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Complement activation in the plasma and placentas of women with different subsets of antiphospholipid syndrome

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Abstract

Problem: As antiphospholipid antibody-positive women with adverse pregnancy outcomes have higher plasma complement activation product levels, and the placentas of women with antiphospholipid syndrome (APS) exhibit C4d complement component deposition, complement activation involvement has been hypothesized in APS pregnancy complications.

Method of study: Plasma levels of C5a and C5b-9 complement components of 43 APS non-pregnant patients and 17 pregnant APS women were measured using enzyme-linked immunosorbent assay. The results were compared with those of 16 healthy non-pregnant women and eight healthy pregnant women, respectively. Placenta samples of five APS patients at high risk of pregnancy complications and of five healthy controls were subjected to immunoblotting analysis with specific antibodies to C5b-9 and CD46, CD55, CD59 complement regulators.

Results: The mean plasma C5a and C5b-9 levels were significantly higher in the non-pregnant APS patients with previous thrombosis ± pregnancy morbidity (P = .0001 and P = .0034, respectively) and in the pregnant APS women with adverse outcomes (P = .0093 for both). Similarly, C5b-9 amounts were significantly higher in the adverse pregnancy outcome placenta (P = .0115) than in those associated to a favorable outcome. The mean CD46, CD55 and CD59 amounts were, instead, lower, although not always significantly, in the placentas of all the high-risk APS women with respect to the control placentas.

Conclusion: Data analysis demonstrated that there was significant complement activation in the more severe subset of APS patients and in only the adverse pregnancy outcome APS women. Further studies will clarify whether the lower CD46, CD55, and CD59 expressions in the APS placentas are limited to only high-risk APS patients.

KEYWORDS

antiphospholipid syndrome, complement system, obstetric antiphospholipid syndrome, pregnancy complications, pregnancy outcome

Cinzia Scambi and Sara Ugolini should be considered joint first author.

1 | INTRODUCTION

Antiphospholipid syndrome (APS) is an autoimmune disease characterized by thrombosis and/or pregnancy morbidity associated to the presence in the blood of antiphospholipid antibodies (aPL), such as anticardiolipin, anti-ß2glycoprotein I antibodies, both at medium or high titers, and lupus anticoagulants all measured at least 12 weeks apart.[1] The presently accepted obstetric criteria are as follows: (a) one or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation, and/or (b) one or more premature births of a morphologically normal neonate before the 34th week of gestation due to eclampsia or severe preeclampsia, or recognized features of placental insufficiency, and/or (c) three or more unexplained consecutive spontaneous abortions before the 10th week of gestation in patients in whom maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes have been excluded.[1]

The complement system, which represents one of the operative forces of antibody-mediated immunity, is a part of the innate immune system. The recall of circulating inflammatory cells is mediated by complement anaphylatoxins C3a and C5a, but complement system activation is tightly controlled, in the fluid phase as well as in self-tissues, to prevent its proinflammatory and destructive capabilities. Complement regulators and specifically membrane-bound regulator proteins CD46, CD55, and CD59 control the three major complement activation pathways and inactivate both C3 and C5b-9 terminal complex.[2]

Complement-mediated placental inflammation has been found to play a key role in experimental models of fetal loss.[3,4] While there appears to be an increase in the deposition of C4d in the placentas of antiphospholipid-positive women, no corresponding increase in the deposition of C3 or its split products has been reported.[5-7] Data on the amounts of complement component C5b-9 and on the expression of complement regulatory proteins in the placenta are, instead, controversial or insufficient.[5,7,8] While lower serum C3 and/or C4 complement protein levels have been found in almost half of 201 women with obstetric APS[9] and they seem to be independent predictors of lower neonatal birth weight and of births at an earlier gestational age,[10] a subsequent study failed to uncover any associations between hypocomplementemia and obstetric complications.[11] More recently, since increased plasma Bb and C5b-9 activation product levels have been reported in antiphospholipid-positive women with adverse pregnancy outcomes, it can be hypothesized that complement activation contributes to the pathogenesis of this clinical condition.[12]

The current study aimed to examine the plasma anaphylatoxin C5a and C5b-9 complement terminal complex levels in APS patients. It also evaluated the amount of C5b-9 complex and the expression of complement regulatory proteins CD46, CD55, CD59 in APS placentas.

2 | MATERIALS AND METHODS

2.1 | Study population

Plasma samples from 43 non-pregnant APS patients and from 17 pregnant APS women, all diagnosed according to the International consensus statement criteria, were collected.[1] A control group of 16 healthy non-pregnant women and eight healthy pregnant women age-matched with the two study groups was considered. Placenta samples were collected from five APS patients and five age-matched healthy controls. The study was approved by the Ethics Committee for Clinical Trials of the Provinces of Verona and Rovigo (Italy) and was carried out in accordance with the 1964 Declaration of Helsinki and its later amendments, or comparable ethical standards. Written informed consent was obtained from all the participants.

2.2 | Sample collection

In order to minimize any potential bias, confounder, or other source of variability, the plasma and placenta samples of the patients and controls were processed by the same operator and the laboratory materials such as precast gels and monoclonal antibodies that were used were from the same production lot.

A venous blood (10 mL) was taken from the non-pregnant APS patients at the time of diagnosis, from the pregnant APS women with a favorable outcome during the first trimester of pregnancy and from those with adverse pregnancy outcome at the time of pregnancy complications. The samples were collected in pre-cooled VACUETTE® tubes containing 0.015 mol/L sodium citrate (Greiner Bio-One GmbH) and centrifuged immediately at 4°C. The plasma was separated from the cells by centrifugation at 3000 *g* for 15 minutes at 4°C. The plasma samples were then aliquoted in 1.5 mL Eppendorf microtubes (Sigma-Aldrich®) and stored at −80°C until use. Analysis was carried out on samples thawed only once. The placentas were collected immediately after delivery, cleaned with a pH 7.4 phosphate buffer solution, and kept on ice. All the tissue samples were handled using sterile gloves, forceps and a scalpel under a biohazard hood. Pieces of placentas weighing approximately 200 mg were sectioned from three different sites and transferred to 1.5 mL Eppendorf microtubes (Sigma-Aldrich®), snap-frozen in liquid nitrogen, and stored at −80°C until use. The samples were thawed only once.

2.3 | Enzyme-linked immunosorbent assay

Plasma concentrations of C5a and soluble C5b-9 were assessed using the MicroVue C5a Plus EIA (QUIDEL®) and the MicroVue C5b-9 Plus EIA (QUIDEL®), respectively, following the manufacturer's instructions. In accordance with the manufacturer suggestions plasma samples were assayed for C5a and C5b-9 detection using a 1:20 and 1:10 dilutions, respectively. The optical density was measured in a microtiter plate reader at 450 nm using the TECAN Sunrise III (Tecan). The concentrations, expressed as ng/mL, were calculated using standard curves generated according to specific standards provided by the manufacturer. The calibrators and the patient and control samples were tested in duplicate. The intra- and inter-assay coefficients of variation were <10% for both tests.

2.4 | Western immunoblotting

NaCl, TRIS, Tween-20, $MgCl_2$, EDTA, Na_3VO_4, NaF, HEPES, NP-40, β-mercaptoethanol, glycine, glycerol, bromophenol blue, sodium dodecyl sulfate (SDS), methanol were purchased from Sigma-Aldrich®. The proteins were extracted from the placenta specimens by homogenizing the tissues in 2 mL of Lysis Buffer (1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L Na_3VO_4, 10 mmol/L NaF, 10 mmol/L HEPES pH 7.9, 10% glycerol, 1% NP-40) and kept on ice for 30 minutes. The aliquots of homogenized placenta samples were sonicated for 45 minutes, kept on ice for 5 minutes, and vortexed for 2 minutes, three times, to complete cell lysis. Finally, the samples were centrifuged at 11 000 *g* for 15 minutes at 4°C. Aliquots of 100 µL of supernatant were obtained from homogenized placenta samples and stored at −80°C until use. The extracted proteins were quantified using Pierce™ BCA protein assay kit (Thermo Fisher Scientific). Thirty micrograms of proteins were subjected to electrophoresis in SDS-(12%) Mini-PROTEAN® TGX Stain-Free™ precast polyacrylamide gel (Bio-Rad) under both reducing and non-reducing conditions and then blotted to a PVDF membrane using the Mini Trans-Blot®Cell (Bio-Rad). The membranes were incubated with blocking buffer (3% *w/v* BSA in Tris-Buffered Saline and 0.1% *v/v* Tween-20) and then probed with each of the four monoclonal primary anti-human antibodies (Abcam): 1:1000 anti-human C5b-9, 1:5000 anti-human CD46, 1:25 000 anti-human CD55, 1:50 000 anti-human CD59 shook overnight at 4°C. As certified by the manufacturer all antibodies used to detect the complement components were verified antibodies. The immunocomplexes were detected by chemiluminescence using the ECL Plus Western Blotting Detection Reagents (GE Healthcare Life Biosciences/Amersham Biosciences) after they were washed and incubated with 1:15 000 HRP-conjugated anti-rabbit antibody (Abcam) or 1:5000 HRP-linked anti-mouse antibody (Abcam) for 1 hour at 22°C. Images were acquired using the Image Quant Las Mini 4000 Digital Imaging System (GE Healthcare Life Sciences), adjusting the exposure time depending on the intensity of the protein bands. The blots were stripped by adding a stripping acid solution made in our laboratory (50 mmol/L glycine, 1% *w/v* SDS, 1% *w/v* Tween-20; pH 2.2 with HCl), shook for 30 minutes at 37°C, and re-incubated with 1:500 anti-human ß-tubulin antibodies to confirm the equal sample loading of the gels and the efficiency in electrophoretic transfer. Densitometric analysis of the bands was performed using Quantity One software (Bio-Rad); β-tubulin data were used to normalize results as C5b-9/CD46/CD55/CD59-to-β-tubulin band optical density ratio.

2.5 | Antiphospholipid antibody detection

IgG/IgM anticardiolipin and IgG/IgM anti-β2Glycoprotein I enzyme-linked immunosorbent assay (ELISA) were performed using a home-made method following the European Forum on aPL recommendations, described elsewhere.[13] A series of sera traceable to the Harris standard sera were used as the calibration curves for the IgG/IgM anticardiolipin antibodies. The results were expressed in GPL and MPL units. A home-made standard curve obtained from a pool of positive samples calibrated to Koike's monoclonal antibodies (HCAL for IgG and EY2C9 for IgM anti-β2Glycoprotein I antibodies) was used to detect IgG/IgM anti-β2Glycoprotein I antibodies. The results were expressed in arbitrary units. The cutoff values for the medium-high levels of IgG/IgM anticardiolipin and IgG/IgM anti-β2Glycoprotein I antibodies were calculated as >the 99th percentile using sera from 100 healthy women. Lupus anticoagulant was assessed by means of multiple coagulation tests carried out using platelet-poor plasma samples following updated guidelines and utilizing dilute Russell viper venom and dilute activated partial thromboplastin times as the screening tests.[14]

2.6 | Statistical analysis

The mean plasma levels of C5a and C5b-9 complement components in the non-pregnant APS and pregnant APS patients were compared with those in the non-pregnant and pregnant controls, respectively, using a nonparametric Mann-Whitney *U* test. The same test was used to compare the mean levels of C5b-9, CD46, CD55, CD59 amounts in the placentas of the APS patients with those of the healthy controls, in the placentas of APS patient with favorable outcome with those with adverse outcome and in the placentas of APS patients with involvement of chorionic villi only with those with involvement of both villi and decidua vessels. In particular, the means of the results obtained from the three different placenta portions of APS women were compared with those of the placentas of the healthy controls. A $P < .05$ value was considered statistically significant. Statistical analyses were performed using GraphPad Prism statistical software.

3 | RESULTS

3.1 | The demographic and clinical data of the APS patients

The 43 non-pregnant APS patients had a mean age of 43.2 years ± 8.7 SD. While 14 had a history of pregnancy morbidity alone, 29 had previous thrombosis ± pregnancy morbidity. The 17 pregnant APS women, who had a mean age of 35.5 years ± 4.0 SD, were divided into two groups on the basis of their pregnancy outcome: Ten had a favorable outcome with a mean week at delivery of 36.6 ± 2.2 and a mean birth weight of 2702 g ± 516; seven had an adverse pregnancy outcome characterized by premature birth associated to severe pregnancy complications including preeclampsia in three cases (one leading to neonatal death), intrauterine growth restriction in two, catastrophic APS in one, and hemolysis elevated liver enzymes, and low platelet count (HELLP) syndrome in one. According to the Miyakis' laboratory categories,[1] among women with favorable pregnancy outcome six were in category I (two or more antiphospholipid antibodies), while four in category II (one only antiphospholipid antibody). All seven patients with adverse outcome were in category I.

TABLE 1 Laboratory and clinical characteristics of APS patients whose placentas were examined

	Patient n 1	Patient n 2	Patient n 3	Patient n 4	Patient n 5
Plasma C5a	11.26	22.18	n.a.	8.8	n.a.
Plasma C5b-9	353.02	408.51	n.a.	257.09	n.a.
Placental C5b-9	0.57	4.74	2.79	5.78	6.95
Placental CD46	22.97	2.21	0.94	3.94	2.18
Placental CD55	13.23	6.68	3.95	10.96	1.49
Placental CD59	4.23	0.61	1.53	0.82	0.87
aPL profile	Triple aPL	Triple aPL	Triple aPL	Triple aPL	Triple aPL
Placental histology	Intervillous fibrin deposition	Villous ischemic necrosis due to spiral artery thrombosis	Syncytial knots and thrombosis of spiral arteries	Immature intermediate villi	Syncytial knots and decidual arteriopathy
Previous thrombosis	No	Skin thrombotic microangiopathy	No	Stroke	No
Previous obstetric morbidities	HELLP	Severe preeclampsia	HELLP	HELLP, fetal and neonatal deaths	Stillbirth and fetal death

Abbreviations: aPL, antiphospholipid antibodies; APS, antiphospholipid syndrome; HELLP, hemolysis elevated liver enzymes low platelet count; n.a., not available.

The five APS patients whose placentas were studied had a mean age of 36.8 ± 2.8 SD, their laboratory and clinical characteristics are reported in Table 1. Given the presence of previous severe pregnancy complications, in two cases associated to vascular thrombosis, and to positivity to all the antiphospholipid antibody assays (triple positivity), they were all considered at high risk of pregnancy complications.[15] Four had a favorable pregnancy outcome with a mean week at delivery of 35.0 ± 2.4 and a mean birth weight of 2582 g ± 495; one suffered from premature birth due to intrauterine growth restriction and oligohydramnios.

All the APS patients participating in the study were treated with prophylactic or therapeutic doses of low-molecular weight heparin plus low-dose aspirin (100 mg) during the pregnancy. While low-dose aspirin was administered before pregnancy, heparin was prescribed when the pregnancy test resulted positive. The five APS patients whose placentas were examined in this study, because they were considered at high risk for pregnancy complications[16] were treated, in addition to conventional therapy as follows: one (patient no.5) with low-dose steroids plus hydroxychloroquine starting at the positive pregnancy test and four with weekly plasma exchange plus a fortnightly intravenous immunoglobulin bolus starting from the detection of fetal heartbeat.[17]

3.2 | Complement studies on the plasma samples

As indicated in Table 2 and Figure 1, the mean plasma level of complement anaphylatoxin C5a was significantly higher in the 43 non-pregnant APS patients considered together with respect the non-pregnant healthy controls. The most significant difference was observed in the patients with previous thrombosis ± pregnancy morbidity. As far as the pregnant, APS patients were concerned, only those with adverse outcomes showed a significant prevalence of C5a with respect to the pregnant control women. The mean plasma C5a levels of the non-pregnant APS women with previous pregnancy morbidity alone and the pregnant APS patients with favorable outcomes were not significantly different from those of the healthy controls. Similarly, the mean plasma C5b-9 levels significantly prevailed in the 43 non-pregnant APS patients considered together with respect to the non-pregnant healthy controls and both in the non-pregnant APS patients with previous thrombosis ± pregnancy morbidity and in the pregnant APS women with adverse outcomes (Table 3 and Figure 2).

3.3 | Complement studies on the placentas

The normalized placental amounts of C5b-9, CD46, CD55, and CD59 in APS patients vs healthy controls and in patients with favorable pregnancy outcome vs that with adverse outcome have been reported in Table 4 and 5, respectively. The mean C5b-9 terminal complex amount level was significantly higher in the placentas of the APS women considered together with respect to that in the controls (Table 4). The mean C5b-9 terminal complex amount level significantly prevailed in the placenta of the adverse pregnancy outcome

TABLE 2 C5a complement component in maternal plasma of different subsets of APS patients and healthy controls

APS and subsets	Patients	Controls	Patients Mean ± SD	Controls Mean ± SD	*P*
Non-pregnant APS patients	43	16	11.9 ± 6.3	6.0 ± 2.9	.0007*
With previous pregnancy morbidity	32.6%	16	8.8 ± 5.8	6.0 ± 2.9	.2201
With previous thrombosis ± pregnancy morbidity	67.4%	16	13.4 ± 6.1	6.0 ± 2.9	.0001*
Pregnant APS patients	17	8	12.9 ± 7.8	7.8 ± 2.9	.1535
With favorable outcome	58.8%	8	7.5 ± 4.5	7.8 ± 2.9	.7618
With adverse outcome	41.2%	8	19.3 ± 7.2	7.8 ± 2.9	.0093*

Abbreviation: APS, antiphospholipid syndrome.
*Significant result.

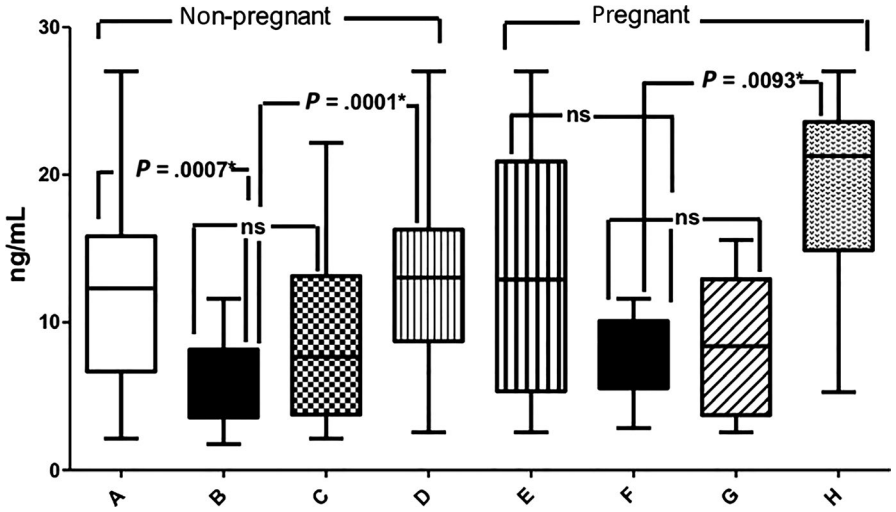

FIGURE 1 Representative images showing the comparison between different subsets of patients with antiphospholipid syndrome and healthy controls of C5a mean levels ± SD detected by enzyme-linked immunosorbent assay in maternal plasma. A, non-pregnant patients with antiphospholipid syndrome considered as a whole. B, non-pregnant healthy controls. C, non-pregnant patients with previous pregnancy morbidity alone. D, non-pregnant patients with previous thrombosis ± pregnancy morbidity. E, pregnant patients with antiphospholipid syndrome considered as a whole. F, pregnant healthy controls. G, pregnant patients with favorable outcome. H, pregnant patients with adverse outcome. *Significant result. ns, not significant result

patient with respect to that in the patients with favorable outcomes (Table 5). Conversely, the mean CD46, CD55, and CD59 complement regulator amount values were lower, even if significantly only CD59, in the placentas of all the APS women with respect to those of the controls (Table 4), and in the placentas of APS patients with adverse outcome, even if significantly only CD55, with respect to those of APS patients with favorable outcome (Table 5). Representative pictures of the normalized placental amounts of C5b-9 complement complex and complement regulators in APS patients vs healthy controls and in patients with favorable pregnancy outcome vs that with adverse outcome are shown in Figures 3 and 4, respectively. Furthermore, Figure 5 shows all plots of Western immunoblotting analysis of the placentas of APS patients and healthy controls concerning the C5b-9 terminal complex, the complement regulators and the respective β-tubulins.

The anatomo-pathologic data of the placentas (Table 1) were subdivided into two categories: involvement of chorionic villi only (patients n 1 and 4) and involvement of both villi and decidua vessels (patients 2, 3 and 5), respectively. The correlation of these histological patterns with the mean amounts of C5b-9 complex and complement regulator deposition showed higher mean values of C5b-9 in the placentas with involvement of both villi and decidua vessels than in those with villus involvement only (4.8 vs 3.2) and lower mean values of all complement regulators in the placentas with involvement of both villi and decidua vessels compared to those with villus involvement only (CD46 1.7 vs 13.4, CD55 4.0 vs 12.1, CD59 1.0 vs

TABLE 3 C5b-9 complement complex in maternal plasma of different subsets of APS patients and healthy controls

APS and subsets	Patients	Controls	Patients Mean ± SD	Controls Mean ± SD	*P*
Non-pregnant APS patients	43	16	353.9 ± 329.6	170.8 ± 92.0	.0065*
With previous pregnancy morbidity	32.6%	16	237.2 ± 121.6	170.8 ± 92.0	.1400
With previous thrombo-sis ± pregnancy morbidity	67.4%	16	410.2 ± 382.1	170.8 ± 92.0	.0034*
Pregnant APS patients	17	8	430.0 ± 398.9	229.6 ± 92.2	.2104
With favorable outcome	58.8%	8	214.7 ± 134.1	229.6 ± 92.2	.6334
With adverse outcome	41.2%	8	693.9 ± 502.0	229.6 ± 92.2	.0093*

Abbreviation: APS, antiphospholipid syndrome
*Significant result.

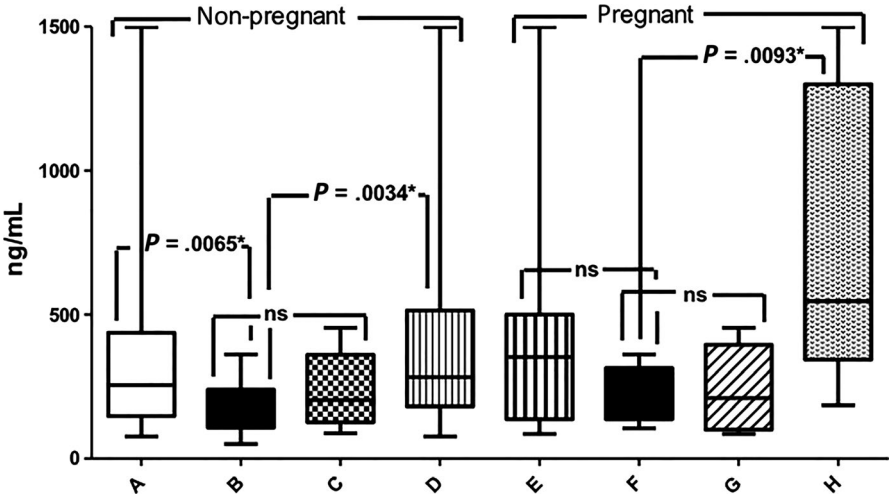

FIGURE 2 Representative images showing the comparison between different subsets of patients with antiphospholipid syndrome and healthy controls of C5b-9 mean levels ± SD detected by enzyme-linked immunosorbent assay in maternal plasma. A, non-pregnant patients with antiphospholipid syndrome considered as a whole. B, non-pregnant healthy controls. C, non-pregnant patients with previous pregnancy morbidity. D, non-pregnant patients with previous thrombosis ± pregnancy morbidity. E, pregnant patients with antiphospholipid syndrome considered as a whole. F, pregnant healthy controls. G, pregnant patients with favorable outcome. H, pregnant patients with adverse outcome. *Significant result. ns, not significant result

2.5). However, CD46 and CD55 only were also significantly lower (*P* = .0016 for both).

4 | DISCUSSION

The current analysis uncovered significantly higher mean plasma complement activation C5a and C5b-9 component levels in the most severe APS subset characterized by a history of vascular thrombosis ± pregnancy morbidity, a profile which is often associated to poor pregnancy outcomes when conventional heparin/aspirin therapy is used.[16] The mean C5a and C5b-9 levels in the patients with previous pregnancy morbidity alone, whose profile is generally associated with a favorable pregnancy outcome during conventional treatment,[16] were not significantly different with respect to those of healthy control women. High plasma C5a and C5b-9 levels would seem, in this context, to be predictive markers of adverse pregnancy outcome during conventional treatment.

Study data showed that only the pregnant APS patients with adverse pregnancy outcome characterized by severe pregnancy complications (preeclampsia, intrauterine growth restriction, catastrophic APS and HELLP syndrome) who were unresponsive to conventional treatment had significantly higher mean plasma C5a and C5b-9 levels with respect to the healthy controls. As an activation

TABLE 4 Normalized placental amounts of C5b-9 complement complex and complement regulators in APS patients and healthy controls

Placental amounts	APS patients	Healthy controls	APS patients Mean ± SD	Healthy controls Mean ± SD	*P*
C5b-9	5	5	4.17 ± 2.42	1.68 ± 0.95	.0037*
CD46	5	5	6.45 ± 8.76	14.2 ± 20.43	.062
CD55	5	5	7.24 ± 5.1	16.7 ± 13.45	.081
CD59	5	5	1.57 ± 1.5	3.01 ± 1.92	.0032*

Note: The mean value ± standard deviation was calculated from three values for each patient obtained in three different placental sites.
Abbreviation: APS, antiphospholipid syndrome.
*Significant result.

TABLE 5 Normalized placental amounts of C5b-9 complement complex and complement regulators in APS patients according to pregnancy outcome

Placental amounts	Favorable outcome	Adverse outcome	Favorable outcome Mean ± SD	Adverse outcome Mean ± SD	*P*
C5b-9	4	1	3.47 ± 2.2	6.95 ± 0.20	.0115*
CD46	4	1	7.52 ± 9.6	2.18 ± 0.93	.516
CD55	4	1	8.70 ± 4.65	1.49 ± 0.52	.0115*
CD59	4	1	1.80 ± 1.60	0.87 ± 0.22	.516

Note: The mean value ± standard deviation was calculated from three values for each patient obtained in three different placental sites.
Abbreviation: APS, antiphospholipid syndrome.
*Significant result.

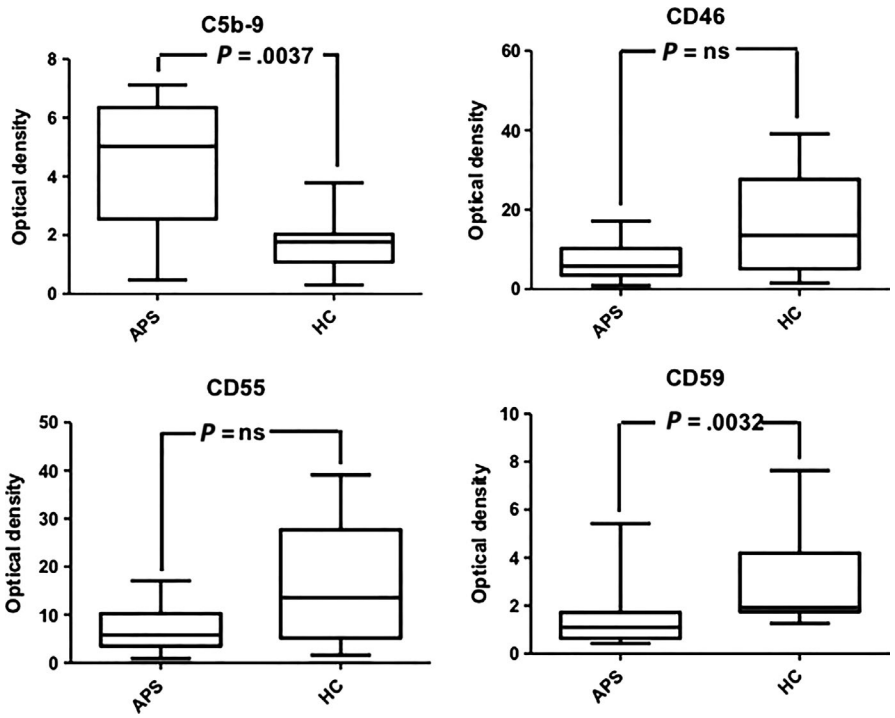

FIGURE 3 Levels of C5b-9, CD46, CD55, and CD59 in patients with antiphospholipid syndrome (APS) and in healthy controls (HC). The amount of Western blot detected proteins was normalized as C5b-9/CD46/CD55/CD59-to-β-tubulin band optical density ratio. The boxes and the middle lines correspond to the values from the lower to upper quartiles and medians, respectively

of complement cascade seemed to be present in these patients and given their severe clinical conditions, treatments prescribed in addition to conventional therapy, which currently include intravenous immunoglobulins, low-dose steroids, plasma exchange and hydroxychloroquine, could be administered, taking into account their anti-complement activity and potential side-effects.[17]

In the effort to obtain reliable results, the current study measured the complement components for three different portions of the placentas. Mirroring the results obtained for the plasma samples, the mean C5b-9 placenta amount level significantly prevailed in the adverse pregnancy outcome patient with respect to those in the women with favorable outcomes and thus confirmed

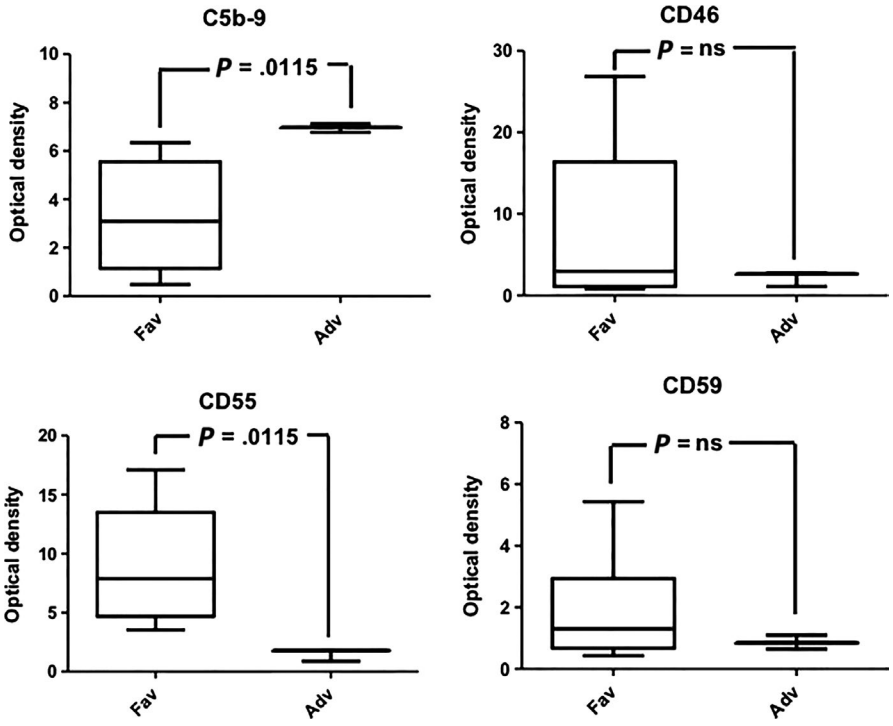

FIGURE 4 Levels of C5b-9, CD46, CD55, and CD59 in APS patients with favorable (Fav) and those with adverse (Adv) pregnancy outcome. The amount of Western blot detected proteins was normalized as C5b-9/CD46/CD55/CD59-to-β-tubulin band optical density ratio. The boxes and the middle lines correspond to the values from the lower to upper quartiles and medians, respectively

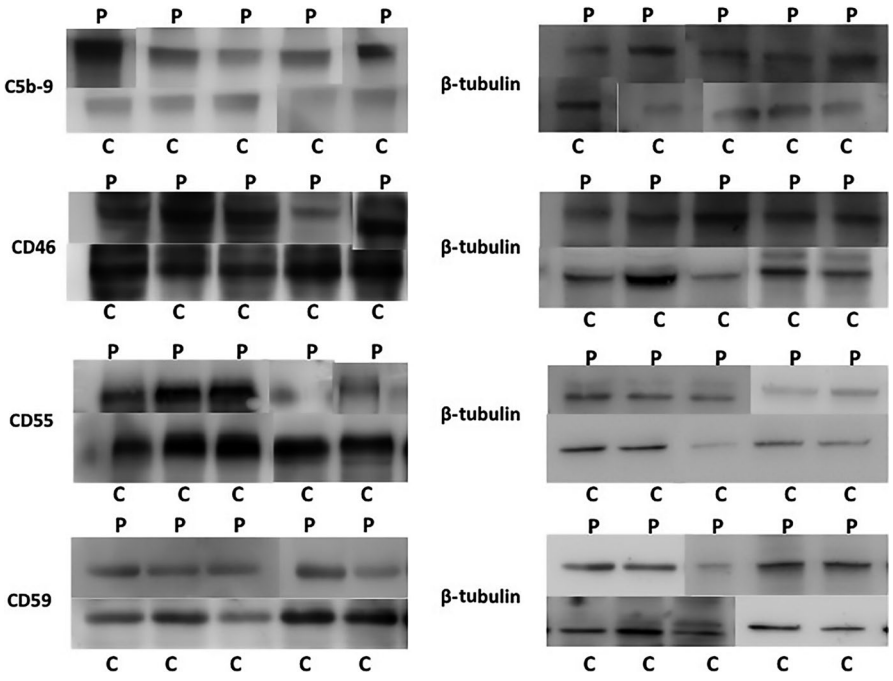

FIGURE 5 All plots of Western immunoblotting analysis of the placentas of APS patients (P) and healthy controls (C) concerning the C5b-9 terminal complex, the complement regulators (CD46, CD55, and CD59) and the respective β-tubulins

that there is more complement activation in the presence of severe pregnancy complications. Only two studies have investigated C5b-9 amount in the placenta thus far. In the first, the presence of C5b-9 in APS placentas was significantly lower or not significantly different from that found in control placentas.[5] In the second, C5b-9 deposits were found on the surface of syncytiotrophoblast with additional distribution on the intervillous fibrin in the placentas of APS patients but not in those of the controls.[8] As the pregnancy outcomes were reported in neither of the studies, it is impossible to verify if the C5b-9 placental expression was higher in the placentas of the APS women with adverse pregnancy outcomes.

In accordance to another report,[8] we observed signs of complement activation in both the plasmas and placentas of pregnant APS women although being treated with heparin, known for its capacity to inhibit complement activation and to prevent complement-mediated pregnancy loss in a mouse model of obstetric APS.[18] The placentas of our APS patients showed a significant deposition of C5b-9 terminal complex and a decrease in complement regulators with respect to the placentas of healthy controls, thus, the important

complement activation in APS patients could have overwhelmed the potential direct inhibitory effects of heparin on the complement cascade. A correlation between placental pathologic features and complement deposition (C4d) in the trophoblastic cytoplasm, cell membrane, and basement membrane has been demonstrated in placentas of.aPL-positive women.[5] It is possible that the binding of complement activation products to restricted placental areas may cause tissue alterations that marginally affect the regular progression of pregnancy, instead a widespread placental involvement could be associated to adverse outcome of pregnancy.

The mean complement CD46, CD55, and CD59 regulator levels were lower, although only CD59 was significantly reduced, in the placentas of all the APS women studied, independently of the pregnancy outcome, with respect to those of the controls. The lower CD46, CD55, and CD59 placenta expressions in APS patients can impair local protection, promoting complement hyperactivation and consequently leading to inflammation, thrombosis and tissue damage. In fact, lower levels of CD46, CD55, and CD59 regulator was found, although only CD55 was significantly reduced, in the placentas of women with adverse pregnancy outcome with respect to those of patients with favorable outcome. This finding would agree with the results of a study demonstrating the presence of risk variants in the complement regulatory proteins in the aPL-positive patients who developed preeclampsia.[19] However, as the difference in the mean complement regulator levels between the APS and the control populations was not significant for all regulators considered in the study and in view of the small number of placentas examined, the authors are unable to draw definitive conclusions. Amounts of complement regulators CD46 and CD55 on the placentas of APS patients were investigated by another study using the same Western blot analysis for their detection.[20] As the authors found significantly higher levels of CD46 and CD55 proteins ($P < .001$ for both) in the placentas of the APS patients with respect to those in the controls, they hypothesized that there is a mechanism which increases CD46 and CD55 expression in placentas as an effect of the proinflammatory cytokines present in APS thrombophilia.[20] The level of risk in the APS women was, unfortunately, not specified by the authors of the study. In our case, the history of severe pregnancy complications and the presence of triple antiphospholipid positivity that characterized all our patients made them all at high risk of pregnancy complications.[17] One, in fact, developed adverse pregnancy outcome despite additional treatment with low-dose steroids and hydroxychloroquine.

Concerning the correlation between placental histology and complement/protein deposition, APS placentas with a more severe histology (chorionic villus and decidual vessel involvement) had higher C5b-9 complex and lower complement regulator mean amounts than those with less severe anatomo-pathological lesion (villus involvement only). In addition, levels of CD46 and CD55 deposition were also significantly lower in placentas with more severe histology. These data, even if obtained in a small number of cases, could further support the role of the complement in the obstetrical complications of the APS.

Clearly, this study's primary limit is linked to the low number of cases studied, while its strength lies in the homogeneity of the results demonstrating that the activation of the complement cascade in both the pregnant and non-pregnant APS patients was associated to severe clinical conditions.

5 | CONCLUSIONS

A heparin/aspirin combination constitutes the conventional treatment protocol for pregnant APS women. As these strategies fail in approximately 20%-30% of cases,[21] uncovering other options for women refractory to conventional treatment or at high risk of pregnancy complications has become an urgent undertaking.[15] Several experts are convinced that in association with conventional therapy, these high-risk APS patients should be prescribed additional treatments in the effort to improve live birth rates and/or reduce pregnancy complications.[22]

In the future, if these results are confirmed by further large-scale studies, it will become possible to identify those patients in whom complement inhibitors are likely to prevent or modify the inflammatory-related sequelae associated with adverse pregnancy outcomes. Detection of high plasma C5b-9 complex and C5a levels in APS women could be considered predictive or the first signs of adverse pregnancy outcome and lead to the decision to prescribe a targeted treatment. Eculizumab, a humanized monoclonal antibody that binds to the C5 component of complement and inhibits terminal complement activation, for example, could represent one such possibility. Prescribed to two pregnant patients at high risk of developing devastating APS-related complications, it was found to be a safe treatment option throughout the rest of the pregnancy.[23,24] Although other cases depicting benefits in pregnant APS patients are lacking, studies on Eculizumab remain an exciting and promising area of research.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

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