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The combination of thrombophilic genotypes is associated with definite antiphospholipid syndrome

Ricardo Forastiero, Marta Martinuzzo, Yolanda Adamczuk, María Luisa Iglesias Varela, Gonzalo Pombo, Luis Oscar Carreras
Department of Haematology, Thrombosis and Haemostasis, Favaloro University and Institute of Cardiology and Cardiovascular Surgery, Favaloro Foundation, Buenos Aires, Argentina

Correspondence: Prof. Luis Oscar Carreras, Hematología, Universidad Favaloro, Solís 453, (C1078AAI) Buenos Aires, Argentina.
Phone: international +54.11.4378114-43781145. Fax: international +54.11.43810323. E-mail: carreras@favaloro.edu.ar

Background and Objectives. Thrombosis and pregnancy morbidity are clinical features of the definite antiphospholipid syndrome (APS). These clinical complications are also associated with the presence of inherited thrombophilias. Interactions between acquired and genetic risk factors are becoming increasingly related to a higher thrombotic risk. The aim of our study was to determine the prevalence of four common gene polymorphisms in patients with antiphospholipid antibodies (aPL).

Design and Methods. A series of 105 consecutive unselected patients with aPL grouped as having APS (n= 69) and not having APS (n= 36) was studied. A control group of 200 healthy subjects was also investigated for the presence of factor V Leiden (FVL), the 20210A allele of the prothrombin (PT-20210A) gene, the thermolabile variant (677TT) of methylenetetrahydrofolate reductase (MTHFR), and the 4G/4G genotype of the plasminogen activator inhibitor (PAI-1) promoter.

Results. Two patients who belong to the APS group carried the FVL while PT-20210A was found in 6 patients with APS (8.7%) and in 1 of the non-APS group (2.8%). The prevalence of FVL was similar to that found in the control group whereas PT-20210A was significantly more frequent in APS patients than in normal controls (2.0%, p=0.02). The MTHFR-677TT was found in 22.0%, 15.1% and 13.0%, and the PAI-1 (4G/4G) in 27.5%, 22.8% and 23.5% of APS, non-APS patients and normal controls, respectively. Furthermore, combinations of PT-20210A or FVL with PAI-1 (4G/4G) were significantly more frequent in APS patients (5.8%) than in normal controls (0.5%, p=0.016). This difference was not found between non-APS patients and normal subjects.

Interpretation and Conclusions. Present data indicate that testing for heritable thrombophilia would be important to identify aPL subjects with an increased risk of developing APS.

Key words: antiphospholipid syndrome, antiphospholipid antibodies, thrombophilia, thrombosis

The antiphospholipid syndrome (APS) is regarded as an autoantibody-mediated thrombotic disorder. Thrombosis in venous and arterial territories as well as pregnancy morbidity are clinical features of the definite APS.[1] However not all patients with antiphospholipid antibodies (aPL) develop APS, suggesting that other factors may influence the manifestation of such clinical features. In the last years several genetic polymorphisms have been identified as established or candidate risk factors in heritable thrombophilia.[2] The single point mutation (G1691A) in the factor V gene (factor V Leiden, FVL) is the most common cause of venous thromboembolism and responsible for resistance to activated protein C.[3] The G to A transition at nucleotide 20210 in the 3'-untranslated region of the prothrombin gene has been associated with increased plasma levels of factor II.[4]

Another common polymorphism is the C677T in the methylenetetrahydrofolate reductase (MTHFR) gene.[5] The thermolabile variant (677TT) of the MTHFR has been shown to exert a potential effect on plasma homocysteine levels but most studies have now concluded that this genotype is not per se a risk factor for thrombosis.[2,6] A 4G/5G polymorphism in the promoter region of the type 1 plasminogen activator inhibitor (PAI-1) gene has been related to PAI-1 plasma levels in some studies.[7]

Thrombosis is now recognized as a multifactorial disorder. Interactions between acquired and genetic risk factors contribute to thrombosis development.[8,9] The prevalence of the above genetic defects in patients with aPL has been reported by some groups.[10-14] However, most studies have evaluated only one or two of such polymorphisms. Recently, Galli *et al.*[15] studied FVL, prothrombin and MTHFR gene polymorphisms in a series of 152 patients with lupus anticoagulant (LA) and concluded that only FVL was associated with the thrombotic risk of patients with LA.

This case-control study was performed in a series of 105 consecutive unselected patients with persistent positivity of LA and/or anticardiolipin antibodies (aCL). Our main purpose was to assess the prevalence of single and combined gene polymorphisms in aPL patients grouped as having APS and not having APS.

Design and Methods

Patients and blood collection

The study was carried out on 105 consecutive unselected patients attending the Department of Hematology of the Favaloro Foundation from May 1998 to January 2001 who were found to be positive for aPL (LA and/or aCL). Our institution is a thrombosis reference center in our country and patients were referred for thrombophilia investigation because of their history of thrombosis. There were also patients who had been referred for aPL screening because of abnormal coagulation tests or a previous pregnancy loss. The presence of aPL was verified on at least two occasions three months apart. All patients were interviewed using a standardized questionnaire in order to collect information about personal and familial history of thrombotic events, pregnancy losses, current medication and circumstantial risk factors predisposing to thrombosis. The diagnosis of definite APS was made according to current criteria.[1] Thus, patients were grouped as having definite APS (APS group) and not having APS (non-APS group). Their main clinical data are shown in Table 1. Thrombotic events were documented by venography, Doppler ultrasound angiography, computerized tomography scan, and/or magnetic resonance imaging. Thirty patients had had recurrent thrombotic events before entering the study. Women with a history of at least three unexplained consecutive abortions before the 10th week of gestation or one or more unexplained fetal deaths at or beyond the 10th week of gestation were considered to have aPL-related obstetric complications for diagnosis of definite APS. In the non-APS group, there were 5 women who had had only one or two abortions (Table 1). Twenty-five patients were receiving oral anticoagulant therapy at the time of the study.

Table 1. Clinical and laboratory features of 105 aPL patients.

	APS	*Non-APS*
Number of patients	69	36
Sex (male/female)	25/44	11/25
Median age in years (range)	43 (15 to 77)	42 (12 to 84)
Previous thrombosis, n	61	-
Venous	38	-
Arterial	30	-
Previous obstetric complications, n	15	5
Transient cerebral ischemia, n	4	3
Cardiac valve disease, n	-	2
Systemic lupus erythematosus, n	7	7
Thrombocytopenia ($<150\times10^9$/L), n	12	4
Livedo reticularis, n	2	1
Hemolytic anemia, n	-	2
LA + aCL, n	41	13
LA alone, n	14	8
aCL alone, n	14	15
Anti-β_2 glycoprotein I antibodies, n	37	18
Anti-prothrombin antibodies, n	34	11

LA: lupus anticoagulant; aCL: anticardiolipin antibodies.

Two hundred unrelated healthy subjects (112 males, 88 females, median age 40 years, range 19 to 66) with no history of thrombosis or autoimmune disorders but from the same geographic area and ethnic background were recruited as control group. All the investigated individuals were Caucasians. Patients and controls gave informed consent to the study, which was approved by the Ethics Committee of our institution.

Blood samples were drawn by clean venipuncture and collected into plastic tubes containing 0.109 M trisodium citrate in a ratio of 9 parts of blood to 1 part of anticoagulant. Platelet-poor plasma was prepared by centrifugation (twice at 3,000 g for 10 min) at room temperature and assayed immediately. For sera preparation, blood was collected into glass tubes and allowed to clot at 37°C, and then centrifuged at 2,000 g for 10 min and stored at –70°C until use. For DNA isolation, whole blood samples were collected into tubes containing EDTA under sterile conditions and stored at -20°C until DNA extraction by standard procedures.

Studies for aPL and anti-protein antibodies

The presence of LA activity was investigated by means of several screening tests, mixing studies and confirmatory procedures as described in detail

before.[16,17] LA was diagnosed according to previously defined criteria.[16,18] Anticardiolipin antibodies (IgG and IgM isotypes) were measured using a standardized home-made ELISA technique.[19] Results were expressed as standard units for either IgG (GPL) or IgM (MPL). Levels above 20 GPL or MPL units were considered positive for diagnosis of APS.

The home-made ELISA for anti-β_2 glycoprotein I antibodies ($a\beta_2$GPI) was performed as previously reported[17] using microtiter plates (Nunc MaxiSorp, Kamstrup, Roskilde, Denmark) irradiated by an electron beam at 100 kGy and coated with purified human β_2GPI (Diagnostica Stago, Asnières, France). Anti-prothrombin antibodies were measured by ELISA as described before[17] using γ-irradiated plates (Nunc MaxiSorp) coated with human purified prothrombin (Diagnostica Stago). Results of both assays for IgG and IgM isotypes were expressed in arbitrary units (U). Values exceeding 15 U were regarded as positive.

Genetic analysis

All polymerase chain reactions (PCR) were carried out in a Perkin-Elmer 2400 Thermocycler (Perkin-Elmer, Norwalk CT, USA). Factor V Leiden (G1691A mutation) was evaluated by digestion with Mnl I (New England, Biolabs, Beverly, USA) of an amplified gene fragment obtained by using previously described primers.[3] The polymorphism (C677T) of the MTHFR gene was identified by digestion with Hinf I (Promega Corporation, Madison, USA) of the amplified fragment.[5] To detect the G20210A transition in the prothrombin gene and the 4G/5G polymorphism of PAI-1 gene promoter, we used allele-specific PCR as described elsewhere .[20,21]

Statistical analysis

Fisher's exact test was used to analyze categorical data. Odds ratios (OR) and 95% confidence intervals (CI) were calculated as estimates of relative risks by logistic regression models that controlled for age and sex. A two-tailed probability (*p*) less than 0.05 was taken to indicate statistical significance. Statistical evaluation was performed by means of the SPSS 10.0 package for Windows (SPSS Inc, Chicago USA).

Results

Among the 105 aPL patients, 69 were diagnosed as having definite APS whereas the remaining 36 comprised the non-APS group as shown in Table 1. The prevalence for each gene polymorphism observed in both groups of aPL patients is presented in Table 2. There were 2 heterozygous carriers of FVL among patients with APS, one with a history of recurrent deep venous thrombosis and the other with cerebral arterial thrombosis. Table 3 shows the details of the laboratory and clinical characteristics of aPL patients carrying FVL or PT-20210A. Among aPL patients carrying the PT-20210A, 6 had definite APS and 1 belonged to the non-APS group (all heterozygotes). This prothrombin variant was present in two APS patients, one of whom had experienced recurrent intrauterine fetal death and the other, four spontaneous abortions. Four out of 6 APS patients bearing the PT-20210A had vascular thrombosis, 2 a history of venous and 2 arterial thrombotic events. In two cases, the thrombotic episodes were recurrent. The frequencies of FVL, MTHFR-677TT and the 4G/4G genotype of the PAI-1 were not different either between the aPL groups and normal controls or between APS and non-APS groups. However, PT-20210A was significantly more frequent in APS patients than in normal controls (OR 4.67, p=0.02). In addition, this genetic variant was more prevalent in patients with APS (8.7%) than in those belonging to the non-APS group (2.8%) although the difference did not reach statistical significance (Table 2). We did not perform a separate analysis of prevalence of gene polymorphisms for the different clinical manifestations (venous thrombosis, arterial thrombosis and obstetric complications) because of the limited number of patients in each subgroup.

The two well-established thrombosis risk factors

Table 2. Prevalence of genetic polymorphisms in aPL patients with (n= 69) or without (n= 36) definite antiphospholipid syndrome and in normal controls.

	APS n (%)	Non-APS n (%)	Normal controls n (%)
Factor V (G1691A)			
Heterozygous	2 (2.9%)	0 (0%)	6 (3.0%)
Normal	67	36	194
Prothrombin (G20210A)			
Heterozygous	6 (8.7%)*	1 (2.8%)	4 (2.0%)
Normal	63	35	196
MTHFR (C677T)			
Homozygous	15 (22.0%)	5 (15.1%)	26 (13.0%)
Heterozygous and normal	53	28	174
PAI-1 (4G/5G)			
Homozygous	19 (27.5%)	8 (22.8%)	47 (23.5%)
Heterozygous and normal	50	27	153

**OR 4.67, 95%CI 1.28-17.07, p=0.02 (APS versus normal controls).*

Table 3. Laboratory and clinical data of the 9 aPL patients with well-established thrombotic risk factors.

Patient	*Age at the first event*	*Sex*	*LA*	*ACL uGPL/uMPL*	*$\alpha\beta_2$GPI (U) IgG/IgM*	*Anti-PT (U) IgG/IgM*	*FVL*	*PT-20210*	*Clinical manifestations*	*Diagnosis*
1	36	F	+	130/21	110/14	49/1	GA	GG	DVT	APS
2	53	M	-	37/2	15/5	3/1	GA	GG	CAT	APS
3	36	M	+	120/2	110/1	34/18	GG	GA	DVT	APS
4	30	F	+	8/8	1/3	7/4	GG	GA	RSA	APS
5	18	F	+	2/1	3/5	1/5	GG	GA	-	Non-APS
6	29	F	+	88/1	51/8	9/4	GG	GA	DVT	APS
7	70	F	+	8/6	7/1	1/5	GG	GA	CAT	APS
8	45	M	+	21/6	15/8	33/1	GG	GA	MI	APS
9	21	F	-	8/110	6/40	1/1	GG	GA	IFD	APS

LA: lupus anticoagulant; aCL: anticardiolipin antibodies; $\alpha\beta_2$GPI: anti-β_2 glycoprotein I antibodies; anti-PT: anti-prothrombin antibodies; FVL: factor V Leiden; PT-20210: prothrombin 20210; GG: normal genotype; GA: heterozygous genotype; APS: antiphospholipid syndrome; DVT: deep vein thrombosis; CAT: cerebral arterial thrombosis; RSA: recurrent spontaneous abortions; MI: myocardial infarction; IFD: intrauterine fetal death.

(FVL and the PT-20210A variant) and the PAI-gene polymorphism could be examined in 104 aPL patients and in the whole group of normal subjects. The coexistence of these genetic defects was observed in 4 aPL patients (3.8%) and in 1 normal subject (0.5%). Among the cases, 4 patients with definite APS carried both PT-20210A and the 4G/4G genotype of the PAI-1. The only healthy control with multiple genetic defects had FVL and PAI-1 (4G/4G). As indicated in Table 4, the prevalence of combined inherited prothrombotic risk factors in APS patients (5.8%) was significantly higher than in normal controls (p=0.016). In contrast, no difference was observed between non-APS patients and normal subjects. There was also a positive trend to a higher proportion of multiple genetic defects in APS patients compared with in non-APS ones but the difference did not reach statistical significance. The clinical features of these 4 APS patients with multiple genetic defects were as follows (Table 3; patients #7, 3, 9, 4): recurrent cerebral arterial thrombosis in 1, recurrent deep vein thrombosis in 1, two intrauterine fetal deaths in 1, and four spontaneous abortions in 1 patient.

Discussion

Antiphospholipid antibodies are closely related to the thrombotic diathesis in APS and several pathogenic mechanisms have been suggested involving effects on different cells, antithrombotic pathways and fibrinolysis.[22] It is not surprising that numerous mechanisms have been described considering the widely accepted heterogeneity of aPL.[22,23] However, not all patients with aPL develop the clinical features of APS. Thus, the possibility of a *second hit* is now becoming increasingly recognized. The concept of thrombosis as a multifactorial disease has received much attention in recent years.[2,8] The impact of the presence of some common genetic causes for thrombophilia in patients with aPL has been recently evaluated by some researchers.[10-15]

The inclusion of consecutive unselected patients who tested persistently positive for aPL allowed us to perform an evaluation of the prevalence of gene polymorphisms in patients grouped as having definite APS and not having APS. We found that only 2.9% of patients with APS carried FVL. This prevalence was similar to that detected in our normal group. Despite the fact that most of our APS patients had a history of venous or arterial thrombosis, the frequency of the FVL mutation was low. This finding is not in accordance with our previous study[24] that showed FVL as a significant risk factor for venous thromboembolism in the Argentinian population that descends mainly from immigrants from Spain and Italy. Nevertheless, our data are in agreement with most studies reporting a low prevalence of FVL in aPL patients with or without systemic lupus erythematosus.[10,15,25] Ames *et al.*[13] concluded that FVL may contribute to the hypercoagulability occurring in a small proportion of thrombotic aPL subjects. Both aPL patients in our population bearing FVL had experienced thrombotic events, an occurrence also demonstrated by Galli *et al.*[15] in their 5 patients with heterozygous FVL. Taking into account the very frequent *acquired* activated protein C resistance (APCR) phenomenon due

Table 4. Prevalence of combinations of FVL or PT-20210A with the 4G/4G genotype of the PAI-1 in aPL patients and in normal controls.

	Multiple genetic defects	*OR (95%CI)**	*p value*
APS (n= 69)	4 (5.8%)	12.2 (1.3-111.6)	0.016
Non-APS (n= 35)	0 (0%)		
Normal controls (n= 200)	1 (0.5%)		

**Comparison versus normal controls.*

to autoimmune aPL,[26] it is likely that the combination of both congenital defects and acquired APCR may interact to increase thrombin generation.

With respect to PT-20210A, our findings in APS patients show a prevalence in close similarity to that previously reported in our patients with venous thrombosis, regardless of their aPL status.[24] These results are in clear contrast to data published by other authors in patients with APS. Bentolila *et al.*[11] and Bertolaccini *et al.*[12] found only 2 out of 119 APS patients carrying the prothrombin mutation. This discrepancy is difficult to explain but one reason could be the way in which patients were enrolled. In the present study we used inclusion criteria similar to those employed in the Italian report.[15] In this way, all consecutive unselected patients having aPL were enrolled. Galli *et al.*[15] studied the PT-20210A in 145 patients with LA and reported a prevalence of around 6% in patients with a history of venous or arterial thrombosis but a group of healthy subjects was not included. We found a high frequency (8.7%) of heterozygous carriers of PT-20210A in patients diagnosed as having definite APS compared with less than 3% in either non-APS patients or normal controls. This high frequency was also found even when considering only patients with LA (n= 76) with or without aCL as in the Italian report.[15] Our findings are in close agreement with those of a recent report[27] in which the 20210A allele of the prothrombin gene but not FVL was significantly much more prevalent in APS patients than in normal subjects.

The prevalence of MTHFR-677TT as well as the homozygous state (4G/4G) of the PAI-1 in APS patients were slightly higher, but not significantly so, than in non-APS patients and healthy subjects. Regarding the thermolabile variant of MTHFR, our data are similar to those in three published reports.[13,15,27] This is the second study evaluating the 4G/5G polymorphism of the PAI-1 gene in patients with aPL. In the first one,[14] the authors studied a larger population of patients with APS (n= 110) and the major finding was a higher frequency of the 4G allele in APS patients with thrombosis compared with in those without thrombosis. This was mainly attributable to a higher prevalence in patients with arterial events. We also found a trend towards a higher frequency of PAI-1 (4G/4G) in aPL patients with arterial thrombosis versus those with venous thrombosis (data not shown).

Most of the previous studies have analyzed the presence of one or two of the prothrombotic genetic risk factors in cohorts of aPL patients. Up to now, there have been only two reports in which three common gene polymorphisms were simultaneously evaluated.[15,27] So far it has been well-established that combinations of thrombophilic conditions may further increase the risk of thromboembolic events and also of obstetric complications,[2,8,28] but no particular attempt has been made to determine whether multiple genetic factors can be identified in aPL patients. We, therefore, decided to study the simultaneous occurrence of gene polymorphisms which are included in our work-up for thrombophilia. The MTHFR polymorphism was excluded from the analysis of multiple thrombophilic genotypes because of the recent overall opinion that this gene polymorphism does not seem to contribute to thrombotic risk.[2,6] Our data show that a higher proportion of patients diagnosed as having definite APS have the PT-20210A variant combined with the 4G/4G genotype of the PAI-1 than patients with aPL without clinical features of APS and healthy controls. Thus, it is likely that when potential genetic risk factors exert their action simultaneously, these effects may interact and the final effect may exceed the sum of the separate actions. This could be particularly relevant in patients with additional acquired factors, such as autoimmune aPL.

In conclusion, the presence of prothrombotic genetic defects might influence the development of APS-related clinical features in a subpopulation of patients with aPL. Therefore, testing for heritable thrombophilia would be important in order to identify aPL subjects with an increased risk of APS. However, larger cohorts of aPL patients will have to be studied in order to confirm these findings.

Contributions and Acknowledgments

RF, MM and LOC were the main investigators involved in the conception and design of the study, analysis and interpretation of the results, and also wrote the manuscript. YA and MLIV were mainly responsible for gene polymorphism evaluations. GP was involved in the inclusion of cases and controls

and participated in the analysis of the results. All authors gave significant contributions to drafting the article and final approval of the version to be submitted. The order of authorship was a joint decision of the co-authors considering the importance of the contribution to the realization of the whole study from the conception and design to the approval of the final version of the manuscript.

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Disclosures

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Potential implications for clinical practice

Testing for inherited thrombophilic risk factors would be important to identify aPL subjects with an increased risk of developing APS (see also recent papers in this journal on this topic[29-30])

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