterior circumference of the ring, the molecule would be strained because it is necessary for the exterior circumference to be larger than the interior one, producing a molecular reorganization with potential distortion and conformational changes of the molecule due to stretching that might distort the epitopes thus preventing a correct recognition by the antibodies.

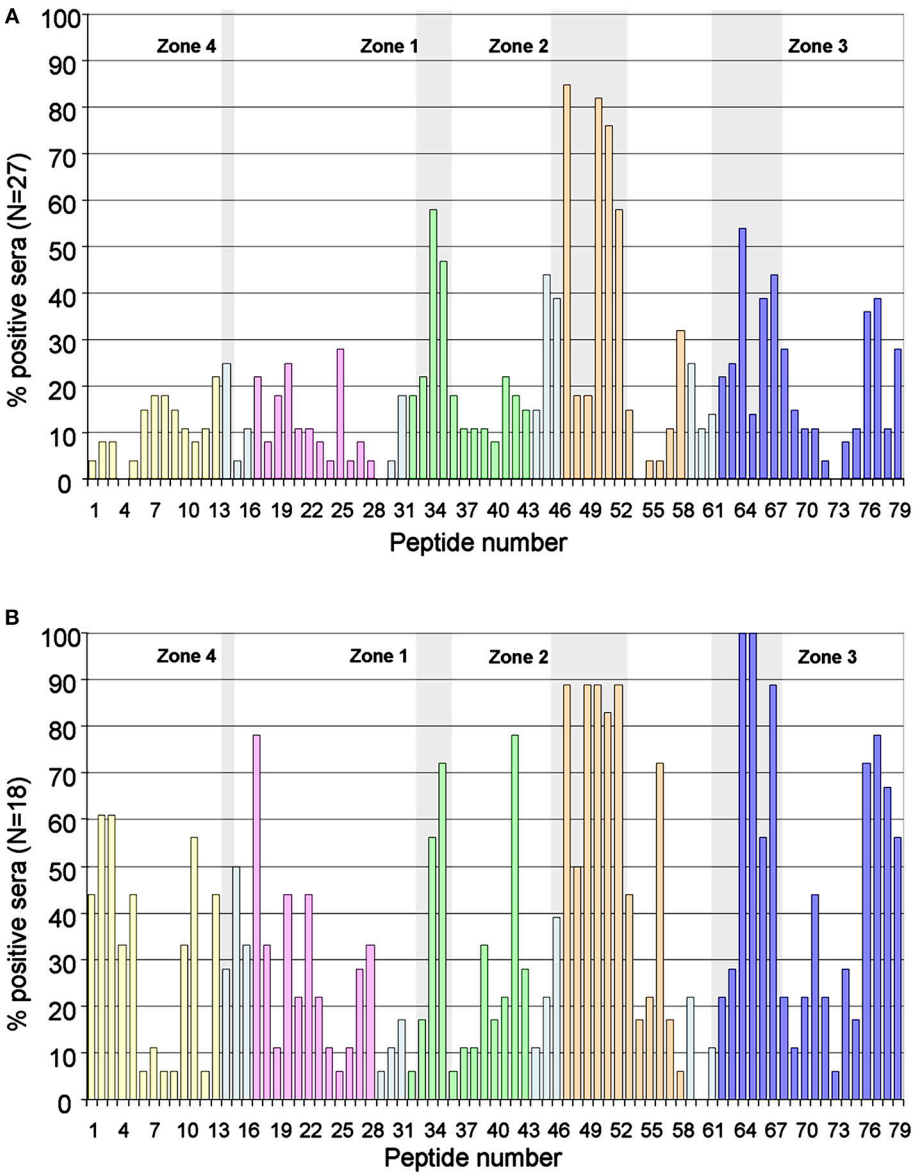


**FIGURE 3 |** Immunodominant regions in the subgroup of patients D4/5 negative and in control group. **(A)** Description of the degree of antigenicity of the 79 peptides in the 27 patients with chronic renal disease and low values of anti-D4/5 antibodies. The four areas with accumulation of positivity are shaded in gray. **(B)** Antigenicity of the 79 peptides in the reference group of 18 asymptomatic carriers of IgA anti-β2GP1 (with other aPL negative). Response in the control group is more heterogeneous, however the three main antigenic zones can be identified. The peptides corresponding to each domain are marked in: Domain 1, Yellow; Domain 2, Pink; Domain 3, Green; Domain 4, Orange; Domain 5, Dark Blue; Interlinking regions corresponding to two domains, cyan.

It is known that in the preparation of the diagnostic systems to measure IgA anti-β2GP1 antibodies, the method used to prepare the antigen and how it is bound to the solid phase is a determining factor for the exposition of the epitopes recognized by the antibodies of the patients. The heterogeneity in the preparation various diagnostic systems can at least partly explain the variations in diagnostic performance and contributes to the often contradictory results in the literature on the relationship of APS clinical signs with the presence of the IgA isotype antibodies (35, 36).

If the localization of various zones with clinical interest is located in the same part of the molecule and within the same face, this can help to overcome the difficulties to implement sufficiently efficient diagnostic systems to detect the presence of the IgA anti-β2GP1 (35, 36). Furthermore, the determination of the spatial relationship between the epitopes recognized by the pathogenic antibodies could help to better understand the function of β2GP1 and APS pathogeny.

It is well-known that the presence of aPL is essential, but not sufficient, to trigger APS events. Additional triggering factors are

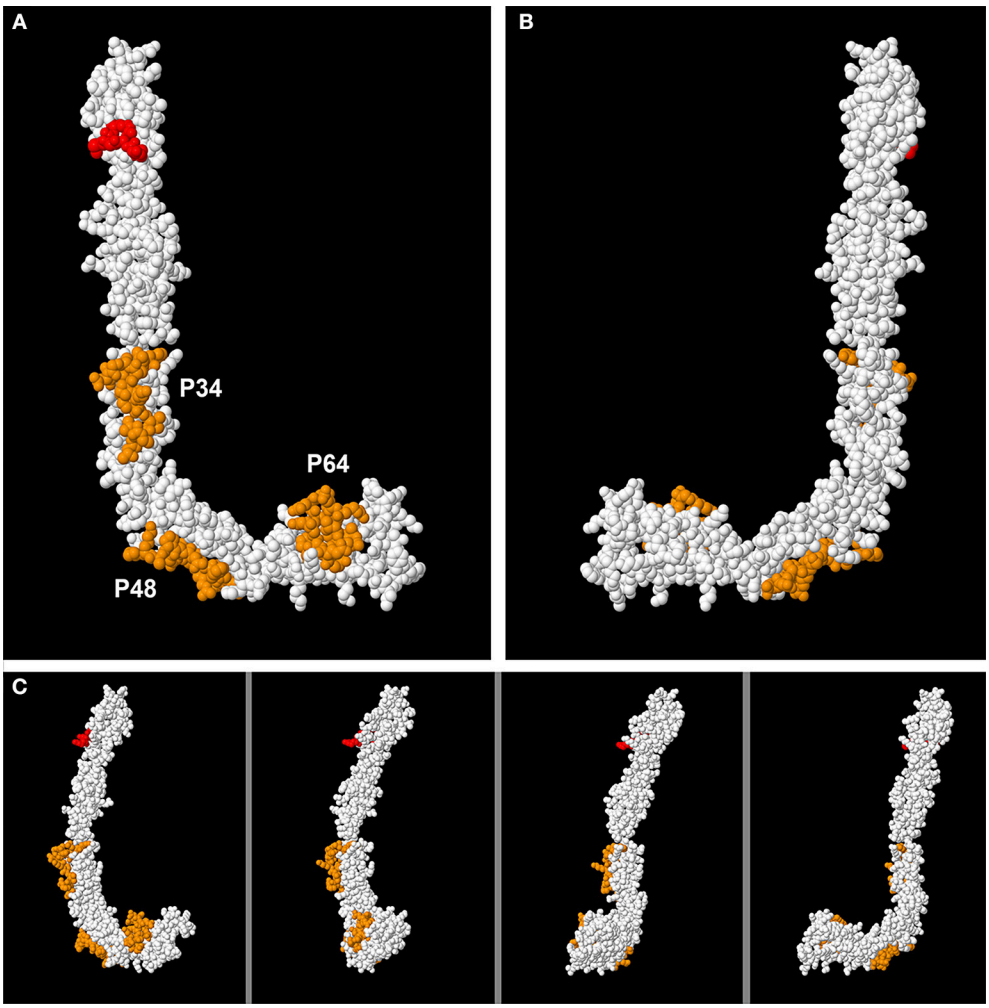


**FIGURE 4 |** Visualization of the 3D structure of B2GP1 from the sides resembling the letter "L" and the letter "J." **(A)** "L side" of B2GP1. Three peptides of high antigenicity (P34, P48, and P64) are indicated in orange. The R39-R43 epitope is indicated in red. **(B)** Visualization of the four previous epitopes in the "J side" of the molecule. Only a part of the P48 corresponding to the angle or "elbow" of the molecule is clearly observed. **(C)** Visualization of the epitopes of high antigenicity in 4 intermediate steps of the rotation process from the form in L to the form in J. In this process, it can be verified that the part of the P64 observed in the J face corresponds to a group of amino acids that protrude and that are really located in the L face.

probably needed to initiate the thrombogenic activity (second hit theory) (37). The hypothesis of the multiple cryptic epitopes could explain a possible second hit. The union of β2GP1 that circulates physiologically through the blood in circular form to anionic superstructures from microorganisms, or from injured endothelium in situations such as infections, surgery or traumatisms could result in a change in the conformation to the fishhook form and exposing the cryptic epitopes of the Face L. To further test the validity of this hypothesis, it is essential to have crystallographic data of the circular conformation, a task that will require assistance from the research community.

Identification by the antibodies of the IgA isotype of these zones of the molecule previously described in aPL of consensus and clearly related with the clinical signs of APS in animal models reinforces the findings in prospective studies of the strong association of the presence of these antibodies with the occurrence of APS events (11, 13, 38).

It also gives even more arguments to support that the IgA anti-β2GP1 antibodies are no longer the "Cinderella" (39) and that it is advisable to assess the possibility of including them in the APS classification criteria.

One of the limitations of our study is that we have used linear peptides that have allowed us to partially identify antigenic regions. In subsequent studies, a more detailed analysis needs to be performed, analyzing peptides having a smaller size and dissecting the sequences of amino acids with specific variations of the amino acid sequences to identify the zones of extensive affinity. The lack of access to crytallographic data of the circular and "S" shapes of β2GP1 has prevented us from making an analysis of the localization of the antigenic zones in these conformations.

In summary, we have described that the determination of the IgA against D1 and D4/5 separately does not improve the detection capacity of the systems using the complete protein to detect patients with IgA anti-B2GP1. Our results suggest that

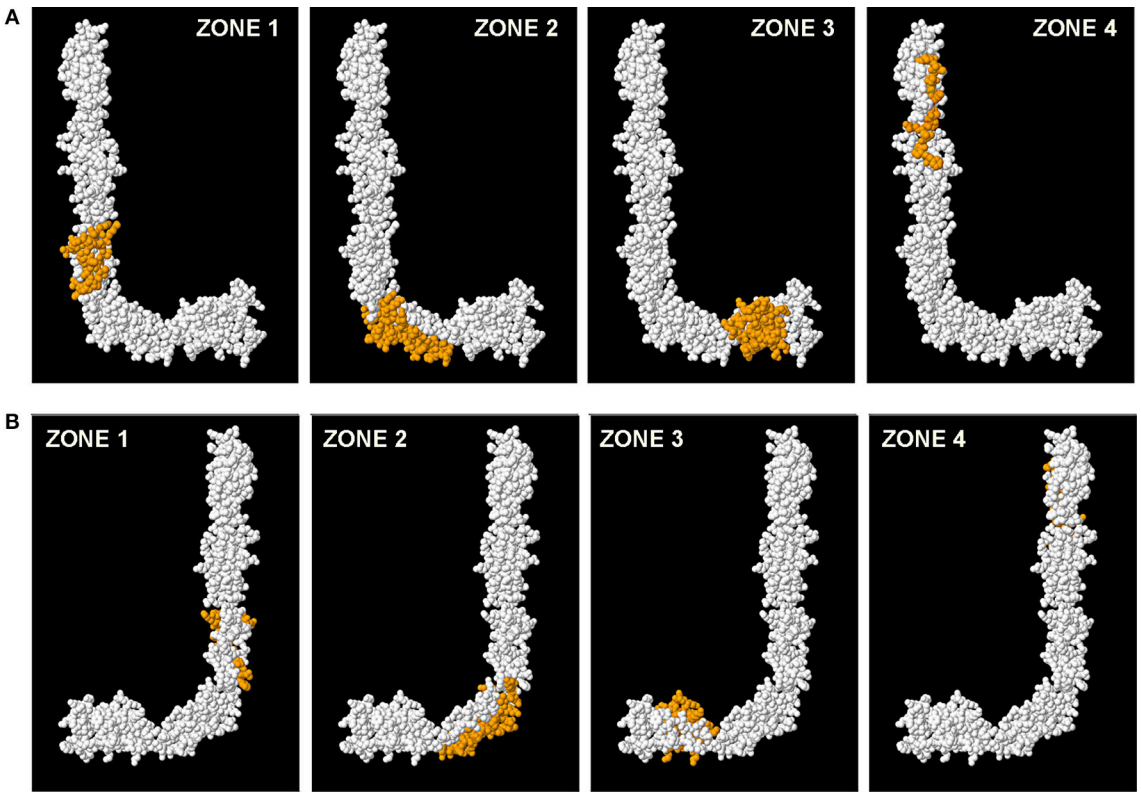


**FIGURE 5 |** Localization of the four immunodominant zones in the structure of B2GP1. Visualization of the amino acids that correspond to each zone (marked in orange). **(A)** Observing the L face. **(B)** Observing the J face.

the anti-β2GP1 antibodies of the IgA isotype found in patients with clinical signs of thrombosis recognize zones of the molecule previously identified by the aPL monoclonals from patients with APS that are capable of inducing APS in animal models. The localization of these epitopes in face L of the molecule opens a new route to begin to understand the pathophysiology of the APS and to propose new alternatives and therapeutic targets.

## ETHICS STATEMENT

The study was approved by the Hospital 12 de Octubre Ethics Committee for Clinical Research (Reference Numbers CEIC PI13/405, CEIC 14/354 and CEIC 15/008). The patients were not asked to sign an informed consent because the Spanish legislation does not require it for observational studies without intervention in which genetic material is not used.

## AUTHOR CONTRIBUTIONS

AS, JM-F, and MS designed the research. MS, JM-F, and JM were responsible for the patients' care and clinical data collection. JM-F, LN, and MS performed the determinations of anti-phospholipid antibodies. MS, JM-F and LN performed the determinations of IgA autoantibodies over the peptide-arrays. GN was responsible of the assays of Anti-D1 and Anti-D4/5 antibodies. AS and MS wrote the manuscript. AS conceived and directed the project. All authors discussed the results, contributed to the data interpretation, reviewed the manuscript, and agreed with the final version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01031/full#supplementary-material

**Supplementary Figure 1 |** Selection of patients and study disposition.

**Supplementary Video 1 |** 3-D motion of B2GP1 with the three most antigenic peptides (orange) and the "classical" D1 epitope (red).

**Supplementary Video 2 |** 3-D motion of B2GP1 with the three zones that accumulate peptides with the highest degree of antigenicity and the zone 4.

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# Cardiac Manifestations of Antiphospholipid Syndrome With Focus on Its Primary Form

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Antiphospholipid syndrome (APS) is a multisystem autoimmune disease most commonly associated with recurrent arterial and venous thromboembolism and recurrent fetal loss. Other possible antiphospholipid antibody (aPL)-related clinical manifestations include cardiac involvement. The heart can be involved through immune mediated and /or thrombotic mechanisms. Mortality due to cardiovascular problems is elevated in APS. However, the cardiovascular risk in patients with primary APS (PAPS) compared with lupus-related APS is yet to be established. Cardiac symptoms of APS include valve abnormalities (thickening and vegetations), coronary artery disease (CAD), myocardial dysfunction, pulmonary hypertension, and intracardiac thrombi. Heart valve lesions are the most common cardiac manifestation, observed in approximately one third of PAPS patients and usually do not cause hemodynamic significance. Deposits of immunoglobulins including anticardiolipin (aCL), and of complement components, are commonly observed in affected heart valves from these patients. This suggests that an inflammatory process is initiated by aPL deposition, eventually resulting in the formation of valvular lesion. aPL may have a direct role in the atherosclerotic process via induction of endothelial activation. Multiple traditional and autoimmune-inflammatory risk factors are involved in triggering an expedited atherosclerotic arterial disease evident in APS. It is imperative to increase the efforts in early diagnosis, control of risk factors and close follow-up, in the attempt to minimize cardiovascular risk in APS. Clinicians should bear in mind that a multidisciplinary therapeutic approach is of paramount importance in these patients. This article reviews the cardiac detriments of APS, including treatment recommendations for each cardiac complication.

**Keywords: primary antiphospholipid syndrome, secondary antiphospholipid syndrome, APS antiphospholipid antibodies, cardiac manifestations, cardiovascular disease, heart valve disease, myocardial infarction, pulmonary hypertension**

## INTRODUCTION

The antiphospholipid syndrome (APS) is an autoimmune disease characterized by the presence of antiphospholipid antibodies (aPL) leading to arterial and venous thrombosis and pregnancy morbidities (recurrent fetal loss and placental insufficiency) (1). The most frequently found subgroups of aPL are Lupus anticoagulant (LAC), anticardiolipin antibodies (aCL), and anti-$\beta_2$-glycoprotein I ($\beta_2$ GPI) antibodies. aPL are not only diagnostic but also pathogenic autoantibodies. The presence of aPL, though necessary, is not sufficient by itself to induce clotting, therefore an involvement of an additional trigger is expected. The most common second trigger identified is the inflammation secondary to infectious agents (2).

APS was initially considered as an autoimmune coagulopathy, but the extensive accumulated data has clarified that it is in fact a complex and systemic autoimmune disease. APS occurs as a primary disorder (primary APS, PAPS) or as a detrimental manifestation secondary to another autoimmune disease (secondary APS, SAPS), most commonly systemic lupus erythematosus (SLE). The annual risk of thrombosis among individuals with aPL in the setting of SLE is likely <4 percent, and the risk in individuals with aPL without SLE is likely <1 percent (3).

Due to its vascular nature, various organs and tissues may be affected, including the cardiac system. The cardiac involvement in APS is multifactorial: thrombosis plays an important role as well as immune-mediated injury (4, 5). The most common cardiac manifestations are valvulopathies, ranging from valve thickening through non-bacterial thrombotic endocarditis (NBTE; Libman-Sacks endocarditis) to regurgitation and severe valvular damage, and coronary artery disease (CAD). Valvulopathies and CAD are the main cardiac manifestations in APS, while other less common cardiac manifestations include myocardial dysfunction, pulmonary hypertension and intracardiac thrombus. As expected, older age is yet another risk factor for occurrence of overall cardiac manifestations in PAPS patients (6). Similarly to treatment of other aspects of APS, the cornerstone of therapy in most APS-related cardiac manifestations is anticoagulation.

This article will review the cardiac involvement in APS, including criteria and non-criteria cardiac manifestations and treatment recommendations, with a focus on PAPS (**Table 1**).

## CLASSIFICATION CRITERIA AND NON-CRITERIA MANIFESTATIONS IN APS

The revised Sapporo APS Classification Criteria defines that APS is present in patients who match at least one clinical and at least one laboratory criteria (1). Classification criteria are an important tool for appropriate inclusion of homogeneous groups of patients in observational and therapeutic studies. A number of other features associated with APS can be exhibited, but these are termed "non-criteria" because they are not included in the revised classification criteria. In addition, non-criteria only, even when combined with persistent aPL, does not provide a definite diagnosis of APS. However, the recent consensus conference in Australia accepted heart valve disease (HVD) as an integral manifestation of APS, yet not as a diagnostic criterion (1).

Even though there are classification criteria for APS and for SLE, it is challenging to determine between the two since they share some manifestations. A few articles brought up the question of whether APS and SLE are distinct diseases or not. Some authors concluded that these two autoimmune diseases are part of a spectrum ranging from PAPS only through overlap SLE-APS to aPL-negative-SLE (8). Approximately 40% of SLE patients have aPL but about one third of them will eventually develop clinical events (9). The incidence of also developing SLE is reported in 4–10% of PAPS patients (10), and the switch can occur many years after the initial diagnosis has been established (7). A 10 year-follow-up in the Euro-Phospholipid project revealed that eight patients initially diagnosed with PAPS, developed elevated titers of anti-dsDNA antibodies and were diagnosed as lupus-like syndrome. Three additional patients were diagnosed as overlap SLE-APS (11). It is of major importance for clinicians to define which patients are at higher risk for developing full blown SLE, and closely follow-up these patients (12). A recent review found that 28% of PAPS patients could have been mistakenly classified as SLE based upon the Systemic Lupus Erythematosus International Collaborating Clinics (SLICC) criteria, and were thus subjected to inappropriate hazardous treatments. None of these PAPS patients developed SLE during the median follow-up period of 12 years. The authors thus emphasized the need to develop classification criteria for PAPS that will enable distinguishing it from SAPS (12).

## TYPES OF CARDIAC INVOLVEMENT IN APS

### Heart Valve Disease (HVD)

HVD in APS patients is defined by the presence of valve lesions and/or moderate to severe valve dysfunction in the absence of a history of rheumatic fever or infective endocarditis (1). Valvular involvement was the earliest reported cardiac manifestation of APS (13) and is the most common cardiac manifestation of APS. It includes valvular thickening and valve vegetations (also referred as Libman-Sacks endocarditis) (14). Valve lesions in APS are characterized by localized valve thickness >3 mm involving the proximal or middle portion of the leaflets and irregular nodules on the atrial area of the mitral valve or the vascular side of the aortic valve are typical. The most commonly affected valves are the mitral valve, followed by the aortic valve (15, 16). In general, different studies have shown that approximately one third of PAPS patients have HVD when evaluated by transthoracic echocardiography (TTE), and that the prevalence was significantly higher compared with healthy individuals in whom valve lesions were evident in 0 to 5 percent (14, 17–23). However, the prevalence of HVD in PAPS ranges from 10% to more than 60% in various studies (24). Due to the different imaging techniques (transthoracic [TTE] or transesophageal [TEE] echocardiography) utilized for assessing HVD. Another important point is the comparison of HVD prevalence in PAPS and SLE-associated APS. Only few studies have directly compared these two groups. It was shown that

**TABLE 1 |** Summary of cardiac involvement in APS and treatment recommendations.

| Cardiac abnormality | Prevalence—PAPS | Prevalence—SAPS | Recommended management for PAPS patients |
|---|---|---|---|
| ***Coronary artery disease*** | | | 1. *Aggressive control of cardiovascular risk factors*<br>2. *Consider statins, HCQ*<br>3. *In the presence of thrombosis - anticoagulation*<br>4. *Consider PCI and/or CABG* |
| *Asymptomatic atherosclerosis* | ∼15% | ∼30–35% | |
| Myocardial infarction | 1.2%* | 3.8%* (in APS-SLE) | |
| ***Valvular disease*** | *∼33%*** | *∼40–50%*** | 1. *TTE for every APS patient as initial screening*<br>2. *Asymptomatic patients: prophylactic low-dose aspirin*<br>2. *Symptomatic patients: stroke prophylaxis with anticoagulation,*<br>3. *Consider Surgical consultation* |
| Non-bacterial thrombotic vegetations | | | |
| Valvular fibrosis and thickening | | | |
| Valvular regurgitation | | | |
| ***Myocardial dysfunction*** | *No reliable data* | *No reliable data* | 1. *Anticoagulation*<br>2. *Standard therapy for systolic heart failure* |
| Diffuse cardiomyopathy | | | |
| Diastolic dysfunction | | | |
| ***Intracardiac thrombosis*** | *No reliable data* | *No reliable data* | 1. *Intense Anticoagulation (target INR 3–4)*<br>2. *Cardiac surgeon consultation* |
| ***Pulmonary hypertension*** | *1–5.7%*** | *0.5–14%*** | 1. *Anticoagulation*<br>2. *Surgical thromboendarterectomy* |

**According to the Euro-phospholipid cohort, 10 year follow-up (7).*
***When assessed by TTE.*

the average prevalence of valve involvement was found to be significantly higher in the secondary APS group (41 vs. 27%, $P = 0.007$) (25, 26). In parallel, SLE patients who are aPL-positive compared to aPL- negative exhibit a significantly greater prevalence of valvular abnormalities (27). In a meta-analysis of aPL-associated HVD in SLE patients, the risk of HVD was higher in patients with LAC or IgG aCL (OR 6) compared with IgM aCL (OR 3) (23). Moreover, nearly 90% of SLE patients with HVD had positive aPL compared to 44% of SLE patients without HVD. Valve impairment in PAPS is usually asymptomatic but it can lead to significant dysfunction. A progression to severe valvular regurgitation requiring surgery is found in ∼4–6% of APS patients with HVD (15). A history of arterial thrombosis increases the co-morbidity of HVD in APS patients (51%) compared with a history of venous thrombosis (42%) (20, 21, 28, 29). Aortic valve lesions, confer an increased risk of stroke (27). A retrospective study of 284 APS patients, 159 of whom diagnosed with PAPS, found significant correlations between HVD and CNS manifestations (epilepsy, migraines, cerebrovascular accidents, and transient ischemic attacks), while patients with SAPS had no such correlations. Thus, CNS manifestations in PAPS may occur in the presence of valvulopathy (25).

The histopathological characteristics of aPL-associated valvulopathy are non-specific and include fibrosis, calcification, vascular proliferation, verrucous thrombosis on endocardial valvular surfaces, and thrombosis of intravalvular capillaries (19, 28, 30). Microscopy of deformed heart valves from APS patients revealed depositions of immunoglobulins including aCL (mainly IgG) and complement components, located along the exterior of the leaflets and cusps. Such deposits were not evident in control valves from aPL-negative patients and APS patients without valve involvement (31). Possibly, aPL may cause a sub-endocardial inflammatory process through an interaction with antigens on valve surfaces (32). The mechanisms of thrombosis and inflammation subsequently lead to fibrosis and calcification and eventually to valve deformation (19). There is some evidence in the literature of an association between the valve lesions in rheumatic fever (RF) and the presence of aPL. A study which evaluated the serum of 90 patients with RF and of 42 patients with APS, showed that 24% of patients with rheumatic heart disease had positivity for anti-$\beta_2$GPI and that patients with APS had anti-streptococcal activity, recognizing the M protein in 16.6% of cases. The authors concluded that there is considerable overlap of humoral immunity, supporting the hypothesis of common pathogenic mechanisms in development of valvular manifestations in APS and RF (33). Another work showed that 80% of RF patients were positive for aCL antibodies during active phase of the disease (34). More studies are needed to evaluate a potential association between RF-associated valve lesions in RF and the presence of aPL.

## Coronary Artery Disease

### Atherosclerosis

The pathogenesis of premature accelerated atherosclerosis has been assessed in APS patients (35), despite a similar incidence of traditional Framingham risk factors in APS patients compared to the general population (5). Evaluation of subclinical atherosclerosis of the carotid and femoral (by ultrasound [US]) in 86 patients with primary APS or SLE/APS compared to patients with diabetes mellitus and healthy controls revealed 28% of

APS patients had carotid atherosclerotic plaques compared to 23% of SLE/APS patients and 30% of patients with diabetes mellitus (36). The relative risk for cardiovascular disease was 2.5. In SLE patients who are aPL positive, plaques were observed in 6–31% of patients (36). Another study included 197 SLE patients and 197 matched controls who underwent carotid US. Atherosclerotic plaques were observed in 37.1% of the SLE population vs. 15.2% of controls ($p < 0.001$). The prevalence of any aPL was not significantly different between SLE patients with or without a carotid plaque. However, aCL antibodies were significantly more common in patients with no plaque compared to those with a plaque—thus, no correlation was found between aCL antibodies and carotid atherosclerosis (37). Atherosclerosis leading to major cardiovascular morbidity and mortality remains an important concern, predominantly in the younger population (5, 38). Other methods to detect subclinical atherosclerosis include evaluation of early endothelial dysfunction, circulation impairment and atherosclerotic plaques (36). However, studies, assessing the presence of an accelerated atherosclerosis in PAPS patients, were inconclusive. Discrepancies were demonstrated with respect to the number of atherosclerotic plaques in the carotid arteries by measurements of the intima media thickness (IMT), which is an early sensitive marker of generalized atherosclerosis (38, 39). Among 28 PAPS patients, an increased prevalence of carotid artery plaques by IMT measurements and a reduced lumen diameter were demonstrated and could be a significant risk for stroke (38). Contrary to these results, another study found similar results on IMT in PAPS patients without CAD risk as compared to controls, and therefore concluded that PAPS itself may not contribute to the risk of premature atherosclerosis. The authors suggested that previous reports that identified endothelial dysfunction by IMT in PAPS, did not exclude CAD risk or aging, which may explain their different findings (40).

The various types of aPL may play different roles in thrombosis formation and atherogenesis. In a retrospective study of 1,519 aPL-positive individuals, 637 patients were considered to have APS. Venous thrombotic events prevailed in patients with LAC, while peripheral, coronary and carotid thrombosis were more frequent in patients with significantly elevated titers of IgG or IgM aCL (41). A study which evaluated whether the presence of aPL was in any way associated with the IMT of carotid arteries demonstrated that IMT values in all tested carotid segments could be independently predicted by the IgG-aCL titer (42). This study further supports the link between the accelerated atherosclerotic process and aPL, specifically aCL and its cofactor, $\beta_2$GPI in these patients (42–44). Premenopausal women with APS were compared with age-matched groups of patients with SLE or rheumatoid arthritis and healthy participants for cardiovascular risk factors and early atherosclerosis. Among premenopausal women with APS, carotid, and femoral plaques were more frequently observed in the absence of other atherosclerotic risks. However, in contrast to the previously mentioned studies, this study could not establish a connection between aCL or anti-$\beta_2$GPI antibodies and atherosclerosis (45). Similar results on the lack of association between aCL and anti-β2GPI antibodies and expedited atherosclerosis were revealed in a cohort of rheumatologic patients who underwent coronary bypass (46).

It has been suggested that the pro-inflammatory and pro-coagulant activity of aPL on vascular endothelial cells might be directly related to atherosclerosis in PAPS. The indirect impact through inflammatory and immune mechanisms was also proposed. These mechanisms are assumed to induce autoantibody-mediated thrombosis (5, 40, 41) through an autoantibody cross-reaction (5), by oxidative stress (47–50). *In vitro* studies from patients with primary or secondary APS depict β2GPI that binds to oxidated low-density lipoprotein (LDL), but not to native LDL. This non-dissociable complex formation is associated with the process of autoimmune-mediated atherogenesis (49, 51). Different studies have confirmed the presence of autoantibodies directed against serum lipoproteins; a high percentage of these antibodies also cross-react (52, 53).

## Myocardial Infarction

The occurrence of acute myocardial infarction (AMI) in APS has been known since the syndrome was first described (54). CAD may be the first presentation of APS (55). The association between aPL and AMI is more frequent in women (56). There is some evidence that ∼2.8–5.5% of cases with AMI in young individuals are secondary to APS. AMI in patients with APS usually occurs in the fourth decade of life (57). MI in the young is usually associated with normal or nearly normal coronary angiographic studies (56, 58). In the Euro-phospholipid cohort, which contains the largest series of 1,000 APS patients, there were significantly more MI events in the APS-SLE group (3.8%) compared to the PAPS group (1.2%) over a 10-year follow up period. In regard to the causes of death depending on the underlying disease, 10% of PAPS patients died of MI in contrast to no patients in the SLE group, but without statistical significance of the underlying disease (PAPS or SAPS) (11). A recent systematic review included 40 patients with acute MI secondary to APS, of whom 33 patients were classified as PAPS. MI was the first presentation of APS in 80% of these cases. Cardiac catheterization revealed normal coronaries in 75% of patients, whereas 25% had evidence of obstructive atherosclerotic stenosis (59).

Accelerated atherosclerosis of the coronary arteries as well as microvascular injury or coronary thromboembolism may lead to ischemia. Coronary embolism is presented most commonly as one of the major causes of MI in the young (60). A strong correlation between thrombophilic conditions and MI in APS patients exists, but not for other thrombophilic disorders (61). LV regional wall motion abnormalities (WMA) correlated with high titers of aPL (62). The LV WMA might be related to MI as a result of CAD, or microvascular injury leading to myocardial necrosis. The specific role of each aPL subtype in APS is yet to be elucidated. In a study of 214 young patients, titers of five criteria and non-criteria aPL were measured. These included aCL anti-phosphatidylserine (aPS), anti-phosphatidylinositol (aPI), anti-phosphatidylcholine (aPC), and anti-phosphatidylethanol amine (aPEA). The titers of aPL in patients with MI were significantly elevated in the younger age group under 50 years old (63). The

presence of elevated aCL antibody titers was found to be an independent risk factor for MI or cardiac death in a cohort of middle aged dyslipidemic men (64). Several studies have found significant correlations between aCL and anti-β2GPI levels and the incidence and severity of acute coronary syndrome (ACS). An association between elevated titers of anti- β2GPI antibodies and a significant risk for MI in young menopausal women was evaluated in 172 patients hospitalized for a first MI before the age of 45 years and compared to 172 matched controls. The contribution of elevated anti- β2GPI antibody titers and the risk of MI was independent of other risk factors, including coronary atherosclerosis (36, 65). In a prospective study of CAD and stroke in 8006 American men, elevated titers of aCL antibodies as a risk factor for MI was examined by using stored frozen sera obtained from subjects enrolled in the study and followed up for 20 years. The results suggested that β2GPI-dependent aCL of the IgG class was a significant predictor of future MI in men (66). Notably, since the IgA isotype was increased in unstable angina (UA) and in MI with ST elevation (STEMI), but not in non-STEMI, increased titers of anti-β2GPI antibodies of the IgA isotype may play a role in the onset and outcome of ACS (67, 68). The prevalence of aCL was found to be ∼10% in patients with acute MI. In young MI survivors under 45 years of age and elevated aCL titers in at least two out of three measurements (3, 12, and 36 months following the event), aCL was found to be 2-fold higher (21%) (65, 69). In patients without APS, the prevalence of elevated titers of aCL was found to be ∼10% of patients with acute MI, and twofold higher (21%) in post-MI patients with persistently elevated titers of aCL and under 45 years of age (64, 68). In a large multi-center case-control study that enrolled women under 50 years of age for their first ischemic stroke or MI, LAC was associated with a significantly increased risk of MI. However, neither elevated titers of aCL antibodies nor antiprothrombin antibodies alone increased the risk of MI. There were no indications that the presence of more than one subtype of aPL affected the risk of MI. Additional cardiovascular risk factors (e.g., oral contraceptives, smoking) further increased the risk of MI in women with LAC positivity (69). Hence, the association between high titers of aCL and anti-β2GPI antibodies are associated with an increased risk of CAD and MI is still a matter of controversy. An important point is that the classification of APS does not exclude coexisting risk factors for thrombosis. The lack of discrimination for risk factors (e.g., age, smoking, lipid parameters), may explain the discrepancy in results in regard to the association between aPL and MI.

## Myocardial Dysfunction

Many prevalent conditions can impair myocardial function (e.g., diabetes mellitus, hypertension, IHD). Thus, distinguishing primary myocardial disease from other common causes of myocardial injury is quite challenging. Antibodies in the myocardium may cause ventricular dysfunction in APS. Coronary artery thrombosis and/or microvascular thrombosis may also lead to APS-related ventricular dysfunction by causing myocardial ischemia (70, 71).

Patients with SLE-APS displayed more left ventricular (LV) systolic dysfunction as compared with PAPS (72, 73). In a comparative evaluation of diastolic filling by echocardiography in 10 PAPS patients and matched healthy controls, diastolic dysfunction in PAPS patients was more prominent. All subjects were free of any cardiac risk factors or pathology that could lead to diastolic dysfunction. On the other hand, LV systolic function was normal in all these patients (74). The abnormal LV filling patterns seen in APS may be a preceding sign of myocardial involvement and systolic dysfunction. The mechanism in which aPL might cause impaired diastolic function directly is not fully understood.

A few cross-sectional studies have shed light on echocardiography findings of the right heart chambers in APS and SLE (75, 76). These studies have shown that in APS, right ventricular (RV) diastolic dysfunction occurs as a primary process independent of concomitant HVD or systolic dysfunction. In a large cohort, a more severe RV diastolic impairment was demonstrated in patients with PAPS compared with SAPS. In PAPS, the right ventricle might be involved more frequently (76). Some reports indicate that endomyocardial fibrosis may lead to severe right heart failure in APS patients (71).

Diffuse cardiomyopathy is a less frequently reported manifestation in APS. However, there are several reports describing a possible histopathological association between dilated cardiomyopathy and the presence of aPL, in the absence of conventional risk factors (13, 76–79). In these cases, autopsies revealed widespread occlusive microthrombosis of small myocardial arterioles and areas of micro-infarction surrounding affected arterioles, which led to extensive myocardial necrosis. On histopathological examination there were no vasculitic characteristics thus supporting the idea that aPL induces a direct thrombotic effect rather than by means of underlying defects in blood vessel walls (18). In one study, there was a significant correlation between high titers of aCL IgM and heart failure (6).

## Pulmonary Hypertension

Venous thromboembolisms are among the most common manifestations of APS, with a very high recurrence risk. APS-associated pulmonary embolism (PE) frequently leads to chronic thromboembolic pulmonary hypertension (CTEPH) (80), which is a prevalent non-valvular cardiac manifestation of APS. The sole presence of PHT in SLE patients worsens prognosis (81). However, in APS patients this has yet to be established. For instance, PHT was not reported as a major cause of death in the large cohort of 1,000 APS patients followed for 10 years, where only 22 patients had PHT ($n = 22$) in this cohort and hence no significant conclusions can be drawn (11). Over time, right ventricular dilation, isolated tricuspid valve regurgitation, and right heart failure may lead to secondary PHT. Different pathogenetic mechanisms, such as recurrent embolism from the veins of the lower limbs (82), *in situ* thrombosis, embolization from the right cardiac chambers (83), and immune mediated damage of the pulmonary vasculature leading to endothelin-1 related vascular remodeling (84) can lead to the development of CTEPH. A European multi-center study evaluated 114 patients with APS using echocardiographic studies, and found the prevalence of PHT to be 3.5% in PAPS and 1.8% in SAPS (85).

In a cohort of 53 APS patients, including primary and SLE-APS, three PAPS patients (5.7%) were diagnosed with PHT at baseline TTE (86). In another cohort of 70 patients with PAPS, two were diagnosed with PHT (3%); in one patient the PHT developed following recurrent PE, whereas the second patient did not show any sign of thromboembolic disease (87). Another work analyzed the echocardiographic characteristics of 29 PAPS patients using TEE, and found five patients to have PHT (17%) (88). This high prevalence of PHT is probably related to the high frequencies of HVD (76%) and PE (31%) observed in this series, which are associated to an increased risk of PHT (89). There has been an increased interest in regard to the pathogenesis of CTEPH in the last 2 decades with a few studies describing an association between aPL and CTEPH. A retrospective analysis of 24 patients with CTEPH found that in 75% of patients, at least one thrombophilic risk factor could be identified, with the most common being aPL in 50% of cases (90). LAC and/or elevated titers of aCL were the most common abnormalities in this study. Conversely, thrombotic risk factors included elevated titers of aPL in patients with primary PHT and CTEPH, 10% of the patients with primary PHT and 20% with CTEPH (91). A study of a series of 54 patients with PHT (primary, secondary or CTEPH) confirmed, applying multivariate analysis, that elevated titers of LAC or IgG aCL were independent risk factors for the development of CTEPH (92). These findings support the need for routine screening for thrombophilia and aPL profile assessment in patients with CTEPH.

## Intracardiac Thrombus

Intra-cardiac thrombus is a rare but potentially life- threatening manifestation of APS. In a cohort of 53 primary and secondary APS patients, intra-cardiac thrombus was evident in only one patient with PAPS. The overall prevalence of intra-cardiac thrombi was 1.8% in this series, though not different from controls ($P = 0.4$) (26). One report described a left ventricular thrombus in a 38 year old SLE-APS female patient with elevated levels of aCL IgG and IgM antibodies who presented with fever and confusion. ECHO revealed a large mobile apical thrombus, normal valves, and decreased left ventricular systolic function. Despite anticoagulation, the patient subsequently underwent a massive stroke and the thrombus was no longer present on a repeat ECHO study (13). Some reports describe thrombi appearing in all four cardiac chambers. An unusual case report described a 39-year-old male who presented with dyspnea and fever. TEE demonstrated three intra-cardiac masses. The diagnosis of APS was confirmed by an aCL assay. Despite anticoagulation therapy, the patient died 2 weeks after surgical resection. An autopsy revealed intra-cardiac thrombi in all four chambers (93). Another report described a 57-year-old woman, who was admitted for progressive dyspnea. TTE revealed a mural thrombus attached to the apex of a normally contracting LV. Cardiac magnetic resonance (CMR) examination was characteristic of ischemic injury, with no obvious LV WMA. Hence, the diagnosis of PAPS was confirmed. The authors concluded that thrombotic cardiac microvasculopathy caused myocardial ischemia which triggered clot formation (94). However, mural thrombosis in a normally contracting LV remains unusual. This case demonstrates the subtle interplay of microvascular and intra-cardiac thrombotic phenomena in APS. Severe myocardial dysfunction itself can be a risk for the formation of a left ventricular thrombus. However, findings of primary intra-cardiac thrombosis in other cardiac chambers suggest a possible role of aPL in this process. High levels of aCL IgG were found to be related to intra-cardiac thrombus formation ($P = 0.007$) (6). CMR is recommended for the assessment of intra-cardiac masses (95).

# MANAGEMENT OF APS-RELATED CARDIAC DISEASE

## Heart Valve Disease

Most clinicians do not routinely screen APS patients for involvement of cardiac valves unless the patient is symptomatic or a new murmur is appreciated on physical examination. In APS patients, who also exhibit previous thrombosis, mainly in cases of arterial involvement, a TEE is recommended (14). In this context, it is worth noting that other clinicians recommend performing an echocardiographic study using TTE in every APS patient as an initial evaluation (95).

In a 2003 consensus report on cardiac disease in APS, the following recommendations for treating aPL-associated HVD were set (96):

1. Prophylactic low-dose aspirin alone is advised for asymptomatic patients without previous thrombosis and with no echocardiographic evidence of valvular vegetation or dysfunction.
2. Anticoagulation, to target International Normalized Ratio [INR] of 2.0–3.0 for patients with valvular vegetations and/or systemic embolization secondary to HVD.

There are conflicting data regarding the effect of antiplatelet agents and warfarin on regression of the valvular lesions, but these treatments may prevent embolic events (92). Some studies found no benefit in regard to regression of valvular vegetations with antiplatelet and/or anticoagulant therapy in PAPS patients (29, 97). In addition to anticoagulation therapy, some experts may add other treatments, such as low-dose aspirin, statins, and hydroxychloroquine (HCQ) (98). A recently published case report described a PAPS patient receiving longterm rivaroxaban who was diagnosed with asymptomatic NBTE. Upon confirmation of diagnosis, the patient was treated with HCQ and corticosteroids (CS) in addition to low-molecular-weight heparin (LMWH). Under this treatment regimen the size of the vegetation decreased by half at 1 week and completely resolved at 6 months (99). Substantial data is lacking on the efficacy of CS in individuals with aPL-associated HVD and remains controversial (100). However, the decline in prevalence of NBTE lesions at autopsies following the introduction of CS, supports their possible beneficial effect (101). One protocol recommends a 1 month course of high dose CS with follow-up echocardiograms in order to determine the appropriate rate of CS taper, along with anticoagulation to prevent embolic stroke (102). CS may expedite healing of vegetations, preventing scarring and eventually valve

dysfunction (22). The task force report published in 2011 (14) provided no recommendations for treating valvular lesions.

Surgical consultation may also be necessary in selected cases of aPL-associated HVD with severe valvular dysfunction and recurrent embolism despite anticoagulation. An increased risk for morbidity and mortality in APS patients following valve-replacement surgery is well-documented. Most complications were due to bleeding and thrombosis (103). In a retrospective analysis of 32 APS patients, who underwent valve replacements, mortality rates were 12.5%, while 50% of patients had an uneventful outcome (104). In other studies, even higher mortality rates (>20%) after valve-replacement surgery were reported (105–107). The type of valve for replacement, whether mechanical or biologic is yet to be established. Biologic valves have a theoretical advantage of less thromboembolic events compared with mechanical prostheses (105). On the other hand, given that these patients are usually young and commonly require anticoagulation for previous thromboembolism, other studies recommend the use of mechanical valves (106). Moreover, some risk might exist for immunologic deterioration of tissue heart valves. One study found no differences in outcome between types of valves utilized (104). More studies are necessary in order to conclude which type of prosthetic valve would be better in this setting, and long-term results are lacking. Heart valve replacement poses significant challenges on the management of APS patients who require this surgery, especially during the perioperative period. Assessing thrombotic and hemorrhagic risks as well as careful monitoring are mandatory procedures to avoid complications. The use of relatively new valve surgery techniques, such as trans-catheter aortic-valve replacement (TAVR), may represent a safer approach for patients with APS. However, longterm results are not available and additional studies are required in order to assess the outcome in this group of patients.

Prophylactic antibiotic therapy for the prevention of developing infective endocarditis in patients with primary or secondary APS and valve lesions is not warranted (108).

## Coronary Artery Disease

There are no substantial additions to therapeutic considerations for APS patients with CAD. However, aggressive monitoring of traditional risk factors should be performed for lifestyle modification and pharmacological treatment. For CAD, Aspirin prophylaxis was not proven to be an added benefit over warfarin therapy alone (58). Pharmacological therapies include anti-platelets, anticoagulants, angiotensin-converting enzyme (ACE) inhibitors, beta-blockers, and statin therapy. Statins have a beneficial effect in various mechanisms in addition to their effect on lipid profile, including their ability to enhance the stability of atherosclerotic plaques, improve the endothelial function (109, 110), decrease oxidative stress (111) and suppress the inflammatory responses (112). All of these mechanisms may contribute to thrombosis prevention in APS patients. HCQ has been reported to exert anti-atherogenic properties. However, recommendations for HCQ use are reported in addition to therapeutic anticoagulation for secondary prevention of arterial thrombotic events in PAPS (57, 113). Therapy with CS in APS-associated ischemic heart disease may be detremental (58). Anticoagulation is optimal with a target INR range of 2.0–3.0 as long term therapy (114, 115), and with escalation to a higher target INR should thrombosis recur (116). Ideally, young APS patients who present with STEMI should undergo primary percutaneous coronary intervention (PCI) (117) and thrombus aspiration in selected cases. In APS patients undergoing PCI a careful balancing between the risk of bleeding and that of thrombosis should be executed. Stenting should be weighed carefully, and kept available for patients with underlying coronary atherosclerosis in the culprit lesion (118). Dual antiplatelet therapy is usually given as short term treatment in ACS with stent implantation. Thrombolysis in hypercoagulable states has also been described in the literature and may be the appropriate treatment for selected APS patients presenting with MI (119). Coronary artery bypass grafting (CABG) is indicated in some APS patients with CAD, and efforts should be directed at assuring adequate perioperative anticoagulation to avoid complications.

## Myocardial Dysfunction

Current guidelines should be implemented in standard medical therapy for systolic heart failure, including ACE inhibitors, beta-blockers, mineralocorticoid receptor antagonists, and ARNI (angiotensin receptor-neprilysin inhibitor). In addition to these agents, APS patients with myocardial dysfunction require long-term anticoagulant therapy to minimize the possibility for thromboembolism of large vessels and lower the risk of thrombotic microangiopathy, which might lead to subsequent cardiac complications. In patients with definite APS and in the absence of contraindications, warfarin is the treatment of choice, with a target INR of 2.0–3.0 while the target INR depends on the causes of the myocardial dysfunction and individual's risk (113). Patient with symptomatic heart failure and reduced ejection fraction despite optimal pharmacological therapy necessitate evaluation for cardiac resynchronization therapy (CRT), left ventricular assist device (LVAD) and heart transplant. These interventions are at a very high risk of failure in APS patients, due to the thrombotic burden of the disease that damages even the device or transplanted heart, and the possible nephrotoxicity of immunosuppressive therapy (120). Nevertheless, there is no consensus on how to manage the myocardial dysfunction in APS (96); it is unclear whether antiplatelet, anticoagulant or other therapies may prevent the associated diastolic dysfunction or dilated cardiomyopathy (18).

## Pulmonary Hypertension

The mainstay of treating PE and CTEPH in APS patients is life-long anticoagulant therapy. Patients with progressive CTEPH should be evaluated by a multidisciplinary team of experts. Surgical pulmonary thromboendarterectomy should be performed to prevent irreversible damage, improve hemodynamics, exercise capacity and survival (58, 121, 122). In symptomatic patients with CTEPH who are ineligible for surgery, or when irreversible pulmonary resistance is already evident, off-label use of drugs approved for pulmonary arterial hypertension, such as prostacyclins and endothelin receptor

antagonists, should be considered. A phase 3 randomized trial compared a group of 173 patients treated with riociguat (a soluble guanylate cyclase stimulator) to a group of 88 individuals who were administered a placebo. The results showed that riociguat significantly improved the clinical outcomes in patients with CTEPH who were ineligible for surgery or who had persistent/recurrent PHT after undergoing pulmonary endarterectomy (122). Since inflammatory mechanisms may contribute to APS-associated CTEPH pathogenesis, the addition of immunosuppressive therapy may be beneficial.

### Intracardiac Thrombus

Adequate maintenance anticoagulation therapy (target INR 3.0–4.0) is needed in APS patients with intra-cardiac thrombi. The role of surgical intervention remains controversial. To prevent the recurrent intra-cardiac thrombotic events, the thrombus should be surgically removed as early as possible and adequate maintenance anticoagulation therapy should be provided. The 2003 expert committee recommended treatment with warfarin anticoagulation along with cardiac surgeon consultation (96). To date, there are no randomized trials comparing different treatment strategies for this rare complication of APS.

The management of APS has been in continuous evolution over the past three decades. Nevertheless, antiplatelet and anticoagulant agents are still the cornerstone of APS treatment. As we discussed in detail, anticoagulation (mainly warfarin or heparin) is also the mainstay of therapy in the various cardiac complications associated with APS. The role of direct oral anticoagulants (DOACs) in managing thrombotic APS manifestations is still unclear and there is insufficient evidence to establish recommendations regarding their use in these patients (123). The current trend aims at providing individually tailored treatment strategies, according to each patient's risk stratification (124).

## SUMMARY

In this review, we have described the cardiac manifestations of APS and the different treatment recommendations, with a close-up on PAPS.

APS is a multi-organ autoimmune disease with various cardiac manifestations. Valve involvement was the first reported cardiac manifestation of APS, including valvular thickening, valve nodules (also referred to as non-bacterial vegetations or Libman-Sacks endocarditis) and valvular regurgitation/stenosis. APS is presumed to be associated with accelerated atherosclerosis of peripheral and coronary arteries: aPL induce activation of endothelial cells, and thus may play a direct role in the process of atherogenesis. Other cardiac manifestations, in the form of myocardial dysfunction, pulmonary hypertension and intra-cardiac thrombi are less common.

To date, a classification system that can distinguish the cardiac manifestations in PAPS from SAPS has not yet been established. We therefor agree with the substantial need to develop classification criteria for therapeutic decisions.

Since the prevalence of valve involvement is higher in SLE-APS than in PAPS, we recommend that all APS patients with evidence of HVD to be screened for SLE. We recommend performing TTE in every APS patient as an initial evaluation.

Due to the high prevalence of cardiac involvement in APS, clinicians should have a high index of suspicion, in the attempt to minimize cardiovascular complication rates. Regarding non-criteria APS manifestations, we believe that APS-related HVD should be integrated into a future revision of APS classification criteria.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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# Immune Complexes of Beta-2-Glycoprotein I and IgA Antiphospholipid Antibodies Identify Patients With Elevated Risk of Thrombosis and Early Mortality After Heart Transplantation

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**Background:** The presence of anti-Beta 2 glycoprotein antibodies (aB2GP1) of IgA isotype is common in patients with functional impairment of the organs in which B2GP1 is elaborated. Pretransplant IgA aB2GP1 has been associated with increased risk of thrombosis in kidney and heart transplanted patients and has also been related with early mortality after heart transplantation. Circulating immune complexes between IgA and B2GP1 (B2A-CIC) have been described in the blood of patients positive for IgA aB2GP1 with thrombotic clinical symptoms. In kidney transplanted patients, B2A-CIC is a biomarker that predicts which patients IgA aB2GP1 positive are at risk of thrombosis events following kidney transplantation and may lead to early prophylactic treatment. The prevalence of B2A-CIC and its relation with outcomes after heart transplantation is not known.

**Methods:** Follow-up study based on 151 consecutive patients who received a heart transplant. Autoantibodies and B2A-CIC were quantified in pre-transplant serum samples. Three groups of patients were followed-up for 2 years: Group-1, positive for IgA aB2GP1 and B2A-CIC ($N = 19$). Group-2, only positive for IgA aB2GP1 ($N = 28$). Group-0 (control group): IgA aB2GP1 negative ($N = 104$).

**Results:** Kaplan-Meir survival analysis showed that mortality in B2A-CIC positive was higher than group-0 at 3 months (HR:5.08; 95%CI: 1.36–19.01) and at 2 years (HR:3.82; 95%CI: 1.54–12.66). No significant differences were observed between group-2 and group-0. Multivariate analysis identified B2A-CIC as the most important independent risk factor for early mortality (OR = 6.12; 95% CI: 1.93–19.4). Post-transplant incidence of thrombosis was significantly higher in B2A-CIC positive patients than in the control group (OR: 6.42; 95%CI: 2.1–19.63). Multivariate analysis identified the presence of B2A-CIC (OR: 6.13; 95%CI: 2.1–19.63) and the pre-transplant habit of smoking actively (OR: 4.18; 95%CI: 1.35–12.94) as independent risk factor for thrombosis. The proportion of

patients who had thrombotic events or died in the first trimester was significantly higher in group-1 (73.7%) than in group-0 (16.3%; $p < 0.001$) and in group-2 (39.3%; $p = 0.02$). Multivariate analysis identified B2A-CIC as the main independent risk factor for early outcomes (mortality or thrombosis) in the first 3 months after heart transplant (OR = 11.42, 95% CI: 1.69–9.68).

**Conclusion:** B2A-CIC are a predictor of early mortality and thrombosis after heart transplant.

**Keywords: antiphospholipid, heart transplant, circulating immune-complexes, anti-beta-2-glycoprotein I, non-criteria aPL, IgA**

## KEY MESSAGES

- Pre-transplant prevalence of B2A-CIC in heart transplanted patients is 12.6%.
- Pre-transplant presence of B2A-CIC is the main independent risk factor for mortality in the first 3 months after heart transplantation.
- About ¾ of B2A-CIC positive patients suffer thrombotic events or death in the first 3 months after heart transplant.
- Positivity of B2A-CIC identifies patients with higher risk of mortality and incidence of thrombotic events after heart transplantation.

## INTRODUCTION

Primary Antiphospholipid Syndrome (P-APS) is an acquired autoimmune disorder characterized by: (1) The presence of recurrent venous or arterial thrombosis and/or gestational morbidity. (2) The presence in the blood of antiphospholipid antibodies (APL). (3) The absence of other systemic autoimmune diseases (1–4).

The autoantibodies included in the classification criteria for antiphospholipid syndrome (APS) are the presence of lupus anticoagulant, or the presence of IgG or IgM isotype antibodies directed against cardiolipin (aCL) or against B2-Glycoprotein I (aB2GP1) (5, 6).

Several authors have suggested that the assessment of new autoantibodies can help to identify the syndrome in patients with APS clinic (7–10). Among the new aPL that have been described associated with APS-events, the IgA aB2GP1 and the anti-phosphatidyl serine/prothrombin (IgG or IgM) are those where a greater association with the APS clinical aspects has been observed (11–13). The clinical relevance of IgA aB2GPI has increased progressively in recent years. In the 13th International Congress on Antiphospholipid Antibodies (2010, Galveston, TX), the task force recommended testing for the IgA aB2GPI in patients with a clinical profile suggestive of APS and negative for the aPL that are included in the APS classification criteria (11).

Our group has described an elevated prevalence of IgA aB2GP1 in patients with chronic kidney disease (CKD) vs. the general population (30 vs. 1.5%) and the association between presence of these antibodies with thrombotic events and mortality in these patients (12). This observation has also been confirmed in other cohorts (13). Patients with CDK positive for IgA aB2GP1 who received a kidney transplant showed a greater incidence of early graft loss, mainly due to thrombosis (14, 15). This fact has been confirmed in a prospective multicenter study (16).

As the presence of these antibodies was not associated with genetic factors (17), type of renal function replacement treatment (12) or base disease that caused renal failure (15), we proposed the hypothesis that misfolded B2GP1 produced in the stressed cells of an unhealthy organ could be identified as a foreign antigen (14). This hypothesis could be supported by the works of Arase et al. that demonstrated that misfolded self-proteins can be incorporated into MHC class II without previous processing and can be transported in their entire form to the cell surface (18). The same group describes the presence of complexes B2GP1- HLA class II as autoantigens in antiphospholipid syndrome (19).

B2GP1 is a plasma protein that is elaborated mainly in the liver, but also in other organs such as the kidney and the heart (20). In this way, we studied the prevalence of IgA aB2GP1 in patients with severe heart failure waiting for heart transplantation, finding a prevalence similar to that observed in CKD as well as a strong association with mortality and thrombosis after heart transplantation (21).

Although the presence of IgA aB2GP1 identifies many patients at risk of developing thrombotic events after kidney transplantation, its predictive value is too low to justify applying preventive treatments to patients who express this biomarker. Searching for new biomarkers that would allow a better identification of the population at risk, circulating immune complexes (CIC) of IgA bound to B2GP1 (B2A-CIC) were described in the blood of patients with APS symptomatology (22) and its presence was associated with the occurrence of acute thrombotic events (23).

In patients who will undergo a kidney transplant, the pretransplant presence of B2A-CIC helps to identify which patients with antiphospholipid antibodies have high risk of developing thrombosis in the first 6 months after kidney

**Abbreviations:** OR, Odds ratio; HR, Hazards ratio; CI, Confidence interval; aB2GPI, Anti-Beta-2 glycoprotein-I antibodies; aCL, Anti-cardiolipin antibodies; CIC, Circulating immune complexes; B2A-CIC, Circulating immune-complexes of IgA bounded to Beta-2 glycoprotein-I; TRB-D, Presence of a severe post-transplant output defined by thrombosis or death of the patient; IABP, Intra-Aortic Balloon Pump; ECMO, ExtraCorporeal Membrane Oxygenation.

transplantation. Patients positive for IgA aB2GP1 who were negative for B2A-CIC had a thrombosis risk similar to the general population (24).

In this work we will assess whether the presence of pre-transplant B2A-CIC allows to better identify the population with higher risk of developing mortality, cardiovascular events and thrombosis in the 24 first months after heart transplantation.

# METHODS

## Study Design

We carried out a 2-year follow-up study using the historical cohort "H12 + 8" that included all patients ($N = 153$) who had received a heart transplant over a period of 8 years (01/01/2004 to 12/31/2011) in the «Hospital 12 de Octubre» (Madrid, Spain) (21).

Aim: To determine the pretransplant prevalence of B2A-CIC in patients positive for IgA aB2GP1 and investigate their possible association with mortality, thrombosis and other cardiovascular events after the transplant.

Main endpoints: thrombosis, vascular events, death, patient survival at three and 24 months.

## Patients

A total of 151 consecutive patients who underwent heart transplantation in a period of 8 years in a single center were enrolled and studied for 24 months or until death. Two patients of the original cohort were excluded: one patient who had received two heart transplants was only included in the second transplant and a second patient lacked a pretransplant serum sample.

Presence of B2A-CIC and aPL was evaluated in the pre-transplant serum sample used for crossmatch. Three groups were formed: Group-0: Control subcohort that includes the patients negative for IgA aB2GP1 ($N = 104$). Group-1: patients positive for both: antibodies IgA aB2GP1 and presence of B2A-CIC ($N = 19$). Group-2: Patients positive for IgA aB2GP1 but negative for the presence of B2A-CIC ($N = 28$).

The disposition algorithm is described in **Figure 1**.

## Ethical Issues

The study was submitted to the Institutional Review Board (ECCR) of Hospital 12 de Octubre and received a favorable report (Reference Number CEIC-15/008). Since this was a non-interventional observational study and no genetic material was used, following Spanish regulations, informed consent was not required.

## Database

The recipient database includes pretransplant characteristics, these being age, blood type, body mass index, original disease and other associated diseases, cardiovascular risk factors (arterial hypertension, hyperlipidemia, diabetes, and smoking) and immunological data. Posttransplant characteristics included data related to donors' features, immunosuppressive treatment, incidence of thrombotic and cardiovascular events, enablers factors for thrombotic events, patient survival and causes of mortality.

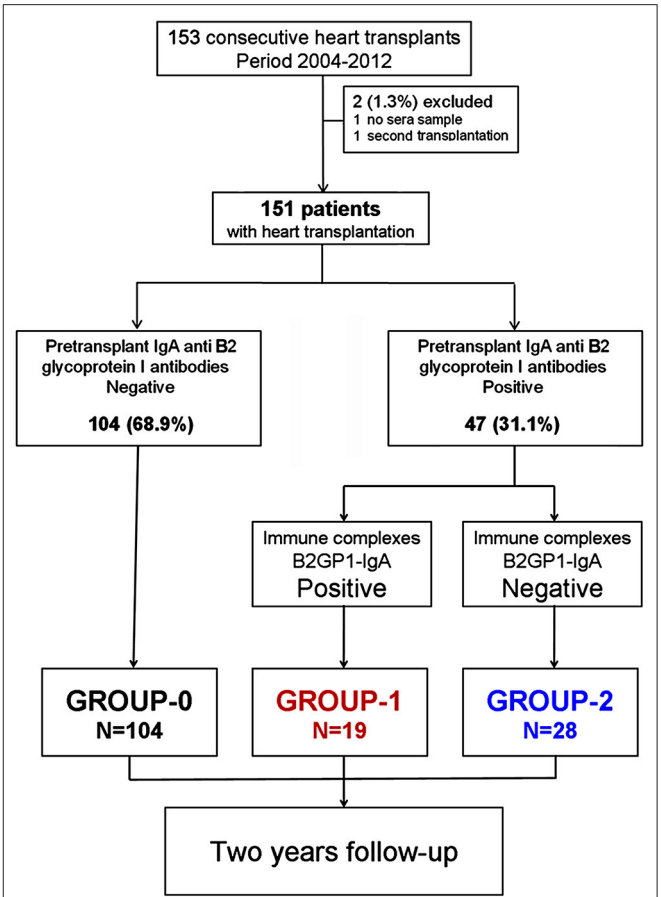


**FIGURE 1 |** Disposition and groups of study.

## Post-operative Immunosuppressive Treatment

This consists in: (1) Two intravenous bolus of basiliximab (20 mg) on days 0 and 4 after transplant. (2) Cyclosporine (CsA), 5– 8 mg/kg per day during the first year (to maintain serum CsA level within the range of 250–350 ng/mL). (3) Mofetil mycophenolate (MMF) 2–3 grams per day. (4) Steroids intravenously, methylprednisolone 500 mg, before and during surgery. After operation, 125 mg every 8 h for 3 doses. This therapy was followed by oral prednisone (1 mg/kg per day, tapered by 0.1 mg/kg on alternate days to 0.2 mg/kg per day and reduced to 0.1 mg/kg per day after 1 year).

## Definitions

Early Graft Failure (EGF): a graft endpoint produced by the death of the patient or a re-transplant associated with graft failure within the first 30 days after transplantation.

Thrombotic events: venous, arterial or intracavitary thrombosis, pulmonary thromboembolism, thrombotic microangiopathy, transient ischemic attack or acute stroke. Events were diagnosed by imaging techniques or histologic study.

Enablers for thrombotic events: mechanical support (IABP, ECMO), atrial arrhythmias, surgery and catheters, infections or sepsis.

Cardiovascular events: myocardial infarction, coronary revascularization, thrombotic events, death or re-transplant.

## Laboratory Determinations

Autoantibodies were measured in pre-transplant serum used for crossmatch or in a serum sample obtained in the month before transplantation. Anti-cardiolipin or anti-B2GPI of IgG and IgM isotypes were evaluated using the BioPLex 2200 multiplex immunoassay system (Biorad, Hercules CA, USA). Antibody levels above 18 U/mL were considered positive (99th percentile of healthy population).

IgA aCL and aBGPI antibodies were quantified by enzyme-linked immunosorbent assays (ELISA) using IgA-aCL and IgA-aB2GPI QUANTA Lite (INOVA Diagnostics Inc., San Diego, CA, USA). Antibody levels above 20 U/mL were considered positive. Cutoff were established with the 99th percentile of a healthy population in our hospital and coincided with the cutoff suggested in the assay manufacturer's guidelines (25).

Mean levels of anticardiolipin antibodies were: IgG 1.9 ± 0.2 and IgM 1.7 ± 0.3 IU/ml. Antibodies anti-B2GPI mean levels were: IgG 2.0 ± 0.4 IU/ml and IgM 1.8 ± 0.4 IU/ml. No significant differences were observed between the percentage of patients who were positive for anti-cardiolipin or anti-B2GPI (IgG/IgM isotypes) vs. healthy people. No association was found between the presence of IgG/IgM aPL and post-transplant mortality.

## Quantification of Immune Complexes B2GP1-IgA

Determination of B2A-CIC levels was performed as previously described (22). Briefly, 96 wells Nunc maxisorp™ plates (A/S Nunc, Kamstrup, Roskilde, Denmark) were coated with mouse monoclonal antibody anti-human B2GP1 H219 (Mabtech AB, Nacka Strand, Sweden) at 2 μg/mL in PBS pH 7.4 and incubated 16 h at 4°C. The coated plates were washed three times (PBS 0.1% tween 20) and blocked 30 min at room temperature with PBS + 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) (RT). Blocked plates were washed (PBS 0.1% tween 20, 3X) and patients serum diluted at 1:100 in PBS were dispensed (100 μL/well; duplicates) and incubated 2 h at room temperature. After incubation, plates were washed (PBS 0.1% tween 20, 3X) and Anti-human IgA HRP-conjugate (INOVA) was used to detect B2A-CIC. The concentration of B2A-CIC of each serum was obtained by interpolating the mean optical density values with a calibration curve. Cutoff for B2A-CIC (21UA) was established with the 99th percentile of a healthy population in our hospital. These levels were practically identical to those calculated through the analysis of ROC curves (22). All the procedures were performed in a Triturus® Analyzer (Diagnostics Grifols, S.A. Barcelona, Spain).

## Statistical Methods

Association between qualitative variables was determined with Pearson's Chi-square test or Fisher's exact test, when appropriate. Results were expressed as absolute frequency and percentage. The relative measure of an effect was expressed as odds ratio.

Prior to the comparison of scaled variables, assessment of normality distribution with Kolmogorov-Smirnov test was performed. The Student's *T*-test was used for comparisons of scaled variables that followed a normal distribution and the results were expressed as mean ± standard error. Mann-Whitney U test was used for comparisons when the outcome was not normally distributed. Results were expressed as median and interquartile range.

Patients' survival probability and incidence of events were calculated using the Kaplan-Meier method. The differences between the survival distributions were evaluated with the Log-rank test and the relative measure of a condition on survival was expressed as hazard ratio (HR).

Odds Ratio calculations and Multivariate analysis were performed using logistic regression model. Multivariate analysis of 3-months mortality-associated risk factors was also performed using logistic regression because it is a more effective model than the Cox regression for events concentrated in a short period of time (26). Adjustment of *p*-values for multiple comparisons were obtained by the false discovery rate method (27). Probabilities under 0.05 were considered significant.

Data were processed and analyzed using MedCalc for Windows version 18.9 (MedCalc Software, Ostend, Belgium) and the "R" programming language (R Foundation for Statistical Computing, Vienna, Austria) (28).

# RESULTS

## Pre-transplant Characteristics of the Three Groups

Of the patients positive for IgA aB2GP1, a total of 19 had levels of B2A-CIC above the cutoff point (Group-1), the remaining 28 were negative for the presence of B2A-CIC (Group-2). No significant differences in the pre-transplant characteristics among patients in the three Groups were found (**Table 1**).

A higher incidence of thrombocytopenia was observed in groups 1 and 2 compared to Group-0. Although the differences were initially significant, the significance was lost (not shown) after performing the adjustment for multiple comparisons.

## Post-transplant Survival Evolution

When the survival of the three groups was compared at 2 years after transplantation, a significantly higher mortality was found in Group-1 vs. Group-0 patients. This mortality was especially concentrated in the first trimester (**Figure 2**). Non-significant differences were observed in the causes of death among the 3 groups (not shown). The Kaplan Meier 2-year survival analysis for the patients in Group-1 vs. Group-0 showed a Hazard ratio (HR) of 3.82 (95% CI: 1.54–12.66). No significant differences were observed between the patients in Group-2 vs. Group-0 (HR: 1.93; 95% CI: 0.75–5.01) (**Figure 2** and **Table 1**).

When we performed the Kaplan Meier survival analysis focusing on the first 3 months after transplantation, the mortality in Group-1 vs. Group-0 was still higher than that observed at 2 years: HR 5.08 (95% CI: 1.36–19.01). Mortality of patients

**TABLE 1 |** Pre-transplant characteristics of patients on Group-1, Group-2, and Group-0 and main outcomes after transplant.

| | Group-1 *N* = 19 | | Group-2 *N* = 28 | | Group-0 *N* = 104 | | Significance | | |
|---|---|---|---|---|---|---|---|---|---|
| | *N*/median | %/IQR | *N*/median | %/IQR | *N*/median | %/IQR | G1 vs. G0 | G2 vs. G0 | G1 vs. G2 |
| Sex (female) | 4 | (21.1%) | 8 | (28.6%) | 16 | (15.4%) | N.S. | N.S. | N.S. |
| Age | 55.0 | 44.0–64.0 | 53.5 | 39.0–58.5 | 49.5 | 39.0–58.5 | N.S. | N.S. | N.S. |
| Body mass index | 23.3 | 21.1–28.6 | 23.6 | 21.4–26.5 | 24.6 | 22.4–26.4 | N.S. | N.S. | N.S. |
| Blood type | | | | | | | | | |
| Group 0 | 8 | (42.1%) | 12 | (42.9%) | 48 | (46.2%) | N.S. | N.S. | N.S. |
| Group A | 9 | (47.4%) | 13 | (46.4%) | 41 | (39.4%) | N.S. | N.S. | N.S. |
| Group B | 2 | (10.5%) | 3 | (10.7%) | 10 | (9.6%) | N.S. | N.S. | N.S. |
| Group AB | 0 | (0%) | 0 | (0%) | 5 | (4.8%) | N.S. | N.S. | N.S. |
| Rh positive | 18 | (94.7%) | 25 | (89.3%) | 85 | (81.7%) | N.S. | N.S. | N.S. |
| Causes of heart dysfunction | | | | | | | | | |
| Ischemic | 7 | (36.8%) | 10 | (35.7%) | 27 | (26%) | N.S. | N.S. | N.S. |
| Idiopathic | 6 | (31.6%) | 7 | (25%) | 45 | (43.3%) | N.S. | N.S. | N.S. |
| Restrictive | 0 | | 4 | (14.3%) | 5 | (4.8%) | N.S. | N.S. | N.S. |
| Valvular | 4 | (21.1%) | 1 | (3.6%) | 6 | (5.8%) | N.S. | N.S. | N.S. |
| Others | 2 | (10.5%) | 6 | (21.4%) | 21 | (16.4%) | N.S. | N.S. | N.S. |
| Pathologies and risk factors | | | | | | | | | |
| Renal dysfunction | 3 | (15.8%) | 4 | (14.3%) | 21 | (20.2%) | N.S. | N.S. | N.S. |
| HTA antecedents | 4 | (21.1%) | 6 | (21.4%) | 33 | (31.7%) | N.S. | N.S. | N.S. |
| Diabetes | 1 | (5.3%) | 6 | (21.4%) | 25 | (24%) | N.S. | N.S. | N.S. |
| Dyslipidemia | 7 | (36.8%) | 9 | (32.1%) | 33 | (25.8%) | N.S. | N.S. | N.S. |
| Hyperuricemia | 3 | (15.8%) | 1 | (3.6%) | 14 | (13.5%) | N.S. | N.S. | N.S. |
| Hyperbilirubinemia | 2 | (10.5%) | 7 | (25%) | 25 | (24%) | N.S. | N.S. | N.S. |
| ALT/AST High levels | 3 | (15.8%) | 4 | (14.3%) | 30 | (28.8%) | N.S. | N.S. | N.S. |
| Active smoker | 4 | (21.1%) | 7 | (25%) | 24 | (23.1%) | N.S. | N.S. | N.S. |
| Ex-smoker | 4 | (21.1%) | 8 | (28.6%) | 29 | (27.9%) | N.S. | N.S. | N.S. |
| No-smoking | 11 | (57.9%) | 13 | (46.4%) | 51 | (49%) | N.S. | N.S. | N.S. |
| Patients with thrombotic antecedents* | 0 | | 1 | (2.1%) | 6 | (5.8%) | N.S. | N.S. | N.S. |
| Deep venous thrombosis | 0 | | 1 | (2.1%) | 3 | (2.9%) | N.S. | N.S. | N.S. |
| Pulmonary embolism | 0 | | 1 | (2.1%) | 4 | (3.8%) | N.S. | N.S. | N.S. |
| Previously anticoagulated | 13 | (68.4%) | 15 | (53.6%) | 56 | (53.8%) | N.S. | N.S. | N.S. |
| Other vascular diseases | | | | | | | | | |
| Thrombosis A/V | 0 | | 1 | (3.6%) | 8 | (7.7%) | N.S. | N.S. | N.S. |
| Thrombophebitis | 0 | | 0 | | 2 | (1.9%) | N.S. | N.S. | N.S. |
| Ethnicity Caucasian | 18 | (94.7%) | 27 | (97.4%) | 100 | (96.2%) | N.S. | N.S. | N.S. |
| Ethnicity: others | 1 | (5.3%) | 1 | (2.6%) | 4 | (3.8%) | N.S. | N.S. | N.S. |
| Post-transplant main outcomes | | | | | | | | | |
| Dead in 2 years | 8 | (42.1%) | 7 | (25%) | 14 | (13.5%) | $P = 0.009$ | N.S. | N.S. |
| Dead in 3 months | 8 | (42.1%) | 5 | (17.9%) | 10 | (9.6%) | $P = 0.006$ | N.S. | N.S. |
| Dead from month 4–24 | 0 | | 2 | (9% of 23) | 4 | (4% of 94) | N.S. | N.S. | N.S. |
| Patients with thrombotic events* | 10 | (52.6%) | 13 | (46.4%) | 27 | (26.0%) | $P < 0.001$ | $P = 0.060$ | N.S. |
| Intracavitary thrombus | 4 | (21.1%) | 2 | (7.1%) | 0 | (0%) | $P < 0.001$ | N.S. | N.S. |
| Stroke | 3 | (15.8%) | 2 | (7.1%) | 3 | (2.9%) | N.S. | N.S. | N.S. |
| Deep venous thrombosis | 0 | (0%) | 0 | (0%) | 2 | (1.9%) | N.S. | N.S. | N.S. |
| Pulmonary embolism | 0 | (0%) | 1 | (3.6%) | 1 | (1%) | N.S. | N.S. | N.S. |
| Arterial Thrombosis | 1 | (5.3%) | 1 | (3.6%) | 1 | (1%) | N.S. | N.S. | N.S. |
| Patients with thrombosis or dead | 14 | (73.7%) | 11 | (39.3%) | 17 | (16.3%) | $P < 0.001$ | $P = 0.013$ | $P = 0.020$ |

*IQR, interquartile range; G1, Group 1; G2, Group 2; G0, Group 0; N.S., not significant. *Some patients have more than one event. P-values were adjusted for multiple comparisons.*

in Group-1 was higher than in Group-2 (HR: 2.65) but these differences were not significant (HR 95% CI: 0.56–12.54). After the first trimester (from the fourth month to the end of the second year), no significant differences were observed in the survival among the three groups (**Table 1**).

When we compared the differences between deceased patients in the first trimester vs. those who survived (**Table 2**), we found that there was a significantly higher proportion of women, blood type group A and positivity of B2A-CIC in the dead patients. No significant differences were observed in the rest of the

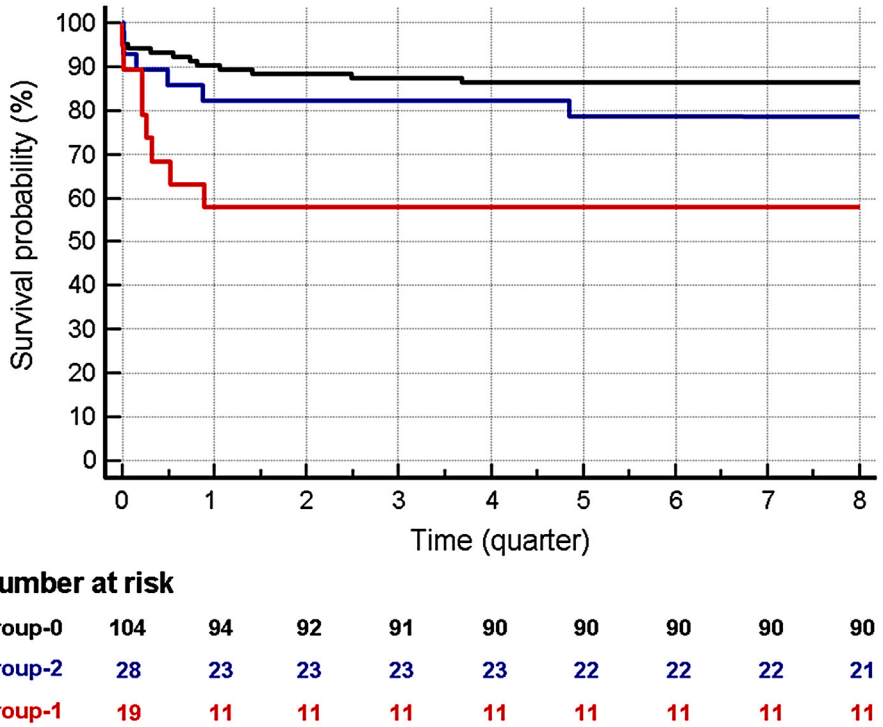


**FIGURE 2 |** Survival at 2 years in patients in the three groups. Red: Group-1. Blue: Group-2. Dark: Group-0. The time is indicated in quarters. Mortality in group-1 is higher than in group-0, both at 3 months (HR:5.08; 95% CI: 1.36–19.01) and at 2 years (HR: 3.82; 95% CI: 1.15–12.66). No significant differences were observed between patients in Group-2 vs. Group-0 both at 3 and 24 months.

characteristics, including the proportion of patients who were positive for IgA aB2GP1 but negative for B2A-CIC.

When these factors associated with mortality were subjected to logistic regression multivariate analysis, it was found that the presence of B2A-CIC (OR: 6.13; 95% CI: 1.93–19.40), blood type A (OR: 4.05; 95% CI: 1.44–11.43) and female gender (OR: 4.18; 95% CI: 1.35–12.94) were independently associated with early mortality (**Table 2**).

## Post-transplant Thrombotic Events

Thrombotic events were only observed in the first trimester after transplantation. After this period, no event consistent with the APS clinical classification criteria were observed. The Group-1 had a significantly higher incidence of thrombotic events than Group-0 (OR: 9.53; 95% CI: 2.75–33.07). No significant differences were observed in patients in Group-2 vs. Group-0 or Group-1 (**Table 1**). The post-transplant thrombosis observed most was intracavitary Cardiac thrombus with a significantly higher incidence in Group-1 vs. Group-0 (OR: 60.7; 95% CI: 3.12–1182).

Patients who suffered thrombotic events in the first 3 months compared with patients who did not suffer these events (**Table 3**) had a significantly higher proportion of B2A-CIC positive patients (OR: 6.42; 95% CI: 2.10–19.63) and pre-transplant active smokers (OR: 3.14; 95% CI: 1.13–8.72). No significant differences were observed in the remaining clinical characteristics (**Table 3**). In the multivariate analysis, it was confirmed that the pre-transplant presence of B2A-CIC (OR: 7.52; 95% CI: 2.30–24.56) and the habit of smoking actively (OR: 3.78; 95% CI: 1.25–11.41) were independently associated with incident of thrombotic events in the first trimester alter heart transplantation (**Table 3**).

## Patients Suffering Thrombosis or Death

If we consider grouping early outcomes (thrombosis and death in the first 3 months) as a new variable: thrombosis or death (TRB-D), 73.7% of patients in Group-1 suffer from TRB-D. This proportion is significantly higher than the 16.3% observed in Group-0 (OR: 15.40; 95% CI: 4.87–48.72) and that 39.3% of Group-2 (OR: 5.04; 95% CI: 14.0–18.14) (**Table 1**).

The Kaplan Meier survival analysis (**Figure 3A**) confirms that the incidence of TRB-D was significantly higher in Group-1 vs. Group-0 (HR: 6.29; 95% CI: 2.22–12.79) and also in Group-2 vs. Group-0 (HR: 2.64; 95% CI: 1.15–6.07).

Evaluating the characteristics of the 40 patients who suffered TRB-D during the first trimester post-transplant and comparing them with the 111 who remained alive and without events, we found a significantly higher presence of positive B2A-CIC (OR: 11.42; 95% CI: 3.77–34.55), pre-transplant active smokers (OR: 2.09; 95% CI: 1.00–4.34) and blood type A (OR: 2.31; 95% CI: 1.03–5.16) (**Table 4**).

A multivariate analysis was carried out with the three factors associated significantly with TRB-D in the univariate analysis and we also added gender. Although gender did not show an

**TABLE 2 | (A)** Pre-transplant characteristics of patients who died in the first trimester after heart transplantation vs. alive patients. **(B)** Logistic regression multivariate analysis of factors associated with mortality in the first trimester after heart transplantation.

| **(A) Pre-transplant characteristics** | **Dead *N* = 23** | | **Alive *N* = 128** | | ***P*-value** |
|---|---|---|---|---|---|
| **Variable** | ***N*/median** | **%/IQR** | ***N*/median** | **%/IQR** | |
| Sex (female) | 8 | (34.8%) | 20 | (15.6%) | 0.030 |
| Age | 52 | 42.3–61.0 | 51 | 39.0–58.5 | N.S. |
| Body mass index | 23.7 | 20.7–27.9 | 24.5 | 22.0–26.6 | N.S. |
| IgA aB2GP1 positive and B2A-CIC positive | 8 | (34.8%) | 11 | (8.6%) | <0.001 |
| IgA aB2GP1 positive and B2A-CIC negative | 5 | (21.7%) | 23 | (18%) | N.S. |
| Blood type | | | | | |
| Group 0 | 7 | (30.4%) | 61 | (47.7%) | N.S. |
| Group A | 15 | (65.2%) | 48 | (37.5%) | 0.013 |
| Group B | 1 | (4.3%) | 14 | (10.9%) | N.S. |
| Group AB | 0 | (0%) | 5 | (3.9%) | N.S. |
| Rh positive | 21 | (91.3%) | 107 | (83.6%) | N.S. |
| Other pathologies and risk factors | | | | | |
| Renal dysfunction | 5 | (21.7%) | 23 | (18%) | N.S. |
| HTA antecedents | 5 | (21.7%) | 38 | (29.7%) | N.S. |
| Diabetes | 3 | (13%) | 29 | (22.7%) | N.S. |
| Dyslipidemia | 8 | (34.8%) | 41 | (32%) | N.S. |
| Hyperuricemia | 1 | (4.3%) | 17 | (13.3%) | N.S. |
| Hyperbilirubinemia | 9 | (39.1%) | 25 | (19.5%) | N.S. |
| ALT/AST High levels | 3 | (13%) | 34 | (26.6%) | N.S. |
| Active smoker | 7 | (30.4%) | 28 | (21.9%) | N.S. |
| Ex-smoker | 5 | (21.7%) | 36 | (28.1%) | N.S. |
| No-smoking | 11 | (47.8%) | 64 | (50%) | N.S. |
| Patients with thrombotic antecedents | 1 | (4.3%) | 6 | (5.8%) | N.S. |
| Previously anticoagulated | 15 | (65.2%) | 69 | (53.9%) | N.S. |
| Other vascular diseases | | | | | |
| Thrombosis A/V | 1 | (4.3%) | 8 | (6.3%) | N.S. |
| Thrombophlebitis | 0 | (0%) | 2 | (1.6%) | N.S. |
| Ethnicity Caucasian | 22 | (95.7%) | 123 | (96.1%) | N.S. |
| Ethnicity: others | 1 | (4.3%) | 5 | (3.9%) | N.S. |

| **(B) Multivariate analysis** | **Univariate** | | **Multivariate** | | |
|---|---|---|---|---|---|
| **Variable** | **OR** | **95% CI OR** | **OR** | **95% CI OR** | ***P*-value** |
| B2A-CIC positive | 5.67 | 1.97–16.33 | 6.13 | 1.93–19.40 | 0.002 |
| Sex (female) | 2.88 | 1.08–7.67 | 4.18 | 1.35–12.94 | 0.013 |
| Blood type: Group A | 3.13 | 1.23–7.92 | 4.05 | 1.44–11.43 | 0.008 |

*IQR, interquartile range; N.S., not significant.*

association with TRB-D in the univariate analysis, it was included because gender was previously independently associated with early death.

The pre-transplant habit of smoking actively (OR: 3.92; 95% CI: 1.29–11.9) and the presence of B2A-CIC (OR: 7.75; 95% CI: 2.35–25.61) were the only independent risk factors for the appearance of death or thrombotic events in the first trimester after transplantation (**Table 5**).

## Influence of Post-transplant Risk Factors for Thrombotic Events

After transplantation, a total of 50 patients (33%) showed risk factors for the appearance of thrombotic events that were not present before the transplant. The proportion of patients with these factors was somewhat higher in patients in groups 1 and 2 compared with Group-0 (**Supplementary Figure 1**), although these differences were not found to be significant after a statistical study of multiple comparisons was conducted (**Supplementary Table 1**).

Kaplan Meier survival analysis of the incidence of TRB-D in patients who were not exposed to risk factors (**Figure 3B**) showed that Group-1 patients (B2A-CIC positive) have a significantly higher risk of developing TRB-D events in the first trimester vs. patients of Group-0 (HR 9.03; 95% CI: 1.28–63.65). No significant differences were observed between patients in Group-2 and those in Group-0 (HR: 1.46; 95% CI: 0.35–6.05).

In the Kaplan Meier survival analysis of patients with risk factors (**Figure 3C**), the differences of incidence of TRB-D in Group-1 vs. Group-O group were significant (HR: 3.27; 95% CI: 1.11–9.64) but lower than in the patients without additional risk

**TABLE 3 | (A)** Pre-transplant characteristics of patients who suffer thrombotic events in the first trimester after heart transplant vs. those without these events. **(B)** Logistic regression multivariate analysis of pre-transplant factors associated with incidence of thrombotic events in the first 3 months after heart transplantation.

| **(A) Pre-transplant characteristics** | **Patients with events *N* = 18** | | **Patients without events *N* = 133** | | ***P*-value** |
|---|---|---|---|---|---|
| **Variable** | ***N*/median** | **%/IQR** | ***N*/median** | **%/IQR** | |
| Sex (female) | 3 | (16.7%) | 25 | (18.8%) | N.S. |
| Age | 50.5 | 40.0–58.0 | 52 | 39.0–59.0 | N.S. |
| Body mass index | 25.5 | 20.9–29.2 | 24.4 | 22.1–26.4 | N.S. |
| **IgA aB2GP1 + and B2A-CIC positive** | 7 | (38.9%) | 12 | (9%) | <0.001 |
| IgA aB2GP1 + and B2A-CIC negative | 5 | (27.8%) | 23 | (17.3%) | N.S. |
| Blood type | | | | | |
| Group 0 | 9 | (50%) | 59 | (44.4%) | N.S. |
| Group A | 7 | (38.9%) | 56 | (42.1%) | N.S. |
| Group B | 1 | (5.6%) | 14 | (10.5%) | N.S. |
| Group AB | 1 | (5.6%) | 4 | (3%) | N.S. |
| Rh positive | 16 | (88.9%) | 112 | (84.2%) | N.S. |
| Pathologies and risk factors | | | | | |
| Renal dysfunction | 2 | (11.1%) | 26 | (19.5%) | N.S. |
| HTA antecedents | 5 | (27.8%) | 38 | (28.6%) | N.S. |
| Diabetes | 2 | (11.1%) | 30 | (22.6%) | N.S. |
| Dyslipidemia | 7 | (38.9%) | 42 | (31.6%) | N.S. |
| Hyperuricemia | 1 | (5.6%) | 17 | (12.8%) | N.S. |
| Hyperbilirubinemia | 1 | (5.6%) | 33 | (24.8%) | N.S. |
| ALT/AST High levels | 7 | (38.9%) | 30 | (22.6%) | N.S. |
| Active smoker | 8 | (44.4%) | 27 | (20.3%) | 0.023 |
| Ex-smoker | 4 | (22.2%) | 37 | (27.8%) | N.S. |
| No-smoking | 6 | (33.3%) | 69 | (51.9%) | N.S. |
| Patients with thrombotic antecedents | 1 | (5.6%) | 6 | (4.5%) | N.S. |
| Previously anticoagulated | 9 | (50%) | 75 | (56.4%) | N.S. |
| Other vascular diseases | | | | | |
| Thrombosis A/V | 2 | (11.1%) | 7 | (5.3%) | N.S. |
| Thrombophlebitis | 0 | | 2 | (1.5%) | N.S. |
| Ethnicity Caucasian | 16 | (88.9%) | 129 | (97%) | N.S. |
| Ethnicity: others | 2 | (11.1%) | 4 | (3%) | N.S. |

| **(B) Multivariate analysis** | **Univariate** | | **Multivariate** | | |
|---|---|---|---|---|---|
| **Variable** | **OR** | **95% CI OR** | **OR** | **95% CI OR** | ***P*-value** |
| B2A-CIC positive | 6.42 | 2.10–19.63 | 6.13 | 1.93–19.40 | 0.002 |
| Active smoker | 3.14 | 1.13–8.72 | 4.18 | 1.35–12.94 | 0.013 |

*IQR, interquartile range; N.S., not significant.*

factor. No significant differences were found between Group-2 vs. Group-0 (HR: 2.43; 95% CI: 0.92–6.43) and between Group-1 and Group-2 (HR: 1.30; 95% CI: 0.37–4.59).

The multivariate analysis including pre-transplant and post-transplant risk factors associated significantly with the occurrence of TRB-D events in the univariate analysis (**Table 5**) demonstrated that the presence of B2A-CIC is the main independent risk factor for the appearance of TRB-D in the three 3 months after cardiac transplantation (OR: 13.13; 95% CI: 3.80–45.36).

The presence of post-transplant risk factors (OR: 5.26; 95% CI: 2.19–12.64) and the pre-transplant smoking habit (OR: 2.65; 95% CI: 1.01–6.94) also behaved as independent risk factors for TRB-D. The area under the ROC curve of this multivariate analysis was 0.818 (95% CI: 0.747–0.876).

## DISCUSSION

In this work, for the first time we have described that the pre-transplant presence of B2A-CIC identifies a subgroup of patients prone to develop the worst outcomes after heart transplant: death and thrombotic events.

The presence of aPL is not enough to trigger a thrombotic event. Additional factors that involve the activation of the mechanisms of innate immunity in the context of processes such as infection or surgery need to converge. It is what is known as

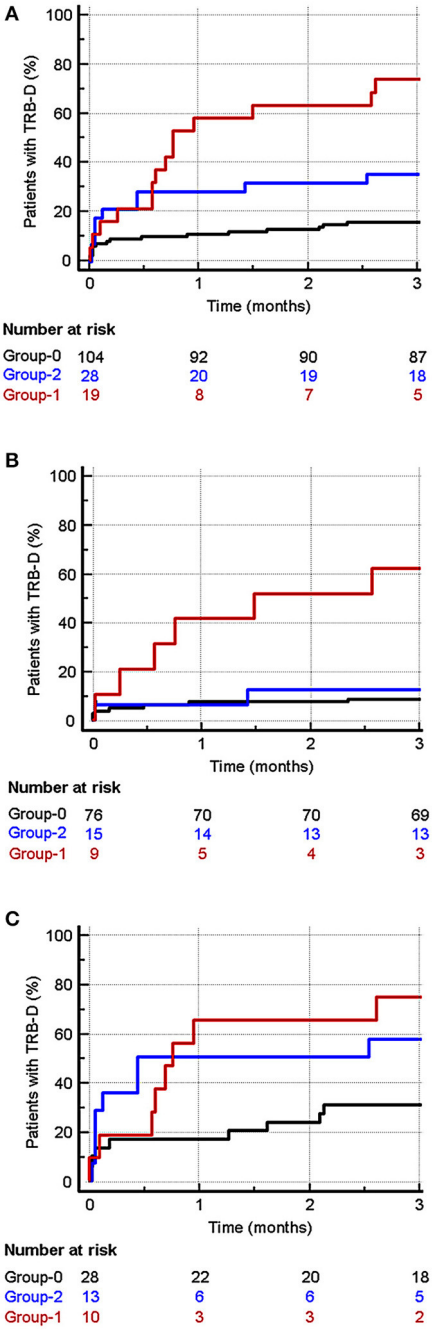


**FIGURE 3 |** Incidence of TRB-D (thrombotic events or death) in the first 3 months after heart transplantation. Red: Group-1. Blue: Group-2. Dark: Group-0. **(A)** Considering all the patients, the incidence was significantly higher in Group-1 vs. Group-0 (HR: 6.29; 95% CI: 2.22–12.79) and also in Group-2 vs. Group-0 (HR: 2.64; 95% CI: 1.15–6.07). **(B)** In patients without post-transplant risk factors for thrombosis, the incidence of TRB-D in the first trimester was significantly higher in Group-1 patients (B2A-CIC positive) vs. patients of Group-0 (HR 9.03; 95% CI: 1.28–63.65). No significant differences were observed between patients in Group-2 and those in Group-0 (HR: 1.46; 95% CI: 0.35–6.05). **(C)** In patients with risk factors for thrombosis, a significant higher incidence of TRB-D in Group-1 vs. Group-0 (HR: 3.27; 95% CI: 1.11–9.64) can also be observed. No significant differences were found between Group-2 vs. Group-0 (HR: 2.43; 95% CI: 0.92–6.43) and between Group-1 and Group-2 (HR: 1.30; 95% CI: 0.37–4.59).

the hypothesis of the two hits to provoke the event: the presence of aPL would be a "first hit" and the concurrent factors would be the "second hit" (29).

The follow-up of patients who are carriers of antiphospholipid antibodies (first hit) and who will undergo transplant surgery (second hit) is an excellent tool to study primary APS because it allows us to have extensive clinical information and serum samples from the stage prior to the triggering of the thrombotic event.

Most patients with primary APS are studied after the event appears. The high prevalence of aPL (first "hit") in patients with end-stage renal disease or severe heart failure, together with their exposure to transplant surgery (a well-known "second hit"), triggers that a high percentage of these patients will have thrombotic events after transplantation. To perform a similar study with patients of the general population, where the prevalence of aPL is ∼2% and the incidence of events/year in aPL carriers is 3% (30–33), it would be necessary to follow-up ∼40,000 individuals for 1 year.

## Pre-transplant Markers and Mortality

The presence of B2A-CIC allows us to identify which IgA aB2GP1 patients have the highest mortality and that the survival in patients B2A-CIC negative does not differ from that observed in the control group. Other independent risk factors for early mortality after heart transplantation are the sex (women) and the positivity of the blood group A.

Female gender is a known risk factor for early mortality in heart transplant patients: in the 23rd official adult heart transplantation report (2006) of the International Registry of Heart Transplantation for adult patients, female gender was associated with higher mortality (34). Although this fact has been attributed to a higher incidence of acute graft failure, the mechanisms responsible for these gender-based differences have yet to be understood.

The presence of group A as a risk factor is a different way of seeing the real fact: non-O blood group patients are at more risk for thrombotics events (35). The greatest risk for patients in groups AB and B could not be demonstrated due to the small sample size. The group of patients have a lower risk of developing thrombotic events which has been attributed to have lower factor VIII and von Willebrand factor blood levels. (36, 37).

## Patients With Thrombotics Events

In this work, we have shown that patients who are positive for IgA aB2GP1 and also present B2A-CIC are those having the highest risk of thrombotic events when they are subjected to a situation capable of triggering the occurrence of thrombotic events such as transplant surgery. The risk of those who are negative for B2A-CIC is only a little higher than that found in the control group.

In the first weeks after kidney transplantation, the most serious thrombotic complication is graft thrombosis, which involves graft loss in the vast majority of patients. In the case of heart transplantation, intracavitary thrombosis, or thrombosis in large vessels can be identified and treated, however multiple thrombosis of small vessels are less evident and can lead to organ

**TABLE 4 | (A)** Pre-transplant characteristics of patients who suffer both, thrombotic events or death (TRB-D) in the first trimester after heart transplant vs. patients who are alive and without thrombotic events at the end of this period. **(B)** Logistic regression multivariate analysis of factors associated with incidence of TRB-D in the first 3 months after heart transplantation.

| **(A) Pre-transplant characteristics** | **Death or events** ***N*** **= 40** | | **Live and without events** ***N*** **= 111** | | ***P*-value** |
|---|---|---|---|---|---|
| **Variable** | ***N*/median** | **%/IQR** | ***N*/median** | **%/IQR** | |
| Sex (female) | 10 | (25%) | 18 | (16.2%) | N.S. |
| Age | 51.5 | 40–61 | 50.0 | 39–58.8 | N.S. |
| Body mass index | 23.9 | 20.8–28.1 | 24.5 | 22.3–26.4 | N.S. |
| **IgA aB2GP1 + and B2A-CIC positive** | 14 | (35%) | 5 | (4.5%) | <0.001 |
| IgA aB2GP1 + and B2A-CIC negative | 10 | (25%) | 18 | (16.2%) | N.S. |
| Blood type | | | | | |
| Group 0 | 15 | (37.5%) | 53 | (47.7%) | N.S. |
| Group A | 22 | (55%) | 41 | (36.9%) | N.S. |
| Group B | 2 | (5%) | 13 | (11.7%) | N.S. |
| Group AB | 1 | (2.5%) | 4 | (3.6%) | N.S. |
| Rh positive | 37 | (92.5%) | 91 | (82%) | N.S. |
| Pathologies and risk factors | | | | | |
| Renal dysfunction | 7 | (17.5%) | 21 | (18.9%) | N.S. |
| HTA antecedents | 10 | (25%) | 33 | (29.7%) | N.S. |
| Diabetes | 5 | (12.5%) | 27 | (24.3%) | N.S. |
| Dyslipidemia | 15 | (37.5%) | 30 | (30.6%) | N.S. |
| Hyperuricemia | 2 | (5%) | 16 | (14.4%) | N.S. |
| Hyperbilirubinemia | 10 | (25%) | 24 | (21.6%) | N.S. |
| ALT/AST High levels | 10 | (25%) | 27 | (24.3%) | N.S. |
| Active smoker | 14 | (35%) | 21 | (18.9%) | N.S. |
| Ex-smoker | 9 | (22.5%) | 32 | (28.8%) | N.S. |
| No-smoking | 17 | (42.5%) | 58 | (52.3%) | N.S. |
| Patients with thrombotic antecedents | 2 | (5.0%) | 5 | (4.5%) | N.S. |
| Pre transplant prophylactic anticoagulation | 23 | (57.5%) | 61 | (55%) | N.S. |
| Other vascular diseases | | | | | |
| Thrombosis A/V | 3 | (7.5%) | 6 | (5.4%) | N.S. |
| Thrombophlebitis | 0 | | 2 | (1.8%) | N.S. |
| Ethnicity Caucasian | 37 | (92.5%) | 108 | (97.3%) | N.S. |
| Ethnicity: others | 3 | (7.5%) | 3 | (2.7%) | N.S. |

| **(B) Multivariate analysis** | **Univariate** | | **Multivariate** | | |
|---|---|---|---|---|---|
| **Variable** | **OR** | **95% CI OR** | **OR** | **95% CI OR** | ***P*-value** |
| IgA aB2GP1 positive and B2A-CIC positive | 11.42 | 3.77–34.55 | 13.25 | 4.14–42.36 | <0.001 |
| Blood type: Group A | 2.09 | 1.00–4.34. | 2.27 | 0.98–5.26 | 0.056 |
| Active smoker | 2.31 | 1.03–5.16 | 2.58 | 1.05–6.31 | 0.038 |
| Sex (female) | 0.72 | 0.72–4.13 | 1.99 | 0.72–5.52 | 0.185 |

*IQR, interquartile range; N.S., not significant.*

failure. Since the heart is a unique organ essential for life, the graft loss necessarily implies the death of the patient. Thus, as in kidney transplantation, the presence of B2A-CIC in the heart transplant is also strongly associated with the graft loss, graft loss being considered the death of patients in the first 3 months after transplantation. This work confirms and validates the described previously conclusion with kidney transplant patients that the presence of B2A-CIC in patients positive for IgA aB2GPI is associated with thrombotic risk. It also confirms the observation that patients positive for IgA aB2GPI who are negative for B2A-CIChave the same risk of developing APS events as patients who are negative for anti-B2GP1 antibodies (24).

Mortality and development of thrombotic events after transplantation are multifactorial processes involving several risk factors present mainly before transplantation and also others that arise after transplantation (such as multiple surgery, mechanical support and infections). The post-transplant evolution in patients receiving a heart transplant is more complex than in

**TABLE 5 |** Logistic regression multivariate analysis ($p < 0.0001$) of pre-transplant and post-transplant predisposing factors associated to TRB-D (thrombosis or death) in the first 3 months after heart transplantation.

| | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| **Variable** | **OR** | **95% CI OR** | ***P*-value** | **OR** | **95% CI OR** | ***P*-value** |
| IgA B2GP1 positive and B2A-CIC positive | 11.42 | 3.77–34.55 | <0.001 | 13.13 | 3.80–45.36 | <0.001 |
| Blood type: Group A | 2.09 | 1.00–4.34. | 0.049 | 1.98 | 0.83–4.73 | 0.122 |
| Pre-transplant active smoker | 2.31 | 1.03–5.16 | 0.042 | 2.65 | 1.01–6.94 | 0.047 |
| Post-transplant predisposing factors | 5.45 | 2.51–11.84 | <0.001 | 5.26 | 2.19–12.64 | <0.001 |

those who undergo kidney transplantation. The failures in graft function in renal transplantation that may arise in the first post-transplant moments can be easily treated with renal function replacement methods (dialysis) that involves a low degree of intervention. However, the appearance of complications with functional repercussion in the heart transplant as well as the use of invasive methodologies (surgery, catheterization, mechanical ventilation, impulse balloons, etc.) to control these situations have repercussions in all the circulatory tree that favor the appearance of thrombotic events. These situations behave as enhancers of thrombotic activity (post-transplant risk factors).

The incidence of TRB-D events in the first trimester in patients without these post-transplant risk factors is very similar to that observed in the first trimester in renal transplants: B2A-CIC negative barely distinguish themselves from the control group (24). However, in patients where these factors are present, although the incidence of TRB-D events in the B2A-CIC positive is still significantly higher, the incidence of TRB-B triples in group-0 and quadruples in group-2.

This clear lower influence of the enhancers in patients with B2A-CIC is due to the fact that most of these patients are already in a pro-thrombotic state and, therefore, the population susceptible to being influenced by these factors is clearly much lower.

## Predictive Value of APL

The predictive value of the aPL presence for occurrence of APS events is low: only a small proportion of patients positive for aPL included in the classification criteria develop thrombotic events (38). The determination of B2A-CIC in carriers of IgA aB2GP1 makes it possible to discard those with a similar risk to the general population (CIC negative) and to focus attention on those with greater risk (B2A-CIC positive) as well as to establish preventive treatments depending on the remaining clinical characteristics.

The value of extra-criteria aPL is on the rise. Its study has allowed us to recognize that there is no simple mechanism by which obstetric complications are triggered in the pathogenesis of PHC but rather diverse pathologic pathways (TLR, NADPH-ox, LRP8) are being linked to aPL (39, 40). In this way, a new pathophysiological pathway of APL is studied through the presence of immune complexes of B2GP1 and aPL (IgG, IgM, or IgA isotype) and its association with acute events (23) and with extra-criteria manifestations of APS (41).

The pathogenic mechanisms and the biological significance of the presence of CIC are not known. In the majority of patients with antiphospholipid antibodies there is a contradictory situation from the point of view of classical immunology: B2GP1 (the antigen) is a relatively abundant blood protein that circulates and shares space and time with the antibody that recognizes it.

Although protein and antibody are present simultaneously in the blood, they paradoxically do not interact or produce pathological situations in most patients, this only occurring in a minority.

Our group initially proposed a possible explanation as to why there are patients with immunocomplexes and high risk of thrombosis and patients without immune complexes and at low risk the existence of aPL with different affinities for B2GP1 in each patient. Patients with high affinity antibodies would be those who are able to form CIC. The presence of CIC would indicate that the aPL are of high affinity and therefore more pathogenic (22). However, this hypothesis does not explain the observation that the presence of CICs vary throughout the evolution of the disease while the antibody titers remain stable (23).

Based on the work of Agar et al. who describe that anti-B2GP1antibodies can bind to fish-hook shape of B2GP1 but do not bind to the circular shape (42), we propose that the variation in affinity would depend on the antigen (B2GP1). The variations in the antigen affinity for the antibody would be conditioned by the exposure of cryptic epitopes that would not be accessible under physiological conditions ("O" Shape of B2GP1). The epitopes would be accessible after changes in the tertiary structure after the "activation" of the B2GP1 molecule in the context of a defensive response, however the validity of this hypothesis must be tested in subsequent studies.

This study has several limitations notwithstanding the experience of 8 years of consecutive heart transplants. It is a single-center study and the number of patients in small. Therefore, multicenter studies with a higher number of patients are mandatory to confirm these findings and to establish the most appropriate therapeutic approach for these patients. At present and based on our knowledge up to now, we could consider that a prophylactic heparin treatment and an exhaustive vigilance in the B2A-CIC positive patients during the first 3 months after transplantation could help to ameliorate the mortality and thrombotic complications.

In summary, the main finding of this study is that 40% of patients with IgA aB2GP1 also present B2A-CIC. Approximately 74% of B2A-CIC suffer thrombotic events or death after heart transplant, so the positivity of this biomarker identifies patients with higher risk of mortality and incidence of thrombotic events

after heart transplantation. There were no significant differences in mortality and incidence of thrombosis between patients who do not have B2A-CIC and those who are negative for aPL of class IgA.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

## ETHICS STATEMENT

This study was submitted to the Institutional Review Board (ECCR) of Hospital 12 de Octubre and received a favorable report (Reference Number CEIC-15/008). Since this was a non-interventional observational study and no genetic material was used, following Spanish regulations, informed consent was not required.

## AUTHOR CONTRIBUTIONS

AS and MS conceived the project, designed the research, and wrote the manuscript. LM and JD were responsible for the patients' care and clinical data collection. MS and JM-F performed the determinations of antiphospholipid antibodies and quantification of immune complexes. EM, DP, and OC-M processed the data from the transplant waiting list, integrated the information with the clinical history, and reviewed the integrity of all the data. MS and LM were responsible for the database. AS and MS carry out the statistical analysis. All authors contributed to the data interpretation, discussed the results, reviewed the manuscript, and agreed with the final version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02891/full#supplementary-material

**Supplementary Figure 1 |** Incidence of TRB-D (thrombosis or death) in the 3 first months after transplantation in function of post-transplant risk factors (enhancer for thrombotic activity). Gray: patients with enhancer factors. White: patients without enhancer factors.

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