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PII: S0049-3848(18)30451-1
DOI: doi:10.1016/j.thromres.2018.08.003
Reference: TR 7112

To appear in: *Thrombosis Research*

Received date: 27 April 2018
Revised date: 17 July 2018
Accepted date: 6 August 2018

Please cite this article as: M. Efthymiou, D.R.J. Arachchillage, P.J. Lane, A.G. O'Keeffe, T. McDonnell, H. Cohen, I.J. Mackie , Antibodies against TFPI and protein C are associated with a severe thrombotic phenotype in patients with and without antiphospholipid syndrome. Tr (2018), doi:10.1016/j.thromres.2018.08.003

Antibodies against TFPI and protein C are associated with a severe thrombotic phenotype in patients with and without antiphospholipid syndrome

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Running Title: TFPI and protein C antibodies in thrombotic patients

Key Words: Antiphospholipid syndrome, TFPI, antibodies, protein C, thrombosis

Abstract word count: 237

Text word count: 5807

Number of Tables and Figures: 3 Tables and 4 Figures

Abstract

Background: Tissue factor pathway inhibitor (TFPI) antibodies, which have been reported in patients with antiphospholipid syndrome (APS), may impair TFPI activity and contribute to hypercoagulability, but their role in APS and in thrombosis remains undefined.

Objective/Methods: We assessed the presence and avidity of TFPI IgG antibodies, associations with protein C IgG antibodies and associations with clinical disease severity, in 50 patients with thrombotic APS and 50 thrombotic control patients, on long term anticoagulation with warfarin.

Results: Thrombotic APS patients had a significantly higher prevalence of TFPI IgG antibodies (40%; 20/50) compared to thrombotic controls (18%; 9/50). TFPI antibodies were predominantly high avidity in APS (50%, 10/20 of positive patients) and strongly associated with a severe thrombotic phenotype (venous and arterial thromboembolism or recurrent thromboembolic episodes despite therapeutic anticoagulation) (odds ratio (OR): 12.0, 95%CI: 2.2-66.1, p=0.004), while thrombotic control patients mainly showed low avidity antibodies (78%, 7/9 of positive patients). Coexistence of TFPI and protein C IgG antibodies, regardless of their avidity, was strongly associated with a more severe thrombotic phenotype in APS patients (OR: 20.2, 95%CI: 2.0-47.0, p<0.0001) and also in thrombotic controls (OR: 75.0, 95%CI 1.2-195, p=0.02).

Conclusions: Coexistent TFPI and protein C IgG antibodies, irrespective of their avidity, may be a useful marker for a severe thrombotic phenotype in thrombotic patients. This suggests a possibly pathophysiological relationship between the two antibodies, predisposing to thrombosis with a possibly more general role in the development of thrombotic complications.

Introduction

Patients with thrombotic antiphospholipid syndrome (APS) inherently differ from other patients with venous or arterial thrombosis due to the presence of antiphospholipid antibodies (aPL), which are known to interfere with a number of haemostatic mechanisms. *In vitro* and animal studies provide evidence of the thrombogenic potential of aPL (1-7). In animal models of thrombosis, both polyclonal (isolated from APS patients), and monoclonal (human and murine) aPL have been shown to enhance thrombus formation (3;5;6). Human derived aPL have also been shown to be directly pathogenic in thrombosis and pregnancy morbidity (8;9).

It has been proposed that activation or upregulation of the tissue factor (TF) pathway is integral to the hypercoagulable state observed in APS, (10-12)mainly due todown regulation of its principal inhibitor, tissue factor pathway inhibitor (TFPI) (12-14). Many *in vitro* studies have shown aPL (and specifically anti-β_2 glycoprotein-1 antibodies; aβ2GPI) mediate the activation of endothelial cells with upregulation of TF expression, both at mRNA and protein level, along with inflammatory cytokines and adhesion molecules (2;5;7;15-17); aβ2GPI has also been shown to suppress TFPI dependent inhibition of the TF pathway of coagulation (13). Several studies have reported the presence of TFPI antibodies in APS patients (12;18-21), suggesting a possible contributory role in the upregulation of the TF pathway of coagulation and the observed hypercoagulability. However, the role of TFPI antibodies in APS or associations with the severity of the clinical APS phenotype is unclear. Associations with protein C antibodies, which we have shown to be associated with increased acquired protein C resistance and a more severe APS thrombotic phenotype (22), are unknown. Accurate definition of TFPI and protein C

antibodies might provide additional information to identify patients with a more severe thrombotic phenotype who could benefit from more aggressive treatment.

The aim of this cross-sectional study was to evaluate the presence and avidity of TFPI IgG antibodies, associations with protein C IgG antibodies and also with clinical disease severity, in a well characterised cohort of patients with thrombotic APS compared to thrombotic patients without APS, on long term anticoagulation with warfarin.

Methods

Subjects and blood samples

One-hundred patients (50 APS and 50 without APS, the latter referred to as thrombotic controls), with a history of venous and/or arterial thrombosis, who had received either standard or high-intensity warfarin treatment (target international normalized ratio [INR] 2.5 and 3.5, respectively) for at least six months since the thromboembolic event were recruited in this cross-sectional study. Patients on any other oral anticoagulants or low molecular weight heparin were excluded. All patients were recruited at specialist Haematology and Rheumatology outpatient clinics at University College London Hospitals (UCLH) NHS Foundation Trust. Patients were randomly recruited as they attended the clinics; APS and thrombotic control patients included in this study were subsequently selected to match mean ages between the two patient groups.

Patients with a target INR target of 3.5 comprised those with a history of recurrent strokes/transient ischemic attacks, or recurrent VTE while on therapeutic anticoagulation (23;24). Despite the lack of an agreed published definition for the

clinical severity of thrombotic APS, patients with recurrent VTE or AT are generally considered to be high risk patients (25;26). In the current study the term 'severe thrombotic phenotype' has been used to identify patients at high risk with VTE as well as AT or recurrent thromboembolic episodes despite therapeutic anticoagulation (warfarin target INR 2.5, range 2.0-3.0) in line with our previous work (27).

One hundred normal controls (NC) were also recruited from staff members (all tested and found to be aPL negative). Written informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. Ethical approval was granted by the Research Ethics Committee NREC (reference: 13/EM/0150) and from the Research and Development office at UCLH (reference: 13/0030).

Patients (APS and thrombotic controls) were excluded if they had heritable thrombophilia (factor V Leiden or the G20210A prothrombin gene mutation, antithrombin, protein S or protein C deficiency), a history of malignancy or myeloproliferative neoplasms. Patients and NC were also excluded if they were receiving estrogen preparations (combined oral contraceptives or hormone replacement therapy) or were pregnant. Patients with APS fulfilled the revised international consensus criteria for APS (28). Clinical and laboratory features of patients are presented in Table 1.

aPL status (lupus anticoagulant (LA), IgG and IgM anticardiolipin (aCL) and aβ_2GPI) were routinely assessed in the hospital laboratory in accordance with international consensus criteria and national guidelines (28-30). LA activity was identified through at least two different screening tests, mixing studies and confirmatory procedures according to the International Society on Haemostasis and Thrombosis (ISTH) (29) and the British Committee for Standards in Haematology (BCSH) (30) guidelines.

The 99th centile was used as a cut off to define aPL positivity in thrombotic APS patients; aCL positivity was defined as: >99th centile= >20 MPLU, and aβ_2GPI positivity was defined as: >99th centile= >20 SGu/SMu. Prothrombin time/INR was assessed with a rabbit brain thromboplastin, PT-Fibrinogen HS Plus on a TOP500 (Werfen, Warrington, UK) with an analyser-specific international sensitivity index (1.14). Factor X activity was measured with an amidolytic assay (Hyphen Biomed, Neuville-Sur-Oise, France) on the CS-2000*i* analyser (Sysmex UK, Milton Keynes, UK) (31). A previously established therapeutic range for amidolytic factor X of 18–33 IU/dL, which corresponds to INR 2·0–3·0, was used to assess anticoagulation intensity (31). The intra-assay coefficient of variation with normal plasma was 8.3%.

Venous blood was collected using a 21 gauge butterfly needle, with minimal venous stasis, into 5 mL Vacutainer® tubes (Becton Dickinson, Plymouth, UK) containing 0.105M citrate. Platelet poor plasma was prepared within two hours of collection by double centrifugation at ambient temperature (2000g for 15 minutes) and stored in aliquots at -80°C. Immediately prior to analysis the samples were thawed in a water bath at 37°C.

Detection of TFPI antibodies with in house-ELISA

96-well micro-plates (Nunc MaxiSorp, Fisher Scientific, Loughborough, UK) were coated with recombinant human full-length TFPI expressed in E. coli (rTFPI; Chiron Corp., Emeryville, USA) diluted in 0.05mol/L carbonate/bicarbonate buffer pH 9.6 to a final concentration of 10µg/mL, and incubated at 4˚C overnight. Following incubation, plates were washed in triplicate with Tris Buffer Saline (TBS)-0.1% Tween (TBST) and then blocked for 2 hours with TBS containing 2.5% BSA (A7030, Sigma Aldrich, Poole, UK). Plates were washed with TBST and duplicate samples,

diluted 1/100 in TBST, were added and incubated for 2 hours. After washing, bound IgG was detected with horseradish peroxidase-conjugated goat anti-human IgG (A-2290; Sigma-Aldrich) and o-phenylenediamine dihydrochloride substrate. Positive and negative controls from patients with/without aPL were run on each plate. Results were expressed in arbitrary units (U/mL), with reference to index plasma from a patient with a high concentration of TFPI IgG antibodies (arbitrarily assigned as 100U/mL). Non-specific binding was eliminated by subtracting the absorbance values from uncoated wells. Inter- and intra-assay CV was determined using the positive (87 U/ml) and negative (6U/ml) controls (8.7% and 9.1%, respectively). The cut-off for TFPI antibody positivity (60.8U/mL) was defined as >99th centile of the values for NC subjects.

Chaotrophic ELISA for determination of avidity of TFPI antibodies

TFPI IgG antibody avidity was assessed by introducing chaotropic conditions to the above ELISA using a method adapted from Cucnik et al (32). All TFPI IgG antibodies positive samples were diluted 1/100 in TBS containing increasing concentrations of NaCl: 0.15, 0.25, 0.5, 1, 2, 4, and 6M and then assayed as above. To distinguish TFPI antibodies with high or low avidity, the initial binding at 0.1M NaCl was compared with binding at higher salt concentrations, and 1M NaCl was arbitrarily selected as the reference concentration. Avidity was expressed as a percentage of maximum binding at 0.1M NaCl which was arbitrarily considered as 100%. High avidity was defined as >60% of the initial binding and low avidity as <25% at 1M NaCl. Samples >25% but <60% binding were defined as intermediate avidity.

TFPI activity

TFPI activity was determined with an amidolytic assay (33). In summary, 25µl of each plasma sample was incubated for 30 minutes at 37°C, in the wells of a microplate (Nunc Polysorp, Fisher Scientific) with 100µl of a mixture of recombinant human TF (1/800 v/v; Innovin; Sysmex UK), recombinant human FVIIa (10nM), bovine FXa (1.1nM) (both Haematologic Technologies Inc., Vermont, USA), I-2882® (Pefabloc®FG) (100µg/ml; Pentapharm, Basle, Switzerland), and $CaCl_2$ (10mM) (all final concentrations in the final reaction mixture of 125µl). 50µl of FX (32nM; Haematologic Technologies), and the substrate S-2222® (1.35mM; Quadratech, Epsom, UK) were added and incubated for a further 30 minutes at 37°C. The reaction was stopped by the addition of 50µl of 50% acetic acid and the optical density was measured at 405nm. TFPI was expressed as percent activity (normal range 76.7-135%). All samples were tested in duplicate and the intra- and inter-assay CV were 10.2% and 7.5%, respectively (at 95% TFPI activity). Samples were considered to have low TFPI activity if levels were <99th centile of the mean activity of the NC (100.8%) established as 66.3%.

Protein C antibodies and determination of avidity

The presence and avidity of protein C IgG antibodies in 77/100 patients were previously reported (22). The remaining 23 patients (16 APS and 7 thrombotic controls) were tested using similar methodology (22). In summary, Costar EIA/RIA high binding plates (Fisher Scientific) were coated with 10µg/mL protein C (Ceprotin, Baxter Healthcare Ltd, Norfolk, UK), test samples were diluted 1:25. Results were expressed in arbitrary units (U/mL), with reference to index plasma from a patient with high protein C antibody levels (arbitrarily assigned as 100U/mL). Non-specific binding was eliminated by subtracting the absorbance values from uncoated wells.

Samples were considered positive for protein C antibodies if values were >99th centile of the NC (36U/mL). The avidity of protein C antibodies was assessed in a similar way to that for TFPI avidity above, by calculating the percentage of maximum binding.

Competitive inhibition assays for TFPI and Protein C IgG ELISA

Competitive inhibition experiments were used to establish whether rTFPI, protein C, or β2GPI could abolish binding of IgG antibodies in patient plasma samples to immobilized TFPI and protein C in the ELISA assays. Briefly, plasma samples from four patients, with >100U/mL for TFPI and protein C antibodies and positive for aβ2GPI) were diluted in assay buffer to a 50% maximal binding in the TFPI and protein C ELISA, as appropriate. The effects of pre-incubation (for 2 hours at room temperature) of the diluted samples with a range of TFPI, protein C, or β2GPI (Enzyme Research laboratories; Swansea, UK) concentrations (0 to 2.5μM), before application to the plates were studied. The percent inhibition for each concentration of inhibitor was determined as follows: % inhibition= [OD of sample with buffer-OD of sample with inhibitor at the given concentration] / OD of sample with buffer x 100.

Serial dilutions of a rabbit polyclonal anti-human TFPI antibody (American Diagnostica Inc, Stamford, USA) were also used to demonstrate concentration dependent binding to the coated microplate; and IgG depleted patient plasma prepared using protein G Sepharose chromatography (Pierce, Thermo-Fisher, Basingstoke, UK).

Competitive inhibition assays were also performed with four patient samples with > 100U/mL for protein C antibodies and positive for aβ2GPI to see whether protein C could abolish binding to immobilized β2GPI. These assays were adapted from

previously described ones (34-36) with slight modifications. Patient plasma samples diluted to a 50% maximal binding dose and were incubated with either protein C or β2GP1 (0-2.5μM) for 2 hours and subsequently added in duplicate into an ELISA plate coated with β2GPI for 1 hour at room temperature before washed with PBST. Bound IgG was detected with an anti-human IgG/HRP conjugate antibody (A6029, Sigma) as described (35).

Increasing concentrations (0-4μg/mL) of a rabbit anti-human aβ2GPI antibody (Dako) were also added in duplicate in both the TFPI and protein C ELISA and binding to the immobilized TFPI or protein C respectively was assessed. This was performed to establish possible reactivity and direct binding to the TFPI and protein C coated plates.

Statistical analysis

Samples were anonymized and the operator was blinded concerning the clinical severity and anticoagulant status of patients. All assays were performed following completion of sample collection. Data analysis was performed using Graphpad Prism 5.0 (Graphpad Software, Inc. La Jolla, California, USA) and STATA (version 14.0). Logistic regression models were used to model the relationship between binary outcomes (e.g. presence of severe phenotype) and exploratory variables. TFPI and protein C antibodies were used in two separate logistic regression analyses, where levels were used as a continuous explanatory variable and as a binary explanatory variable of positive versus negative presence according to the established cut off for positivity if each respective assay. Odds ratios (OR) together with associated 95% confidence intervals (CI) are reported alongside p-values for hypothesis tests based on the fitted models. A p-value <0.05 was considered to be statistically significant. An association was considered relevant when the 95%CI range excluded 1.0 (values

>1.0 denoting a positive association). Bonferroni corrections were applied for multiple comparisons. For continuous outcomes, normality was assessed using a Kolmogorov Smirnov test with comparisons between groups assessed using two-sample t-tests. For non-normal continuous outcomes, Mann-Whitney U tests were used. One-way ANOVA or Fisher's exact tests (FET) were used to compare age and gender or for associations in APS, thrombotic controls and NC.

Results

Subject characteristics

Mean age in NC was 43±12 years with a male/female ratio of 52/49. Patient characteristics and clinical features are presented in Table 1 and laboratory features in Table 2. INR values and amidolytic factor X assays suggested a similar anticoagulant intensity in the two patient groups (Table 2). According to the APS classification (categories: I, IIa, IIb, IIc based on Miyakis et al, 2006) (28), 20/50 APS patients were category I (more than one laboratory criteria present; seven of whom were double and 13 triple aPL positive); 23 were category IIa (LA alone), five were category IIb (presence of aCL antibodies alone); and two patients were category IIc (presence aβ_2GPI alone). Eighteen APS and nine thrombotic control patients were classified as having a severe thrombotic phenotype as defined above for this study.

Diagnosis	APS (n = 50)	Thrombotic controls (n = 50)
Age, mean years ± SD	52.0 ± 14	50.0 ± 15
Sex, male/female, n	16/34	23/27
SLE, n (%)	7 (14)	2 (4)
Age at first thrombotic event, mean years ± SD	42.0 ± 14	48.5 ± 13
VTE only (DVT or PE), n (%)	28 (56)	43 (86)
AT only, n (%)	14 (28)	0 (0)
Severe thrombotic phenotype**, n (%) VTE + AT, n (%) 1 VTE and 1 AT ≥2 VTE and 1 AT 1 VTE and ≥2 AT Recurrent VTEs ≥2 ≥3 Age at first thrombotic event, mean years ± SD	18 (36) 8 (16) 4 (8) 4 (8) N/A 10 (20) 3 (6) 7 (14) 33.7 ± 14.0	9 (18) 7 (14) 7 (14) N/A N/A 2 (4) 2 (4) N/A 41.1 ± 12.6
APS categories ǂ, n (%) I /Double aPL +/Triple aPL + IIa IIb IIc	20 (40) /7 (14)/13 (26) 23 (46) 5 (10) 2 (4)	N/A
Medication Target INR 2.0-3.0, n (%) 2.5-3.5, n (%) 3.0-4.0, n (%)	Warfarin 30 (60) 2 (4) 18 (36)	Warfarin 35 (70) 5 (10) 10 (20)

Table 1: Clinical features of thrombotic patients ± APS: The results columns indicate the number (n) and percentage (%) of patients positive for each parameter indicated. **Severe thrombotic phenotype refers to patients with recurrent thromboembolic episodes despite therapeutic anticoagulation or patients with both venous thromboembolism (VTE) and arterial thrombosis (AT). ǂAPS categories: I, IIa, IIb, IIc based on (28). DVT: deep venous thrombosis, PE: pulmonary embolism

Diagnosis	APS (n = 50)	Thrombotic controls (n = 50)
INR (PT-Fib HS Plus)	2.4, (2.2-2.6)	2.2(2.1-2.4)
Factor X amidolytic activity (IU/dL)	20.6 (18.2-24.1)	20.8 (18.8-22.6)
β2 GPI IgG (GPU)	23.9 (20.6-32.0)	N/A
β2 GPI IgM (MPU)	12.8 (9.8-18.2)	N/A
aCL IgG (GPLU)	18.9 (16.2-26.2)	N/A
aCL IgM (MPLU)	7.5 (5.8-16.8)	N/A
LA, n (%)	46 (92)	N/A
TFPI antibodies IgG, n (%)	20 (40) 53.3 (42.0-71.4)	9 (18) 34.1 (27.5-47.8)
Protein C antibodies IgG, n (%)	11 (22) 36.0 (33.0-0.43.0)	9 (18) 27.0 (24.0-31.0)
TFPI activity (%)	100 (95-102)	106 (100-108)

Table 2: Laboratory features of thrombotic patients ± APS: The results columns indicate the median and 95% confidence intervals (CI) or otherwise indicated for each parameter specified.

TFPI antibodies

29% (29/100) of all thrombotic patients (20 APS and 9 thrombotic controls) were TFPI antibody positive. A greater prevalence of TFPI antibodies (40% versus 18%; p=0.004), and higher levels (Table 2) were observed in APS compared to thrombotic control patients (p=0.007, Fig 1A). The presence of TFPI antibodies was associated with a severe thrombotic phenotype in APS patients (OR: 15.2, 95%CI: 3.7-62.9, p<0.0001). TFPI antibody levels were associated with a severe thrombotic phenotype in both APS (OR: 1.03, 95%CI: 1.01-1.05, p<0.0001) and in thrombotic control patients (1.09, 1.03-1.16, p=0.005).

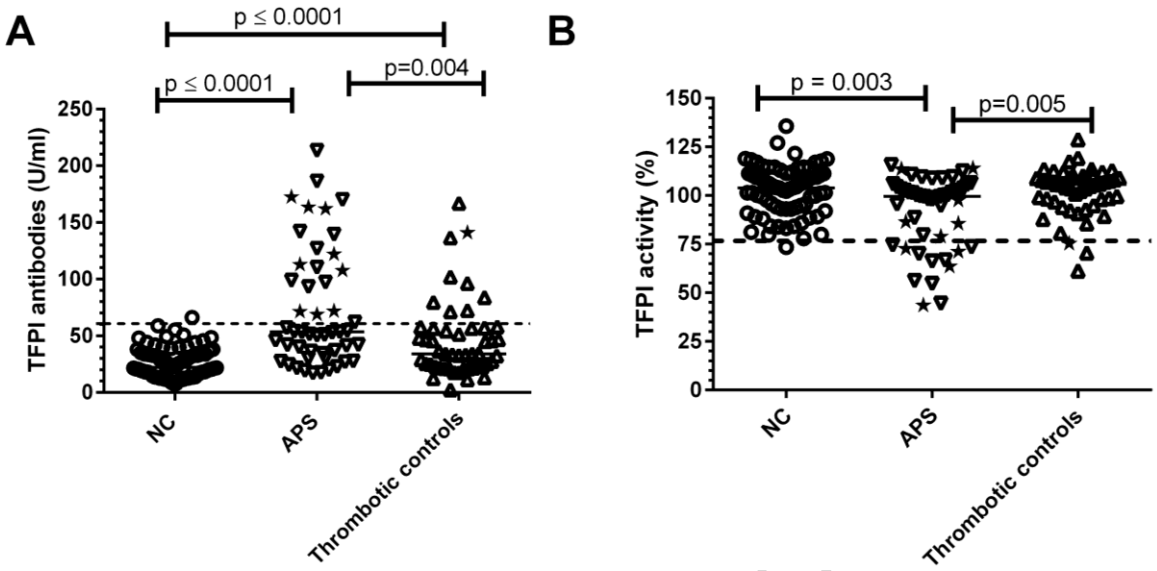

Fig 1: TFPI antibodies and TFPI activity levels **A.** TFPI antibody levels **B.** TFPI activity in normal controls (NC), APS, and thrombotic control patients. Patients with high avidity TFPI antibodies are indicated by an asterisk (*).The horizontal broken line represents the normal cut-off values (60.8 U/mL for TFPI antibodies and 76.7% for TFPI activity).

Of the 20 APS patients positive for TFPI antibodies, high avidity antibodies were detected in ten, intermediate in six and low avidity in four patients (Fig 2). Of the nine thrombotic control patients positive for TFPI antibodies, one patient had high, one had intermediate and seven had low avidity antibodies. TFPI antibody levels were not associated with avidity in either patient group (APS; p=0.36, thrombotic controls; p= 0.60).

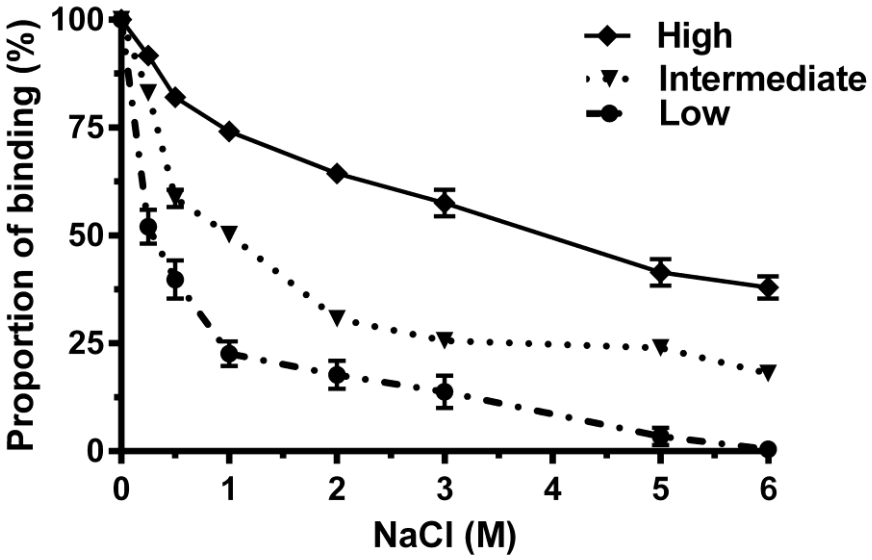

Fig 2: Avidity of TFPI antibodies in APS patients and thrombotic controls

Percentage of maximum IgG binding to TFPI at various NaCl concentrations (mean ±standard error of the mean) for 10 APS patients with high-avidity antibodies, six with intermediate, four APS and nine thrombotic controls with low-avidity TFPI antibodies.

In APS patients, the presence of high avidity TFPI antibodies were strongly associated with a severe thrombotic phenotype and with triple aPL positivity (OR: 12.0, 95%CI: 2.2-66.1, p=0.004); Eight out of the ten APS patients with high avidity TFPI antibodies had a severe thrombotic phenotype (six had both VTE and AT and two had recurrent VTE while on therapeutic anticoagulation) and all eight were triple aPL positive. Three out of the six patients with intermediate avidity were triple aPL positive, while the single thrombotic patient without APS and with high avidity TFPI antibodies exhibited clinically severe thrombotic disease.

TFPI activity

TFPI activity was comparable in NC (mean 100.8, 99-103%) and thrombotic control patients (106.0, 100-108%), but was lower in APS patients (100.0, 95-102%)

(p=0.005 & p=0.048, versus thrombotic controls and NC, respectively); this was mainly due to a group of 12 APS patients with TFPI activity below the normal cut off value. Only three thrombotic control patients had low TFPI activity (Fig1B). In female patients TFPI activity was significantly lower (p=0.006) between female APS (99.4%, 88.5-102.3%) and thrombotic controls (105.8%, 99.2-109.0%) despite both groups having comparable mean ages.

There was no association between TFPI activity and presence or levels of TFPI antibodiesp=0.67, p=0.36, respectively; 9/15 patients (APS and thrombotic controls) with decreased TFPI activity were TFPI antibody positive (Fig 3), while 20 patients with TFPI antibodies had normal TFPI activity. All 15 patients (APS and thrombotic controls) with decreased TFPI activity had a history of at least one AT episode. There was a weak association between the presence of high avidity TFPI antibodies and decreased TFPI activity in APS (5/10 APS patients with high avidity TFPI IgG antibodies had decreased TFPI activity, p=0.03).

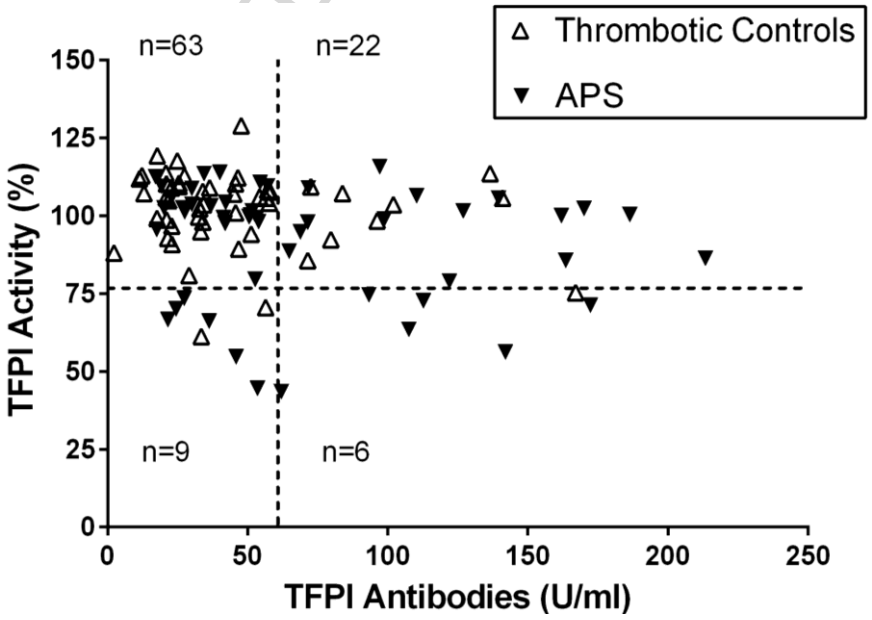

Fig 3: Association between TFPI antibodies positivity and TFPI activity levels in APS patients and thrombotic controls Horizontal broken line represents the TFPI activity cut off value (76.7%) and vertical dotted line represents the cut off value of 60.8U/mL for TFPI antibodies.

Protein C antibodies and avidity

All patients included in this study were also tested for presence of protein C antibodies and the results are presented in Table 2. Of the 100 thrombotic patients, 23 APS and only nine thrombotic control patients were positive for protein C antibodies ($p<0.0001$). In APS patients, both the presence (OR: 15.0, 95%CI: 3.4-65.6, $p<0.001$) and protein C antibody levels (OR: 13.1, 95%CI: 1.37-125.7, $p=0.025$)were identified as strong risk factors for a severe thrombotic phenotype (Eleven out of the 23 APS patients had high avidity protein C antibodies (all experienced a severe thrombotic phenotype) and the remaining 12 had low avidity, while all nine thrombotic control patients had low avidity protein C antibodies. High avidity protein C antibodies were also independently associated with a severe thrombotic phenotype in APS patients (OR: 12.0, 95%CI: 2.2-66.1, $p=0.004$), although the small numbers of patients resulted in an imprecise OR estimate.

Associations between TFPI and protein C antibodies

Eleven out of the 20 APS patients with TFPI antibodies (regardless of avidity) were also positive for protein C antibodies (either low or high avidity) and all 11 had a severe thrombotic phenotype; (Table 3) (OR: 20.2, 95%CI: 2.0-47.0, $p<0.0001$), with coexistence of both high avidity antibodies been an even stronger risk factor for a severe thrombotic phenotype (OR: 34.6, 2.0-47.0, $p<0.0001$), However, the small number of these patients resulted in imprecise OR estimates. Notably, the nine thrombotic control patients positive for protein C antibodies (low avidity) were also

positive for TFPI antibodies, and seven of these exhibited a severe thrombotic phenotype (OR 75.0, 95%CI 1.2-195.0, p=0.02) (Table 3).

TFPI IgG antibodies		Protein C IgG antibodies		Number of patients with Severe Thrombotic Phenotype*
APS Patients				
High Avidity	10	High Avidity	3	3
		Low Avidity	2	2
		Negative	5	3
Intermediate Avidity	6	High Avidity	2	2
		Low Avidity	1	1
		Negative	3	0
Low Avidity	4	High Avidity	2	2
		Low Avidity	1	1
		Negative	1	0
Negative	30	High Avidity	4	4
		Low Avidity	8	0
		Negative	18	0
Thrombotic control patients				
High Avidity	1	Low Avidity	1	1
Intermediate Avidity	1	Low Avidity	1	1
Low Avidity	7	Low Avidity	7	5
Negative	41	Negative	41	0

Table 3: Co existence of TFPI and protein C antibodies, avidity and association with clinical phenotype *Severe thrombotic phenotype as defined in table 1.

Three APS patients negative for protein C antibodies, but with high avidity TFPI antibodies had a severe thrombotic phenotype while four APS patients with high avidity protein C antibodies, but no detectable TFPI antibodies also had a severe thrombotic phenotype. However no associations were established due to the small number of patients. Only seven APS patients had SLE and there was no clear association with the presence of either antibody type.

Associations with severe thrombotic phenotype and aPL

Age was not a significant factor in the development of a severe thrombotic phenotype in APS patients, but was contributory in the thrombotic control patients (OR: 1.11 with 95%CI 1.02-1.21, p=0.014), with gender not having any impact on either of the two groups.

LA was associated with a severe thrombotic phenotype in APS patients (p=0.001), but no association was found for triple aPL positivity (p=0.255), possibly due to the small number of patients.

Out of the 13 triple aPL positive patients, seven were positive for both TFPI and protein C IgG antibodies and had a severe thrombotic phenotype. However, multivariate analysis showed no statistically significant association between triple aPL positivity and severe thrombotic phenotype (OR: 8.0, 1.8-35.1, p=0.06). Of the remaining six triple aPL positive APS patients, two were negative for both TFPI and protein C antibodies, two were only positive for TFPI antibodies and two were only positive for protein C antibodies; these last four patients having a severe thrombotic phenotype.

Of the 4 patients with coexistent TFPI and protein C antibodies that were not triple aPL positive: two were LA positive, one LA and aβ2GPI positive, and one LA and aCL. Multivariate analysis showed that TFPI,protein C antibodies, and LA were independently associated with a severe thrombotic phenotype in APS patients (14.2, 3.0-46.0, p<0.0001 with TFPI antibodies: OR 22.3, p<0.0.001; protein C antibodies: 6.8, p=0.012, and LA: 16.8, p<0.0001).

Specificity and cross reactivity of TFPI and Protein C ELISA with β2GP1

Due to the co-existence of TFPI antibodies, protein C antibodies and aβ2GPI in a number of patients, we performed competitive inhibition assays to evaluate whether there was any cross reactivity in the two ELISAs with β2GP1, TFPI, or protein C. In the TFPI antibody ELISA, pre-incubation of rTFPI with the test sample inhibited antibody binding in all four plasma samples in a concentration dependent manner (Fig. 4A). Pre-incubation with β2GP1, induced less than 12% inhibition and pre-incubation with protein C, less than 10% inhibition, suggesting good specificity for the TFPI IgG ELISA. This was supported by the fact that serial dilutions of a rabbit polyclonal anti-human TFPI antibody demonstrated concentration dependent binding to the coated microplate, and IgG depletion of patient plasma abolished binding in the TFPI antibody ELISA (data not shown).

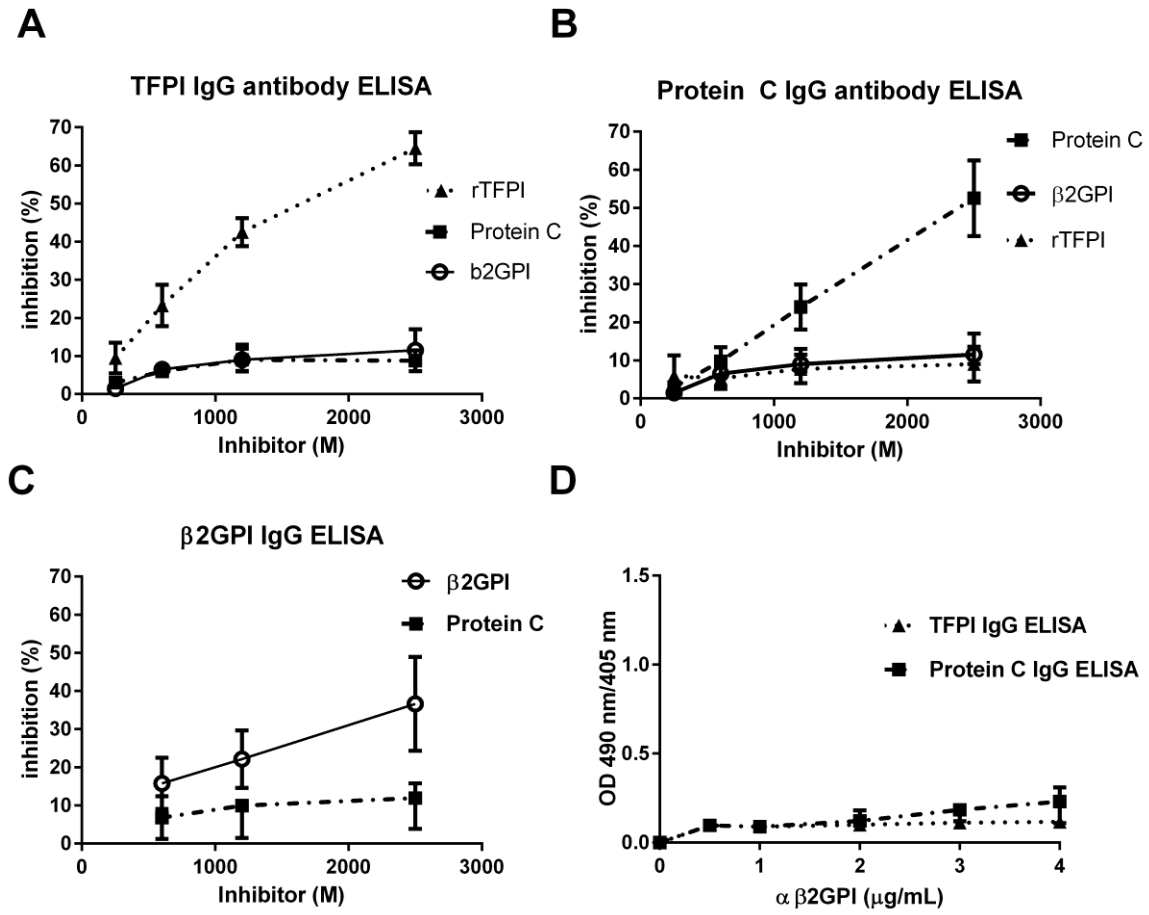

Fig 4: Cross inhibition assays for assessment of cross reactivity with β2GP1, TFPI, and protein C in **A**. TFPI IgG ELISA **B.** Protein C IgG ELISA **C.** B2GPI IgG ELISA **D.** Assessment of a direct binding of rabbit aβ2GPI IgG antibody to TFPI and Protein C IgG ELISA. **A** and **B.** B2GP1, TFPI, and protein C (0-2.5μM-indicated on the horizontal axis) were incubated for 2 hours with patient plasma and inhibition of binding for the coated antigen was assessed as described in the methods section. Results are expressed as percentage inhibition of binding (mean ∓ SD) (y axis). **C.** B2GP1 and protein C (0-2.5 μM-indicated on the horizontal axis) were incubated for 2 hours with patient plasma and inhibition of binding for the coated antigen was assessed as described in the methods; results were expressed as for A and B .**D.** Increasing concentrations of a rabbit polyclonal aβ2GPI IgG antibody (0-4 μg/mL-indicated on the horizontal axis) were assessed in both ELISAs for direct binding. Y axis- optical density at measured wavelength for each ELISA.

Similarly, pre-incubation of test samples with protein C inhibited antibody binding in the protein C antibody ELISA in a concentration dependent manner (Fig. 4B), whereas pre-incubation with either rTFPI or β2GP1 caused no inhibition.

B2GP1 inhibited binding to the β2GP1 immobilized on the plate in a concentration dependent manner, whereas protein C did not inhibit binding (10% inhibition at 2.5 μM) suggesting minimum cross-reactivity (Fig 4C).

Absorbance of increasing concentrations of a rabbit aβ2GPI IgG antibody (0-4 μg/mL) in the TFPI and protein C antibody ELISAs was below the cut-off for positivity in either ELISA suggesting no contamination of the coating antigens with β2GP1 in either of the two ELISAs (Figure 4D).

Discussion

In this cross-sectional study of well characterized patients, we made the novel observation that coexistence of TFPI and protein C IgG antibodies, regardless of avidity, was strongly associated with a more severe thrombotic phenotype (as

defined above), not only in thrombotic APS patients, but also in thrombotic controls. This suggests that there might be a pathophysiological relationship between the two antibodies, predisposing to the development of thrombosis. We established a higher prevalence of TFPI antibodies in thrombotic APS patients (40%; 20/50) compared to thrombotic controls (18%; 9/50). We also demonstrated, for the first time, differences in avidity between the two patient groups: TFPI antibodies were predominantly high avidity in APS (50%, 10/20 of positive patients) and strongly associated with a severe thrombotic phenotype, while thrombotic control patients mainly showed low avidity antibodies (78%, 7/9 of positive patients). The avidity assays used were based on antibody binding at high ionic strength (32), are based on previous work by our group and others (22;32;37;38).

The high prevalence of TFPI IgG antibodies (40% of patients with thrombotic APS), was in agreement with previous studies (prevalence 28.4-65.0% (12;18;19;21)), but we also found that their presence and antibody levels were associated with a severe thrombotic phenotype in APS patients(OR 15.2.and 1.03, respectively $p<0.0001$). Notably, TFPI antibody levels were also detected in thrombotic control patients (18%, 9/50)seven of whom had a more severe thrombotic phenotype (OR: 1.09, $p=0.005$). This suggests that these antibodies may contribute to the overall development of thrombotic complications and might be independent of APS criteria aPL (aCL, aβ2GPI, LA), and other non-criteria aPL (4;39-46).

TFPI function can be impaired in APS patients (12;14;47;48) and the presence of TFPI antibodies has been suggested as a contributory factor in the pathogenesis of the disease (12;14;18-20). In our well defined APS cohort, higher levels of TFPI antibodies were also observed in APS compared to thrombotic control patients (Table1, $p=0.007$). These antibodies, especially when present at high levels, do not occur frequently in patients with infection-related aPL (19),suggesting that they are not transient and could have a distinct pathophysiological significance.

High avidity TFPI antibodies were weakly associated with lower TFPI activity (p=0.03), suggesting an inhibitory effect on TFPI function. Previous studies showed conflicting results for TFPI antibodies and TFPI activity: Adams et al [18] reported no association between TFPI activity and antibodies with slightly higher TFPI activity in APS patients than normal controls, while other studies, using different assay methods, reported reduced TFPI activity (12;49)(or no reduction in TFPI activity in patients with LA (20) . Studies on TFPI antigen levels are also conflicting, with levels reported to be higher (50;51), and lower (52) in APS patients compared to controls. These varying results probably reflect the nature and sensitivity of the TFPI assay employed, which of the various TFPI interactions it measures, variations in experimental reagents and conditions, small numbers of samples, and use of different assay calibrants. There is also controversy about the pool of TFPI that is measured, as most assays only detect free plasma TFPI, which accounts for just 10-50% of total body TFPI; with poor correlation between the methods currently used (53-55). We used a two-stage assay where TF, FVIIa and FXa are inhibited by TFPI and then residual TF/FVIIa is measured in terms of FX activation, using an amidolytic substrate for FXa. However this only assesses plasma TFPI and does not take into account the endothelial surface bound TFPI pool or intracellular, releasable TFPI. Therefore, the current assays employed for either TFPI activity or antigen levels might not be a reliable indicator of the overall anticoagulant function of TFPI in each patient.

We previously have shown (22) that high avidity protein C antibodies are associated with a more severe clinical phenotype. Protein C activity levels, though decreased, were consistent with warfarin anticoagulation, and normal protein C activity was completely restored when patient samples were mixed with normal plasma (to

counteract the effects of warfarin) indicating that the antibodies were not activity neutralizing, and suggesting that protein C clearance is not increased by immune complex formation (22). In the current study we made the novel observation that the presence of both TFPI and protein C IgG antibodies, regardless of the avidity of either antibody type, is associated with a severe thrombotic phenotype in thrombotic patients regardless of APS; suggesting a possible role in the pathogenesis of thrombosis for both sets of antibodies.

Pengo et al showed that triple aPL positivity is associated with an increased risk of thrombosis (56), however this association not present in a multivariate analysis in our cohort possibly due to the small number of patients. However, TFPI and protein C antibodies, as well as LA were all associated with a severe thrombotic phenotype in APS patients). It has not been established whether TFPI inhibition in APS patients is dependent on criteria aPL, or due to the presence of TFPI antibodies. Salemink et al, (13) showed that total IgG, aβ2GPI and aCL isolated from APS patients all increased TF-induced FXa generation in normal plasma, but this enhancing effect was lost when patient plasma was depleted of aβ2GPI or when normal plasma was depleted of β2GPI or TFPI. Lean et al (21), also demonstrated an association between purified aβ2GPI IgG antibodies and increased thrombin generation in the presence of TFPI, suggesting a direct interaction of aβ2GPI with TFPI despite no cross-reactivity being demonstrated between antibodies against the two proteins. Liestol (14) demonstrated that IgG fractions isolated from certain patients with LA had an inhibitory effect on the ability of recombinant TFPI and TFPI released from the endothelium by heparin, to reduce thrombin generation

Coexistence of TFPI and protein C antibodies was also present in thrombotic control patients with no aPL positivity who had a more severe thrombotic phenotype)

suggesting a mechanism of action independent of criteria or not aPL and present in a subgroup of patients (regardless of APS) at high risk of thrombosis.Cross reactivity in the TFPI and protein C antibody ELISAs with β2GPI was excluded with competitive and cross inhibition experiments (Fig 4) reinforcing this theory .

Our results are in agreement with reports suggesting that non-criteria aPL such as antibodies against protein C (22), prothrombin (57), domain I of β2GPI and annexin V (58) might also contribute to the development of thrombosis in APS patients independently from the APS criteria aPL.' More importantly, our results suggest that these antibodies might also have a distinct role in the development of thrombosis in the general population.

The use of assays that detect high avidity antibodies to specific target proteins such as β2GPI, phosphatidylserine/prothrombin, protein C and TFPI, may prove to be more specific for the prediction of the clinical course than the tests currently used for APS diagnosis. In addition, they may prove to be helpful in assessing the anticoagulant requirements of patients with thrombosis who do not fulfil the current APS laboratory criteria and aid in identification of additional patients at high risk of thrombosis. The TFPI domain(s) responsible for TFPI antibody binding also need to be established as this may lead to differential effects on thrombin generation (59).

The strengths of our study are the well-defined and homogeneous population of patients, including a well characterized thrombotic APS population, and the inclusion of thrombotic controls. Our study has some limitations: we did not assay IgM antibodies against TFPI and protein C; we focused on IgG as these antibodies have been shown to have significant correlations with thrombosis (60;61) The chaotropic avidity method used produced valuable results, but future studies with equilibrium methods such as surface plasmon resonance could also be used to confirm our findings.In conclusion, thrombotic APS patients exhibited a high prevalence of TFPI

antibodies, which were mainly high avidity and associated with lower TFPI activity. The association of high avidity TFPI antibodies with severe thrombotic phenotype suggested a possible defect in the regulation of the TF pathway of coagulation. Most importantly, TFPI IgG antibodies may also act synergistically with protein C IgG antibodies, and provide a marker of a severe clinical thrombotic phenotype regardless of APS status or aPL positivity. This supports the view that non-criteria aPL may also be independently contributing in the pathogenesis of APS and in the development of more severe thrombotic complications and could help differentiate APS patients who may benefit from more aggressive treatment. More importantly, th pathophysiological relationship between the two antibodies in thrombotic patients without APS suggests that these antibodies could also be used for identification of thrombotic patients negative for aPL and could have a more general role in the development of thrombosis.

Addendum

M Efthymiou and IJ Mackie designed the study. All assays (except those previously published elsewhere, and the β2GPI competitive inhibition ELISA that was performed by T. McDonnell), data handling, statistical analysis and manuscript preparation were performed by M Efthymiou. AG O'Keeffe provided critical feedback and assisted with all statistical analysis performed for this manuscript. All authors contributed to the critical evaluation and final preparation of the manuscript.

Acknowledgements

This project was supported by a grant from LUPUS UK. Dr Arachchillage was supported by an unrestricted educational grant from Bayer Healthcare (Newbury,

UK). rhAPC and rhTFPI were kind gifts from Eli Lily & Co. (Indianapolis, USA) and Chiron Corp (Emeryville, C, USA), respectively.

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Highlights

- TFPI and protein C (PC) antibodies (abs) have been reported in APS
- We examined prevalence and avidity and associations with severity of clinical phenotype in APS
- TFPI abs show high prevalence in APS (40%); and thrombotic controls (18%)
- Mostly high/intermediate avidity in APS, associated with severe thrombotic phenotype
- Coexistent with PC abs, regardless of avidity, may be a marker for a severe thrombotic phenotype in thrombotic patients