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## Concise report

## Interest of IgG and IgM antiprothrombin autoantibodies in the exploration of antiphospholipid syndrome: a 5-year retrospective study

Daniel Bertin<sup>1</sup>, Abdelouahab Beziane<sup>1</sup>, Noemie Resseguier<sup>2</sup>, Morgane Pelissier<sup>1</sup>, Pierre-Emmanuel Morange<sup>3,4</sup>, Jean Louis Mege<sup>1,5</sup> and Nathalie Bardin<sup>1,4</sup>

## Abstract

**Objectives.** Non-conventional aPL have been described in patients presenting clinical manifestations of antiphospholipid syndrome but negative for conventional markers. Among them, detection of autoantibodies against prothrombin has been proposed to improve diagnosis and management of these patients. However autoantibodies against prothrombin are heterogeneous and their use in clinical practice still remains unclear. The aim of this study was to evaluate the interest of IgG and IgM autoantibodies directed against the prothrombin only (aPT).

**Methods.** We retrospectively studied IgM and IgG aPT results, conventional antiphospholipid syndrome markers and clinical data of a large cohort of 441 patients referred for antiphospholipid syndrome exploration with aPT detection over a period of 5 years.

**Results.** We observed a total prevalence of 17% of aPT-positive patients (75/441). A significant association was found between aPT and thrombosis ( $P = 0.035$ ), with 70% of patients having unexplained thrombosis, aPT representing the sole aPL detected. aPT positivity was significantly more frequent in venous thrombosis than in arterial thrombosis ( $P = 0.004$ ). Interestingly, we demonstrated for the first time that aPT IgG levels were higher in recurrent thrombosis than in isolated thrombosis ( $P = 0.013$ ), leading us to propose a predictive level of recurrence for thrombosis.

**Conclusion.** Our results show that aPT are associated with thrombosis and demonstrate the interest of assessing both IgG and IgM aPT, in particular in venous thrombosis when conventional markers are negative. Quantification of aPT could predict recurrence of thrombosis and influence subsequent treatment strategy. Prospective clinical studies are now required to confirm these results.

**Key words:** anti-prothrombin antibodies, IgG, IgM, autoantibodies, antiphospholipid syndrome, thrombosis

## Rheumatology key messages

- Anti-prothrombin (aPT) antibodies are detected in sera of patients suffering from venous thrombosis.
- aPT antibodies are often found alone without any other conventional markers of APS.
- Measure of aPT levels is important because high levels are associated with recurrent thrombosis.

## Introduction

Antiphospholipid syndrome (APS) is an autoimmune disease characterized by the association of thrombosis and/or obstetric manifestations and the persistent positivity of

conventional aPL including: LA, IgG and/or IgM aCL and IgG and/or IgM anti- $\beta_2$  glycoprotein I autoantibodies [1]. Beside these markers, non-conventional aPL have been described in patients presenting clinical manifestations highly suggestive of APS but persistently negative for conventional markers [2, 3]. Among them, the detection of autoantibodies against prothrombin (aPT), has been proposed to improve the diagnosis and thus the management of these patients [4]. These antibodies against prothrombin have been identified as co-factors of LA and their prothrombotic effect has been described in an *ex-vivo* mice model after active immunization of animals with prothrombin [5]. The prevalence of aPT and their association with clinical features of APS have been investigated in previous studies [6, 7]. But, results are still controversial,

<sup>1</sup>AP-HM, Hôpital de la Conception, Pôle de Biologie, Service d'immunologie, <sup>2</sup>Aix-Marseille Univ, Unité de Recherche EA 3279, Service de Santé Publique, <sup>3</sup>AP-HM, Hôpital de la Timone, Pôle de Biologie, Service d'hématologie, <sup>4</sup>Aix Marseille Univ, INSERM UMR\_S 1263, C2VN and <sup>5</sup>Aix-Marseille Univ, IRD, MEPHI, IHU-Méditerranée Infection, Marseille, France

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Correspondence to: Daniel Bertin, Laboratoire d'immunologie, Pôle de Biologie, Hôpital de la Conception, 147, Bd Baille, 13005 Marseille, France. E-mail: daniel.bertin@ap-hm.fr

especially regarding the diagnostic interest of these antibodies, and the isotype to explore in routine practice.

The aim of the study was to evaluate the interest of IgG and IgM aPT detection in the exploration of APS in a large cohort of 441 patients successively referred to the Immunology laboratory over a period of 5 years.

## Methods

### Patients

We performed a retrospective study of the laboratory results of all patients who underwent IgG and IgM aPT testing at the University Hospital of Marseilles between September 2011 and September 2016. Reasons for testing were the suspicion or presence of autoimmune disorders, recent or previous thrombosis and/or obstetrical complications. A total of 441 assaying results for IgG and IgM aPT were extracted from the results server of the laboratory. All samples were part of a declared Biobank (DC 2012-1704) in compliance with ethical directives. This retrospective study exclusively analysed data issuing from healthcare. It was approved and registered by the institution and fulfilled local requirements in terms of data collection, patients' consent and protection of data.

### Clinical data

Clinical data included demographic parameters (age, gender), occurrence of arterial/venous thromboembolic events, pregnancy morbidity, auto-immune diseases, active infectious disease and neurological diseases including inflammatory diseases of the central nervous system such as multiple sclerosis or myelitis.

Arterial thrombotic events included transient ischaemic attack, cerebrovascular accident or other acute events involving the arterial vasculature.

Venous events included deep vein thrombosis, pulmonary embolism or other acute events involving the venous vasculature.

### aPT ELISA

For the detection of aPT, we used an in-house ELISA previously described [8].  $\Delta OD$  (optical density) for each sample was calculated by subtracting OD of coated well from OD obtained with non-coated well. The aPT levels were reported as a ratio of  $\Delta OD$  of patient/ $\Delta OD$  of a selected control serum with a  $\Delta OD$  at the cut-off value. The result is positive when the ratio is  $\geq 1$ . The cut-off values were calculated as the 99th percentile of  $\Delta OD$  values obtained from analysis of 100 healthy-blood donors' sera.

Inter-assay variability was measured for both aPT IgG and aPT IgM ELISA assays. Coefficient of variation (%CV) of results from one weak positive sample was equal to 9.1% for aPT IgM and 13.2% for aPT IgG.

### Statistical analysis

Comparative analysis between aPT positivity/negativity and the type of disease was performed using chi-squared test when valid (otherwise using Fisher's exact test).

Adjusted *P*-values were provided given the multiplicity of comparisons within each analysis. The Benjamini and Hochberg [9] method was used to control the false discovery rate.

Comparative analysis between aPT antibodies levels and recurrence of thrombosis was performed using Mann-Whitney *U* test.

To define the best threshold value of aPT IgG ratio for discriminating patients with and without thrombosis recurrence, receiver operating characteristic and accuracy curves were established, according to the Youden's method [10] which allows the maximization of both sensitivity and specificity. Accuracy was defined as the proportion of the total number of correct predictions of thrombosis recurrence obtained from the aPT IgG threshold value.

All statistical analyses were performed using R software. All tests were two-sided, and *P* < 0.05 defined statistical significance.

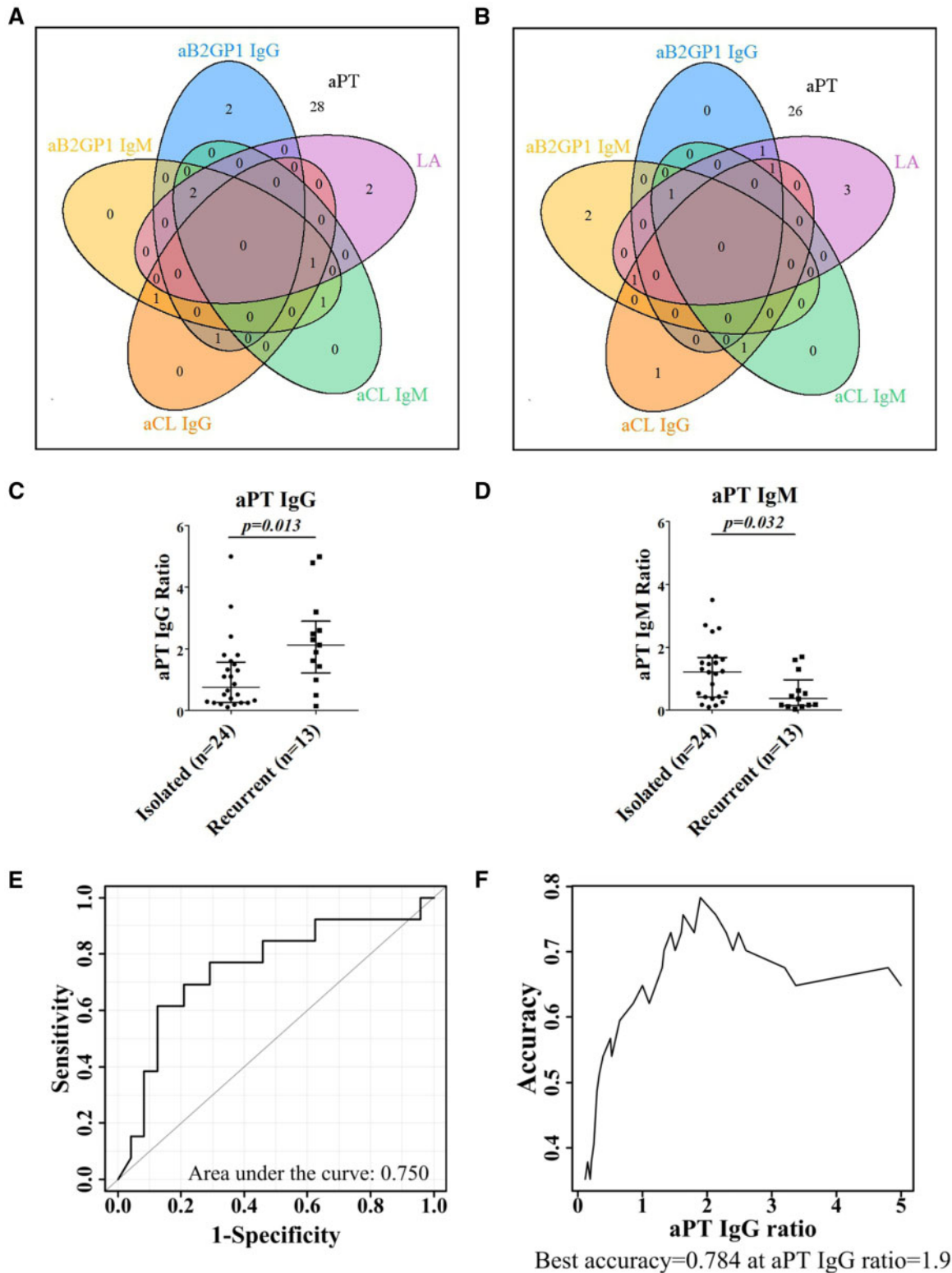
## Results

Detection of IgG and IgM aPT were performed in a total of 441 patients. Patients' serum samples were mostly addressed to our Immunology laboratory by Internal Medicine (39%), Neurology (29%), and to a lesser extent by Paediatric (4%) and Infectious Disease (4%) departments. All patients lived in the southeast of France. Mean (s.d.) age was 45 (18) years and 63.5% of patients were women.

We found a total prevalence of 17% of aPT-positive patients (75/441) that is above the low prevalence of 1% observed in controls (100 healthy-blood donors). Of the 75 aPT-positive patients, 41 were aPT IgG only, 30 IgM only and 4 both IgG and IgM. Inside the aPT-positive patient group, 38 patients (Fig. 1A) had no thrombosis and, among them, 28 patients were aPL-negative, whereas 10 were aPL-positive, of which five were women without obstetrical complication.

To further analyse results, clinical data were collected (Table 1). We found a significant association between aPT positivity and thrombosis (*P* = 0.035) but not with obstetrical complications (*P* = 0.37) or infectious disease (*P* = 0.132). Because thrombosis was the most prevalent context associated with aPT positivity, we further analysed results by focusing on the 37 aPT-positive patients suffering from thrombosis. aPT positivity was significantly associated with venous thrombosis (*P* = 0.004) but not with arterial thrombosis (*P* = 0.799). We observed that both IgG and IgM isotypes were represented (20 patients with aPT IgG only, 15 with aPT IgM only and two with both IgG and IgM aPT). Only 30% (11/37) presented thrombotic events in an APS context, with 82% (9/11) of patients positive for LA and/or anti- $\beta 2$  glycoprotein I autoantibodies (Fig. 1B). In this subgroup only positive for aPT, we observed 14 patients with cerebral stroke, 8 with pulmonary embolism, 20 with deep vein thrombosis of the leg, 3 with renal vein thrombosis and 2 with jugular vein thrombosis. In addition, 27 patients had a single-site thrombosis while 10 patients presented a thrombosis of multiple

Fig. 1 Qualitative and quantitative study of aPT in thrombotic patients



(A, B) Venn diagrams with detailed serological profile of aPL detected in sera of aPT positive non-thrombotic patients (A,  $n=38$ ) and aPT positive thrombotic patients (B,  $n=37$ ); aB2GP1: anti- $\beta 2$  glycoprotein I antibodies; aPT: anti-prothrombin antibodies; LA: lupus anticoagulant (C, D). Comparison of aPT levels for each isotype in patients suffering from isolated or recurrent thrombosis (C, IgG; D, IgM). (E, F) Determination of aPT IgG ratio threshold for predicting recurrence of thrombosis in thrombotic patients with positive aPT IgG (E, receiver operating characteristic (ROC) curve; F, accuracy curve).

TABLE 1 Clinical data of patients according to their serological aPT profile

	aPT quantitative results						aPT qualitative results					
	aPT-			aPT+			aPT-			aPT+		
	IgG	IgM	IgG	IgG	IgM	IgM	aPT-	IgG only	IgM only	IgG and IgM	IgG and/or IgM	P
Venous thrombosis	0.32 (0.27) (0.20) {0.33}	0.33 (0.23) (0.24) {0.33}	1.67 (1.55) (1.3) {1.84}	1.07 (0.91) (0.82) {1.16}	53 (14.5) (19.9)	12 (16) (12)	9 (12) (12)	2 (2.7) (2.7)	23 (30.7) (30.7)	0.0040		
Arterial thrombosis	[0.00, 0.092] 0.27 (0.21) (0.21) {0.22}	[0.02, 0.093] 0.32 (0.24) (0.26) {0.36}	[0.15, 5] 1.27 (0.98) (1.3) {0.23}	[0.09, 3.5] 0.86 (0.81) (0.52) {1.27}	73 (19.9) (19.9)	8 (10.7) (10.7)	6 (8) (8)	0 (0) (0)	14 (18.7) (18.7)	0.7999		
Neurological disorders	[0.02, 0.097] 0.24 (0.18) (0.20) {0.15}	[0.02, 0.094] 0.33 (0.21) (0.29) {0.31}	[0.11, 3.2] 0.42 (0.37) (0.31) {1.34}	[0.03, 2.7] 1.42 (0.64) (1.3) {0.38}	93 (25.4) (25.4)	1 (1.3) (1.3)	5 (6.7) (6.7)	0 (0) (0)	6 (8) (8)	0.0040		
Infectious diseases	[0.02, 0.093] 0.23 (0.18) (0.17) {0.12}	[0.03, 0.93] 0.25 (0.21) (0.22) {0.18}	[0.16, 1.15] 3.78 (1.54) (4.2) {1.88}	[0.52, 2.47] 1.25 (1.84) (0.41) {0.97}	5 (1.4) (1.4)	3 (4) (4)	0 (0) (0)	1 (1.3) (1.3)	4 (5.3) (5.3)	0.1318		
Obstetrical morbidity	[0.07, 0.53] 0.38 (0.26) (0.35) {0.32}	[0.09, 0.6] 0.40 (0.21) (0.41) {0.31}	[1.7, 5] 0.69 (1.48) (0.51) {0.31}	[0.17, 4] 1.31 (1.12) (1.04) {0.71}	32 (8.7) (8.7)	1 (1.3) (1.3)	3 (4) (4)	0 (0) (0)	4 (5.3) (5.3)	0.3724		
SLE	[0.06, 0.93] 0.274 (0.17) (0.21) {0.30}	[0.06, 0.93] 0.26 (0.13) (0.28) {0.17}	[0.33, 1.4] 1.98 (0.84) (2.05) {1.03}	[0.26, 2.9] 0.57 (0.78) (0.21) {0.4}	15 (4.1) (4.1)	5 (6.7) (6.7)	0 (0) (0)	1 (1.3) (1.3)	6 (8) (8)	0.1941		
Other autoimmune diseases	[0.04, 0.5] 0.37 (0.19) (0.33) {0.21}	0.37 (0.23) (0.30) {0.31}	1.13 (0.99) (0.56) {1.61}	2.13 (3.06) (1.48) {1.38}	19 (5.2) (5.2)	3 (4) (4)	5 (6.7) (6.7)	0 (0) (0)	8 (10.7) (10.7)	0.1941		
Other diseases	[0.10, 0.89] 0.30 (0.22) (0.26) {0.24}	[0.09, 0.87] 0.35 (0.22) (0.31) {0.35}	[0.23, 2.6] 1.57 (0.97) (1.25) {0.55}	[0.22, 9.5] 0.62 (0.45) (0.53) {0.52}	76 (20.8) (20.8)	8 (10.7) (10.7)	2 (2.6) (2.6)	0 (0) (0)	10 (13.3) (13.3)	0.1941		
	[0.03, 0.90]	[0.04, 0.95]	[0.55, 3.5]	[0.17, 1.57]								

Quantitative data are given as mean (s.d.) (median) [interquartile range] [range] and qualitative data as number (percentage) values. aPT: anti-prothrombin autoantibodies.

vascular sites. No difference was found in isotype repartition with 16 aPT IgG and 12 aPT IgM.

In our study, we observed that 13 IgG aPT positive patients had recurrent thrombosis. Interestingly, among them, eight were negative for conventional aPL, showing that the majority of patients, 62% (8/13), suffering from recurrent thrombosis with positive IgG aPT, were negative for conventional aPL.

Of interest, we showed that IgG aPT levels were significantly higher in patients with recurrent thrombosis than in patients with isolated thrombosis ( $P = 0.013$ , Fig. 1C) whereas IgM aPT levels were significantly higher in patients with isolated thrombosis ( $P = 0.032$ , Fig. 1D) than in patients with recurrent thrombosis. A calculated aPT IgG index superior to 1.9 was predictive of thrombosis recurrence (Fig. 1E and F). This demonstrates the interest to quantify aPT level in order to predict the recurrence of thrombosis.

## Discussion

In this 5-year retrospective study, we demonstrated the interest of aPT in the exploration of APS in particular when conventional markers are negative and showed that the quantification of aPT level could predict the recurrence of thrombosis.

In recent years, the interest of antibodies directed against PT for the exploration of APS has been poorly studied in the literature and their use in routine testing is still unclear. Several studies showed that these antibodies correspond to an heterogeneous group of antibodies with different contribution in the diagnostic strategy of APS [11]. At least two different kinds of antibodies directed against PT have been described: those targeting prothrombin (aPT) and those targeting anti-phosphatidylserine/prothrombin complex (aPS/PT) [12]. Recent studies are mainly focusing on anti-phosphatidylserine/prothrombin antibodies (aPS/PT) whereas they are redundant with LA [13–16] and have been proposed in cases of difficult LA interpretation [17].

In this study, we found a total prevalence of 17% of aPT-positive patients (75/441). This result is well above the low prevalence reported for healthy subjects in the literature [12]. No difference was found in isotype repartition, highlighting the interest of detecting both IgG and IgM isotypes of aPT. Moreover, the fact that 70% (26/37) of the aPT-positive patients had an unexplained thrombosis without any conventional aPL supports the contention that aPT detection could be systematically investigated to improve the management of this category of patients. With respect to anti- $\beta_2$  glycoprotein I autoantibodies antibodies, which have been well characterized, the interest of aPT detection remains controversial. A previous meta-analysis failed to show any association between aPT antibodies and the risk of thrombosis [18]. More recently, a systematic review of literature aggregating data from 38 studies found that aPT represents a risk factor for thrombotic events [4], in agreement with our results. We showed that aPT are significantly associated with thrombosis, a clinical context related to APS and

more precisely that aPT are significantly associated with deep venous thrombosis.

Lastly, we are the first to show that aPT levels are higher in recurrent thrombosis than in isolated thrombosis ( $P = 0.013$ ), leading us to propose a predictive level of recurrence that could subsequently influence treatment strategy.

The persistent positivity of autoantibodies is one of the definition criteria of APS. It is less described for the non-conventional aPL, often considered as risk factors. Nevertheless, in our study, among the small number of patients with follow-up, >50% of them were persistently positive (data not shown).

In conclusion, we evidenced the interest to investigate both IgG and IgM aPT isotype in the laboratory exploration of APS, in particular in patients with venous thrombosis when conventional markers are negative. Prospective clinical studies are now required to confirm that the quantification of these aPT represents a help for predicting the recurrence of thrombosis.

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D.B. and M.P. gathered and analysed data. A.B. performed the ELISA assays. D.B. and N.R. conceived and performed statistical study of results. N.B. and D.B. wrote the manuscript. J.L.M. and P.-E.M. discussed the results and contributed to the final manuscript.

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