Thrombosis Research 172 (2018) 172–178

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

Lupus anticoagulant identifies two distinct groups of patients with different antibody patterns

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A B S T R A C T

Background: Whether antibodies directed to β2-Glycoprotein I (aβ2GPI) are responsible for LA activity is not well defined. However, in the absence of such antibodies the molecule responsible for LA phenomenon is unknown.
Objective: The aim of this study was the biochemical identification of the target antigen epitope of aPL responsible of LA activity in the absence of aβ2GPI antibodies together with the biological and clinical characteristics of these patients in comparison with classical triple positive patients.
Patients/methods: A comparison of patients with LA without (LA+/aβ2GPI−) and those with (LA+/aβ2GPI+) associated aβ2GPI antibodies was performed. Size exclusion chromatography and analytical chromatography were used to identify the molecule with LA activity in patients LA+/aβ2GPI-.
Results and conclusions: Analytical size-exclusion chromatography revealed a peak of 996Kd with LA activity perfectly overlapping that of IgM anti phosphatidylserine/prothrombin (aPS/PT) antibodies. Similarly, all the 25 LA+/aβ2GPI− patients were positive for aPS/PT antibodies. LA+/aβ2GPI− compared to 33 LA+/aβ2GPI+ patients turned out to be significantly older, with a lower rate of previous thromboembolic events and a weaker LA activity. Search for aPS/PT and aβ2GPI antibodies in patients with LA is useful to identify two subgroups of LA at different risk of thromboembolic events.

1. Introduction

Lupus Anticoagulant (LA) is a laboratory phenomenon characterized by prolongation of phospholipid-dependent coagulation tests. Its presence is confirmed by normalization (or near normalization) of clotting times when an excess of phospholipids is added to the reagent mixture [1]. LA could result from the presence of anti β2-glycoprotein I antibodies (LA+/aβ2GPI+) as a subgroup of these antibodies, those directed against the Domain I of the molecule, were shown to have LA activity [2]. The coexistence of LA and aβ2GPI, together with anti-cardiolipin antibodies (aCL), identifies a group of patients or carriers at high risk for thromboembolic events and pregnancy loss [3–7]. On the other hand, LA may be positive in the absence of aβ2GPI antibodies (LA+/aβ2GPI−) and the target antigen epitope of aPL responsible for LA phenomenon is not well defined. Isolated LA (LA+/aβ2GPI−), however, might be clinically less relevant, as its presence is not a predictor of thromboembolic events [8,9]. In this study, IgM against phosphatidylserine/prothrombin complex (aPS/PT) was identified as the possible causative agent of LA in an aβ2GPI− patient. Moreover, laboratory and clinical characteristics of patients with the two different antiphospholipid antibody profiles (LA+/aβ2GPI− and LA+/aβ2GPI+) were compared.

2. Methods

2.1. Patients

Patient population consisted of consecutive patients with isolated LA positivity and consecutive patients with LA+/aβ2GPI+ positive profile. LA studies in isolated LA positivity were performed for the following reasons: prolonged aPTT prior to surgery, routine laboratory testing in autoimmune disorders, thrombophilia screening before taking estroprogestinic drugs or before pregnancy [10]. Clinical information was obtained by reviewing clinical patient records or by survey questionnaires.

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https://doi.org/10.1016/j.thromres.2018.11.003
Received 6 September 2018; Received in revised form 31 October 2018; Accepted 2 November 2018
Available online 09 November 2018
0049-3848/

Table 1
LA, aCL and aβ2GPI antibodies of patient and control plasma.

	dRVVT (seconds)			
	Screening	Mixing 1:1	Confirm	
Patient	58	53	30	
Control	30	30	28	
Ratio	1.9	1.8	1.1	1.72[a]
	SCT (seconds)			
	Screening	Mixing 1:1	Confirm	
Patient	98	75	38	
Control	30	30	32	
Ratio	3.3	2.5	1.2	2.75[a]

	ELISA			
	aCL IgG (GPL)	aCL IgM (MPL)	aβ2GPI IgG (U)	aβ2GPI IgM (U)
Patient	6	7	5	6
Normal values	< 10	< 8	< 13	< 7

[a] Screening ratio/confirm ratio.

2.2. Coagulation studies

Blood was processed and plasma obtained by double centrifugation; plasma was deep frozen at −80 °C until testing and thawed only once at the time of testing by total immersion of sample content in a water bath at 37 °C for 5 min [1]. LA activity of patient plasma was assessed by the dilute Russell Viper Venom Time (dRVVT) (HemosIL dRVVT, Werfen Group, Milan, Italy) and Silica Clotting Time (SCT) (Werfen Silica Clotting Time, Werfen Group, Milan, Italy) according to guidelines [1], using an automatic coagulation analyzer (TOP 1000, Werfen Group, Milan, Italy). Patients were considered LA positive when the dRVVT and SCT screening, mixing and confirm were positive and thrombin time (TT) within the normal values to check for heparin contamination. The anticoagulant activity of fractions of gel-filtered plasma was measured using 75 µl of reagent (Russell Viper Venom, phospholipids and calcium), 75 µl of normal pooled plasma and 150 µl of tested material. Addition of 150 µl of Tris Buffered Saline (TBS: tris 20 mM, NaCl 0.15 M, pH 7.4) to normal plasma was used as the control value. TT was determined in all positive samples: 100 µl of 3 U/ml bovine thrombin (Hemosil, Instrumentation Laboratory, Orangeburg, NY, USA) were mixed with 100 µl of patient plasma, and the coagulation time was recorded. The cut-off value obtained in 20 normal subjects was 20 s. Three Thrombosis Centers in Italy (Padova, Bologna, and Cremona) sent plasma samples of consecutive patients with isolated LA (LA + /

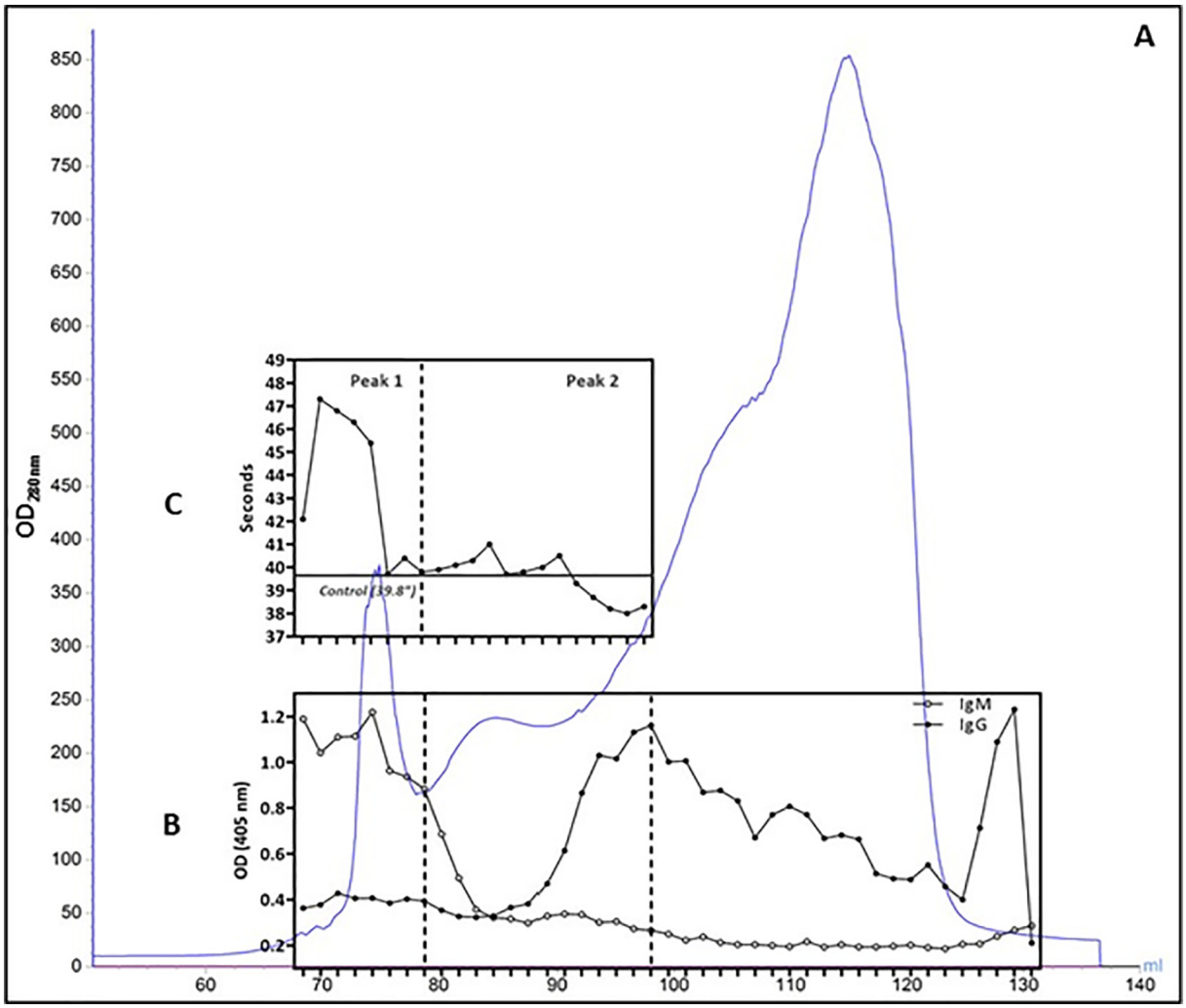


Fig. 1. Sephacryl S-300 size exclusion chromatography of a plasma sample from a patient with strong isolated LA. The protein profile (expressed as OD_{280nm}) is shown in the large frame (A). Proteins above 300 kDa (left to the vertical dotted line) contain mainly immunoglobulin of IgM isotype (insert B) and possess LA activity (insert C). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

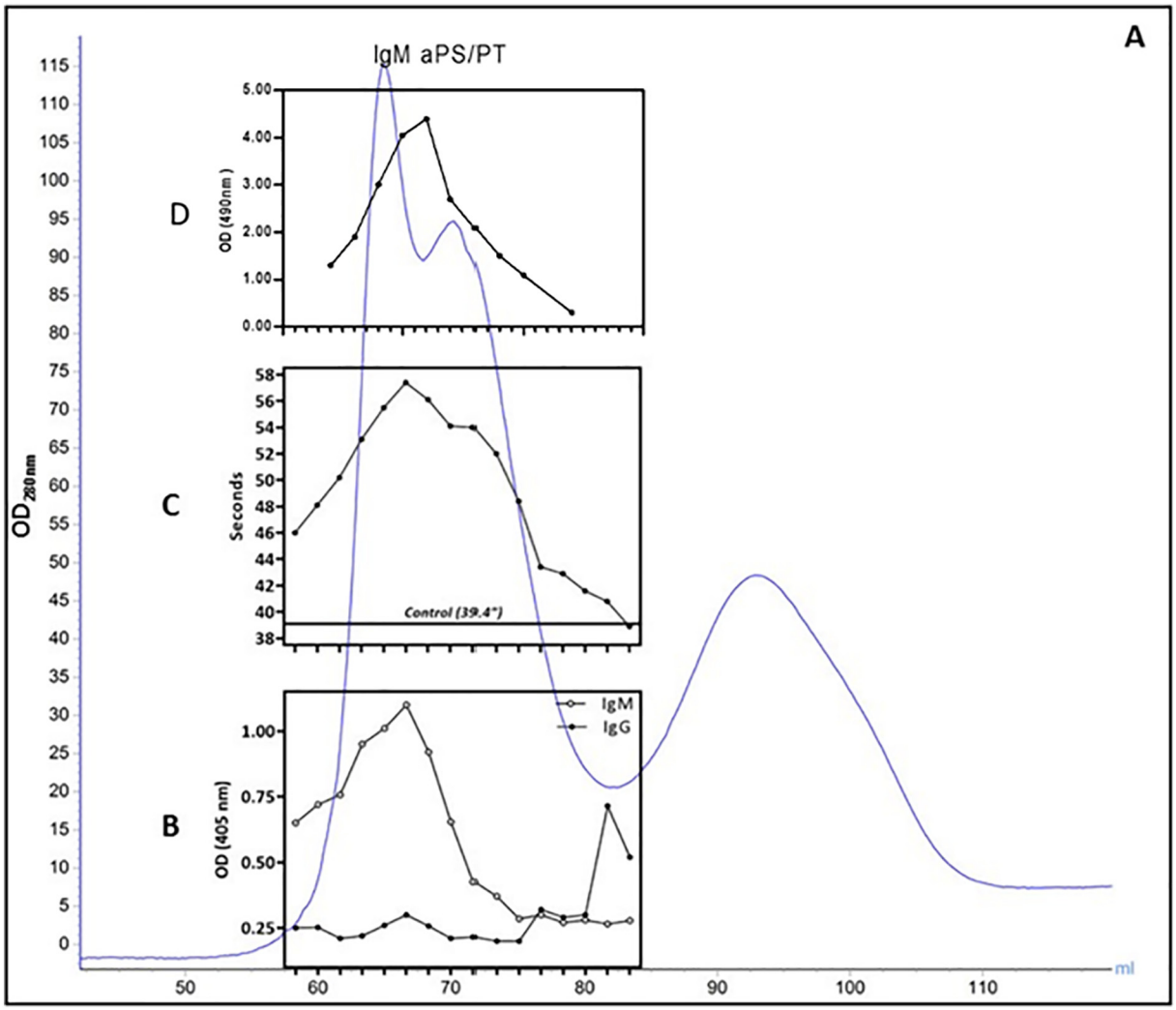


Fig. 2. Sephacryl S-300 size exclusion chromatography of plasma precipitated with PEG 8000 (5% w/v) from a patient with LA + /aβ2GPI − . The peak at shorter retention times contains proteins having a MW higher than 300 kDa (frame A), identified as IgM (insert B) with overlapping LA activity (insert C) and aPS/PT antibodies (insert D).

aβ2GPI −). Data were confirmed after 12 weeks.

2.3. IgM and IgG ELISAs

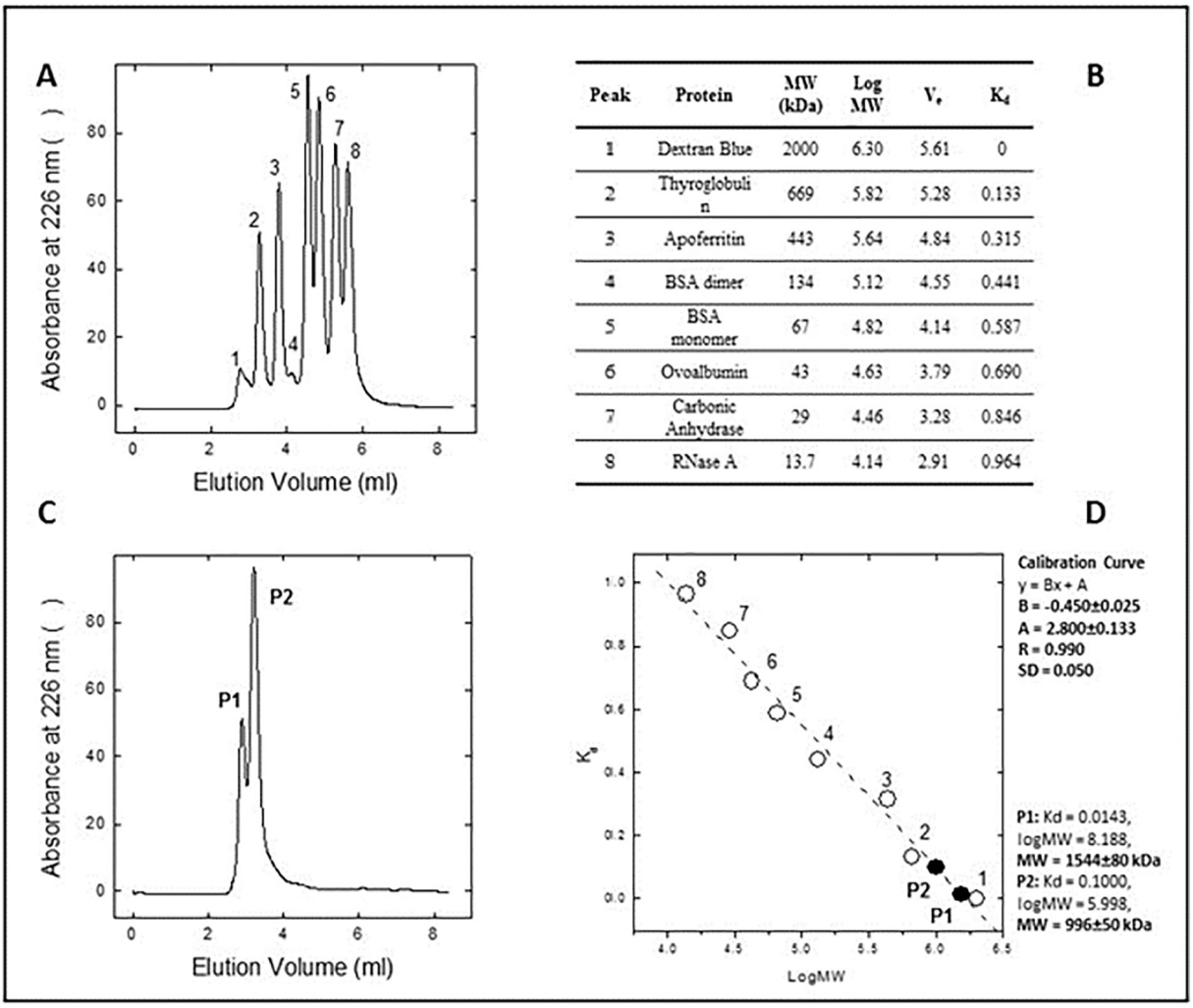
One hundred microliter of fractions of gel-filtered plasma was added to each well of a 96-well microtiter plate (Costar, Corning, NY, USA) and incubated overnight at 4 °C, after which the wells were washed twice with 100 µl of PBS/Tween (PBS: 8 g/l of NaCI, 0.2 g/l of KH,P04. 2.9 g/l of Na2HPO4, 0.2 g/l of KCI, Tween 0.05%, pH 7.4). One hundred microliters of PBS/Albumin 2% was then added to each well and incubated for 2 h to block nonspecific binding. After the plates were washed three times with PBS/Tween, 100 µl of goat anti-Human IgM or anti-human IgG (SIGMA, St Louis, MO, US) alkaline phosphatase conjugated diluted 1:1000 in PBS/Albumin 2% was added to each well and incubated for 1 h. The plates were then washed three times more in PBS/Tween and 100 µl of freshly prepared *p*-nitrophenyl phosphate solution (1 mg/ml in 10% diethanolamine buffer, pH 9.8) was added to each well. After 30 min incubation, the absorbance was measured at 405 nm, using a plate reader (Thermo Scientific, Evolution 60S, Wilmington, DE, US).

2.4. Antiphospholipid antibody (aPL) ELISAs

Anti-β2GPI and aPS/PT IgG and IgM were measured by ELISAs (QUANTA Lite, Werfen Group, Milan, Italy). ELISAs were performed following the guidance from the Scientific Standardization Committee on Lupus Anticoagulant/Antiphospholipid Antibodies of the International Society of Thrombosis and Haemostasis [11].

2.5. Identification of lupus anticoagulant activity in isolated LA (LA + / aβ2GPI −)

Plasma from a single patient with a strong isolated LA was used to identify the protein factor(s) responsible for the prolongation of coagulation time. Sephacryl S-300 (Sigma-Aldrich, Milan, Italy) size exclusion chromatography was used to separate immunoglobulins with a cut–off molecular weight of 300kD. Five ml of plasma were applied to a 2.5 × 90-cm column equilibrated with TBS. Fractions of 2.5 ml were collected and tested for IgG, IgM by ELISA and for lupus anticoagulant activity. Immunoglobulins were purified using the protocol reported by Nikolayenko and co-workers, with some modifications [12]. Briefly, to freshly prepared plasma samples (2 ml), polyethylene glycol (PEG 8000) was added up to a final concentration of 5% w/v and, after stirring (15 min), the mixture was centrifuged at 4000 rpm for 15 min. The supernatant was discarded, the pellet dissolved in TBS (2 ml), and the solution filtered at 0.2 µm cut-off on cellulose filters. Aliquots of this preparation were loaded onto a preparative Sephacryl S-300 (Sigma-Aldrich, Milan, Italy) size exclusion chromatography. An aliquot (10 µl)



Peak	Protein	MW (kDa)	Log MW	V_e	K_d
1	Dextran Blue	2000	6.30	5.61	0
2	Thyroglobulin	669	5.82	5.28	0.133
3	Apoferritin	443	5.64	4.84	0.315
4	BSA dimer	134	5.12	4.55	0.441
5	BSA monomer	67	4.82	4.14	0.587
6	Ovoalbumin	43	4.63	3.79	0.690
7	Carbonic Anhydrase	29	4.46	3.28	0.846
8	RNase A	13.7	4.14	2.91	0.964

Fig. 3. Analytical size exclusion chromatography of the fraction at the highest LA activity eluted from S-300 column (see Fig. 2). (A, B) Elution profile of standard proteins loaded onto a Yarra SEC-3000 column and table that correlates the molecular weight of the proteins with their Kd values. (C) Elution profile of the fraction from S-300 column. (D) Calibration curve with standard proteins (○) and the two main peaks (P1, P2) eluted from S-300 fraction (●). The calibration curve gives a molecular estimation of 1544 ± 80 kDa and 996 ± 50 kDa for P1 and P2, respectively. Peak P2 is positive for LA activity and for IgM aPS/PT.

of the fraction displaying maximal LA activity, and eluted from the S-300 column, was loaded onto a (7.8 × 150 mm) Yarra SEC-3000 column (Phenomenex, CA, USA), eluted in PBS (20 mM sodium phosphate buffer, pH 6.8, 0.15 M NaCl) at a flow rate of 0.5 ml/min. The molecular weight of the proteins eluted in correspondence of the chromatographic peaks was estimated after calibration of the column with a protein standard mixture.

2.6. Statistics

Descriptive statistics are reported as appropriate: categorical data are expressed as frequencies (percentage); continuous data are reported as mean and standard deviation or median and interquartile range for data without normal distribution. Unpaired Student's *t*-test was used to compare means from values with normal distribution. Mann-Whitney test was used to compare values without a normal distribution. The chi-squared test for independence was used to evaluate the association of thrombosis with LA+/aβ2GPI− and LA+/aβ2GPI+. GRAPHPAD-INSTAT 3 (GraphPad Software, CA, USA) was used for the statistical analysis (www.graphpad.com).

3. Results

Initially we came across the plasma of a middle-aged patient with psoriasis and a strong isolated LA at routine screening for the presence of antiphospholipid antibodies (Table 1). Both dRVVT and SCT assays showed a marked prolongation of coagulation times that were not normalized in mixing studies and normalized or nearly normalized in confirming studies. The patient was not on treatment with anticoagulant drugs and TT was normal.

To identify the protein factor(s) responsible for the strong LA activity in one patient, displaying an aβ2GPI− profile, a plasma sample was loaded on size-exclusion chromatography (SEC) Sephacryl S-300 column, allowing separation of proteins in the 10–1.500 kDa molecular weight range (Fig. 1A, blue trace). Fractions were collected and tested for the presence of IgG and IgM, by using specific ELISAs (Fig. 1B), and for LA activity, by dRVVT assay (Fig. 1C). Our data indicate that LA activity, prolonging coagulation time for a maximum of 7.1 s over the control value, is present only in those fractions at short retention times (peak 1), where high-molecular weight (> 300 kDa) IgM antibodies are predominantly eluted.

To confirm these findings, an IgM-enriched plasma concentrate was prepared by fractionated precipitation with PEG-8000[11]. The precipitate was dissolved in TBS, fractionated by SEC on a Sephacryl S-300

Table 2
Characteristics of patients LA + /aβ2GPI − .

P	Age	Sex	Autoimm disease	Obst	Thr	dRVVT mix ratio	SCT mix ratio	aCL IgG GPL	aCL IgM MPL	aβ2 IgG U	aβ2 IgM U	aPS/PT IgG U	aPS/PT IgM U
Normal values						< 1,16	< 1,22	< 10 U	< 8 U	< 13 U	< 7 U	< 30 U	< 30 U
1	64	M	Psoriasis	–	–	1,8	2,5	3	7	2	3	**13**	**250**
2	67	F	–	–	–	2,6	4,2	7	9	1	1	**18**	**250**
3	49	F	SLE	–	–	1,4	1,3	2	2	1	1	**10**	**250**
4	42	F	SLE	+[a]	–	1,5	2,6	4	2	8	1	**155**	**22**
5	42	F	–	–	–	1,4	1,6	1	7	1	8	**13**	**88**
6	57	M	UCTD[a]	–	–	1,18	1,4	1	2	1	1	**16**	**31**
7	57	F	Psoriasis	–	PE[b]	1,4	1,6	5	7	1	1	**149**	**70**
8	83	F	SLE	–	–	1,7	2,7	6	4	2	2	**5**	**250**
9	74	F	UCTD	+[a]	–	1,3	1,7	9	6	12	6	**5**	**100**
10	82	F	–	–	–	2,0	3,6	8	4	10	5	**37**	**11**
11	89	M	–	–	–	2,3	4,0	7	5	6	6	**15**	**180**
12	53	F	–	–	–	1,5	2,3	4	7	8	6	**5**	**250**
13	68	F	–	–	–	2,0	2,5	5	6	11	6	**5**	**250**
14	77	F	–	–	–	2,1	2,6	2	11	1	2	**1**	**250**
15	52	M	–	–	–	1,7	2,2	8	5	10	6	**55**	**64**
16	84	F	–	–	–	1,8	2,4	3	4	6	1	**164**	**10**
17	35	F	–	–	–	1,5	2,4	9	7	12	6	**101**	**56**
18	77	F	UCTD		–	1,3	1,8	1	2	1	1	**6**	**250**
19	62	M	–	–	–	1,7	1,9	4	2	1	1	**36**	**10**
20	7	F	UCTD	–	–	2,9	3,0	1	2	1	1	**138**	**52**
21	62	F	–		–	2,3	3,9	3	5	2	2	**1**	**175**
22	49	F	–	+[a]	–	1,25	1,5	2	3	5	3	**9**	**250**
23	30	M	Spondyl	–	–	1,35	1,69	5	7	1	3	**13**	**250**
24	63	F	UCTD	–	–	1,35	1,7	3	5	3	2	**7**	**90**
25	62	F	Sjogren	–	–	1,3	1,6	8	7	2	3	**9**	**250**

Obst denotes Obstetric complications; Thr denotes Thrombotic complications; UCTD = undifferentiated connective tissue disease.
[a] Pregnancy loss.
[b] PE denotes pulmonary embolism.

Table 3
Comparison between LA + /aβ2GPI − and LA + /aβ2GPI + patients.

	LA + /aβ2GPI − N = 25	LA + /aβ2GPI + N = 33	p
Age (yrs) mean ± SD	59.5 ± 19	50.8 ± 12.3	0.04
Sex (F) %	76	76	–
Autoimmune diseases n (%)	11 (44)	13 (39)	–
Thrombosis n (%)	1 (4)	28 (85)[a]	< 0.01
Pregnancy loss n (%)	3 (16)	6 (24)	–
dRVVT mixing ratio	1.7 ± 0.4	2.3 ± 1.0	0.01

[a] Site of thrombosis: 10 were arterial, 7 were venous, and 11 were both arterial and venous.

column, and fractions tested as above. Once again, the data in Fig. 2 clearly show that fractions eluting with peak 1 contain high IgM titers and possess remarkable LA activity, prolonging the dRVVT of normal plasma by 17 s (Fig. 2B and C). Noteworthy, there is a substantial overlapping between the content of IgM and LA activity and, importantly, when the same fractions were tested for the presence of IgM against phosphatidylserine/prothrombin complexes (aPS/PT) a profile resembling that of LA activity was obtained (Fig. 2D).

To better characterize the protein component(s) responsible for LA activity, an aliquot (10 μl) of the material eluted from the Sephacryl S-300 column as Peak-1, was injected on a highly resolving Yarra-3000 SEC column (Fig. 3). Only two peaks were eluted (P1 and P2, Panel C) from the column and, after column calibration with known protein standards (Fig. 3, panels A, B), a molecular weight of approximately 1.5 and 1.0 kDa was estimated for P1 and P2, respectively (Fig. 3, panel D). Notably, only P2 expressed LA activity and was positive in ELISA test to aPS/PT IgM.

3.1. LA + /aβ2GPI − patients

During the period 2014–2017, 25 plasma samples positive for LA and negative for IgG or IgM antibodies directed to β2GPI were collected and tested for the presence of aPS/PT antibodies. As shown in Table 2, all the patients were positive for aPS/PT antibodies that were predominantly of IgM isotype. Twelve out of 25 patients had associated autoimmune diseases. In three cases (two in patients with associated autoimmune disease), patients reported in their clinical history a pregnancy loss fulfilling the obstetric criteria for APS. One obese patient treated with estroprogestinic drugs had a pulmonary embolism.

During the same period 2014–2017, 33 plasma samples of patients with well characterized LA and positive for aβ2GPI antibodies were collected. A comparison of patients LA + /aβ2GPI − and LA + /aβ2GPI + is shown in Table 3. Mean age was significantly higher in patients with LA + /aβ2GPI − ($p = 0.04$) while gender and the rate of associated autoimmune disease did not differ between groups. There was one thromboembolic event in a LA + /aβ2GPI − patient and 28 thromboembolic events in patients with LA + /aβ2GPI + (4% vs 85%, $p < 0.01$). The strength of LA was significantly higher in LA + /aβ2GPI + patients.

All LA + /aβ2GPI + patients were also all positive for aPS/PT but the isotype was different. In fact, as shown in Fig. 4 (panel A), IgG aPS/PT titer was significantly lower in LA + /aβ2GPI − in comparison to that in LA + /aβ2GPI + (median 13 Units/ml, IQR 5.5–46.0 vs median 103 Units/ml, IQR 64.3–258.5; $p < 0.0001$). On the other hand (Fig. 4, panel B), IgM aPS/PT titer was not different between the two groups (median 175 Units/ml, IQR 54.0–315.0 vs median 111 Units/ml, IQR 49.4–201.0; p = ns).

4. Discussion

Our results show that all patients with LA not associated (isolated LA) or associated with the presence of aβ2GPI antibodies are positive for aPS/PT antibodies. We found that isolated LA (LA + /aβ2GPI −) and LA associated to the presence of aβ2GPI antibodies (LA + /aβ2GPI +) identify two distinct groups of patients with different clinical and

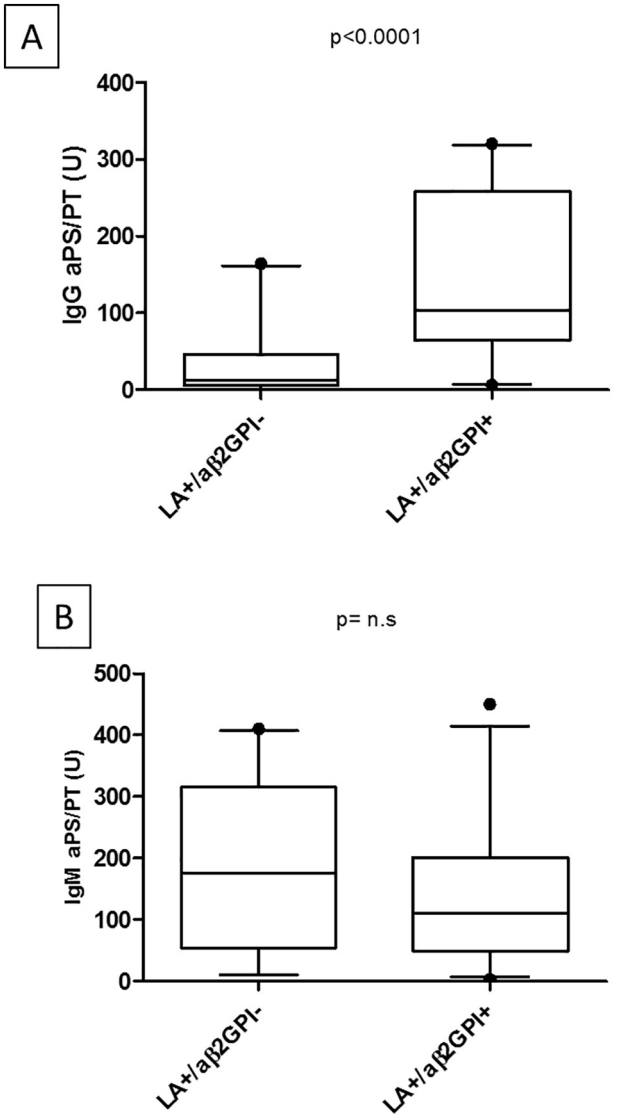


Fig. 4. IgG aPS/PT titer (panel A) and IgM aPS/PT (panel B) in patients with LA according to the presence of aβGPI antibodies.

laboratory characteristics. The two groups of patients showed significant differences with respect to age, history of thrombosis, and LA potency. Thus, only coexistence of LA, aβ2GPI and aPS/PT (mainly of IgG isotype) suggests strong thrombotic risk [13]. Moreover, the laboratory evaluation of the two groups indicates the dilute Russell Viper Venom Time (dRVVT) to be significantly more altered in LA+/aβ2GPI+, as previously reported [14]. Finally, an interesting observation is the prevalence of IgG aPS/PT isotype in patients LA+/aβ2GPI+, confirming the association of this isotype with thromboembolic events, even though we are not aware of the mechanisms behind the contribution of IgG aPS/PT to the development of thromboembolic events. We cannot affirm that these results can be generalized to the entire population of patients with isolated LA although the fact that all our patients are positive in aPS/PT is quite significant. Many reports in the literature provide evidence that phospholipid-dependent coagulation reactions are impaired by two types of LA, one requiring the presence of prothrombin and the other requiring the presence of β2GPI [15,16]. Moreover, it was demonstrated that anti-prothrombin and aPS/PT antibodies are present in most patients with LA [17–19] and could be removed by using insolubilized prothrombin [15]. Nevertheless, LA is still considered as single laboratory test predisposing to thrombosis and pregnancy loss. In this study, we show that there are two distinct types of LA, one associated with the presence of aPS/PT antibodies only and the other associate with both aβ2GPI and aPS/PT antibodies.

Initially we tried to understand which matter, substance or immunoglobulin was responsible for a strong LA activity in a patient testing negative for the presence of antibodies to β2GPI. The patient was a middle-aged man affected by psoriasis with no previous history of thromboembolic events. The LA activity of his plasma was confined to proteins with a molecular weight of > 300 kDa that perfectly overlapped with the detection of IgM aPS/PT. An analytical size exclusion chromatography confirmed that the LA activity was restricted to a protein of 996 kDa molecular weight testing positive for IgM aPS/PT. Following this experiment, we tested a series of patients LA+/aβ2GPI− and all of them were positive mainly for IgM or IgG aPS/PT or both. It is, thus, reasonable to hypothesize that the mechanism underlying the prolongation of phospholipid-dependent coagulation tests in the presence of such antibodies is similar to that described for aβ2GPI antibodies [20]. Antibodies bound to prothrombin on the phospholipid surface may hinder the activation of coagulation factors [21,22]. At variance with LA+/aβ2GPI+, aPS/PT antibodies present in patients LA+/aβ2GPI− are not associated with thromboembolic events [9]. Previous studies report an association between aPS/PT and LA with thrombosis [23–27] without distinguishing the patients according to the presence/absence of aβ2GPI antibodies.

In conclusion, in patients with positive LA is mandatory to search for of aβ2GPI antibodies in order to distinguish between patients at high vs low risk of thromboembolic events. Testing for aPS/PT provides an added value to the current tests exploring aPL antibodies to confirm the presence of an isolated LA.

Conflict of interest disclosures

None to declare.

CRediT authorship contribution statement

V. Pengo: Writing - original draft. **T. Del Ross:** Conceptualization, Data curation, Writing - original draft. **A. Ruffatti:** Conceptualization, Data curation, Writing - original draft. **E. Bison:** Methodology, Visualization. **M.G. Cattini:** Methodology, Visualization, Writing - review & editing. **E. Pontara:** Methodology, Visualization. **S. Testa:** Data curation, Writing - review & editing. **C. Legnani:** Data curation, Writing - review & editing. **N. Pozzi:** Methodology, Writing - review & editing. **D. Peterle:** Methodology, Visualization, Writing - review & editing. **L. Acquasaliente:** Methodology, Writing - review & editing. **V. De Filippis:** Writing - original draft. **G. Denas:** Writing - original draft.

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