



## The effects of tissue factor pathway inhibitor and anti- $\beta$ -2-glycoprotein-I IgG on thrombin generation

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**Background and Objectives.** Recent evidence suggests that autoantibodies to tissue factor pathway inhibitor (TFPI) and/or antiphospholipid antibodies (aPL) may contribute to upregulation of the tissue factor (TF) pathway of blood coagulation and the development of thrombotic complications in the antiphospholipid syndrome (aPS). The aim of this study was to determine the influence of aPL e.g. anti- $\beta$ -2-glycoprotein-I (anti- $\beta$ 2GPI) and anti-prothrombin, on *in vitro* TF-induced thrombin generation in the presence and absence of TFPI.

**Design and Methods.** IgG fractions were collected from subjects with aPL (n=21) and normal controls (n=36). Anti-TFPI activity was determined after incubation of IgG isolated from control or subject plasma with pooled normal plasma using an amidolytic assay for TFPI. The influence of IgG fractions and purified aPL (anti- $\beta$ 2GPI and anti-prothrombin) on TF-induced *in vitro* thrombin generation was determined using a chromogenic assay of thrombin activity.

**Results.** Patients with aPL had significantly elevated thrombin generation (median [interquartile range]) compared to normal controls (112.0 [104.0-124.0]% vs 89.9 [85.7-100.9]%, respectively;  $p < 0.001$ ). Thrombin generation was significantly correlated with anti-TFPI activity in patients with aPL ( $r_s = 0.452$ ;  $p = 0.039$ ). It was also demonstrated that anti- $\beta$ 2GPI, but not anti-prothrombin IgG antibodies, significantly enhanced TF-induced thrombin generation in the presence of TFPI, using both purified and patients' samples.

**Interpretation and Conclusions.** Our findings support the hypothesis that anti- $\beta$ 2GPI IgG antibodies accelerate thrombin generation in the presence of TFPI and may contribute to hypercoagulability in patients with aPS.

Key words: antiphospholipid antibodies, tissue factor pathway inhibitor, thrombin generation,  $\beta$ -2-glycoprotein-I.

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The antiphospholipid syndrome (aPS) is a systemic autoimmune disorder characterized by persistent moderate to high titers of antiphospholipid antibodies (aPL), with venous or arterial thrombosis, pregnancy morbidity and thrombocytopenia.<sup>1</sup> It is widely accepted that the pathogenesis of this disorder is due to the contribution of aPL, although the precise mechanisms are unknown. aPL are a family of heterogeneous autoantibodies directed against phospholipid-binding proteins and include lupus anticoagulants (LA) and anticardiolipin antibodies (aCL).<sup>2,3</sup> The two most common phospholipid-binding proteins targeted are  $\beta$ -2-glycoprotein-I ( $\beta$ 2GPI) and prothrombin.<sup>4-6</sup> They have higher affinities for anionic phospholipids upon binding with certain aPL<sup>7-9</sup> and therefore may interfere with phospholipid-dependent coagulation reactions and contribute to the prothrombotic state. The major pathway of blood coagulation,

the tissue factor (TF) pathway, is triggered by TF in complex with activated coagulation factor VII (FVIIa). Tissue factor pathway inhibitor (TFPI) is a natural anticoagulant that regulates TF: FVIIa proteolytic activity against coagulation factors X (FX) and IX (FIX), by either binding to activated FX (FXa), then to TF: FVIIa, or forming a complex directly with TF: FVIIa: FXa.<sup>10,11</sup> The resulting quaternary TF: FVIIa: FXa: TFPI complex prevents further TF: FVIIa activity and consequentially decreases thrombin generation and fibrin formation. There is increasing evidence that antibodies with activity against TFPI have a significant role in the development of an increased thrombotic tendency observed in aPS patients.<sup>12-17</sup> High titers of anti-TFPI autoantibodies have recently been detected with greater frequency among aPS patients compared to patients with aPL only and healthy controls.<sup>12</sup> Furthermore, an inhibitory component to

TFPI has been identified in the IgG fraction of aPS patients,<sup>13</sup> which is associated with increased *in vitro* TF-induced thrombin generation.<sup>14</sup> It is probable that this inhibitory component is an anti-TFPI antibody and/or another aPL with inhibitory activity against TFPI. Thus, inhibitors of TFPI or interference to TFPI activity, coupled with an aPL-induced increase in TF-like activity from monocytes and endothelial cells,<sup>18,19</sup> may upregulate the TF pathway and contribute to hypercoagulability in aPS.

The aim of this study was to determine the influence of markers of aPS e.g. anti- $\beta_2$ GPI and anti-prothrombin antibodies, on *in vitro* TF-induced thrombin generation. It was hypothesised that aPL would interfere with the control of the TF pathway and be enhanced by the presence of TFPI.

## Design and Methods

### Ethics

This study was approved by the Human Research Ethics Committee of Curtin University of Technology (Approval Number: HR 238/2001).

### Patients and controls

Plasma from 21 patients with aPL (mean age=57.4 years; age range=30-79 years) was collected from Fremantle Hospital (Fremantle, Australia) and Royal Perth Hospital (Perth, Australia). Plasma samples were aliquoted and stored at -80°C until assay. Normal control plasma was collected from 36 volunteers (mean age=29.3 years; age range=19-60 years) with no apparent hemostatic abnormality from the School of Biomedical Sciences (Curtin University of Technology, Perth, Australia). Nine parts of venous blood was collected in one part of 0.109 M tri-sodium citrate anticoagulant and centrifuged for 15 minutes at 3000 g. Plasma was collected and centrifuged again for 15 minutes at 3000 g to obtain platelet poor plasma. Equal volumes of platelet-poor plasma from 20 healthy male donors were pooled to prepare normal pooled plasma (NPP).

### Plasmas, proteins and antibodies

NPP was used as the reference control in the anti-TFPI activity and TF-induced thrombin generation assays. TFPI-depleted plasma (TDP) and recombinant human TFPI (rTFPI) were purchased from American Diagnostica Inc. (Stamford, CT, USA). Purified human prothrombin, purified human  $\beta_2$ GPI, affinity purified polyclonal rabbit anti-human  $\beta_2$ GPI antibodies and affinity purified polyclonal rabbit anti-human prothrombin antibodies were purchased from Hyphen Biomed (Neuville, France). Polyclonal sheep anti-human TFPI IgG antibody was purchased from Hematologic

Technologies Inc. (Essex Junction, VT, USA). Rabbit anti-sheep IgG (Silenus, Hawthorn, Australia), sheep anti-rabbit IgG (Silenus, Hawthorn, Australia) and affinity purified goat anti-human IgG, IgA, IgM and light chain (Biosource International, Camarillo, USA) were alkaline phosphatase-conjugated antibodies used in immunoblotting.

### IgG isolation

IgG was isolated from plasma using a HiTrap™ Protein G 1mL column (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions and as described previously.<sup>14</sup> Isolated IgG (2.5 mL) was buffer exchanged into 3.5mL of 0.9% NaCl or TFPI assay buffer pH 8.0 (0.05mol/L tris, 0.15mol/L NaCl, 0.01M tri-sodium citrate, 0.2g/L polybrene and 0.1% normal serum albumin) using a PD 10 desalting column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 25 mL of PBS. The concentration of IgG was calculated by dividing the IgG eluate absorbance by an extinction coefficient of 1.43 and multiplying by the total volume (3.5mL). Aliquots of IgG were stored at -80°C.

### TF-induced thrombin generation assay

*In vitro* thrombin generation in plasma was measured using a chromogenic assay<sup>20</sup> recently modified by our laboratory.<sup>14</sup> Prior to assay, platelet-poor plasma was heated for 15 minutes at 49° C, cooled on melting ice for 2 minutes and centrifuged for 15 minutes at 3000 g to remove fibrinogen. Defibrinated plasma diluted 1/2 in 0.9% NaCl, or protein (50  $\mu$ L/well) was incubated with IgG or antibody (25  $\mu$ L/well) for 20 minutes at room temperature in the well of a 96-well round bottomed microtiter plate. Thromboplastin (Innovin®, Dade Behring, Marburg, Germany) diluted 1/20 in 0.9% NaCl was added (25  $\mu$ L/well) and the mixture incubated for 10 minutes at 37°C. A chromogenic substrate specific for thrombin (Biophen CS-01<sup>38</sup> Thrombin Chromogenic Substrate, Hyphen Biomed, Neuville, France), diluted to 1 mmol/L in 0.9% NaCl was then added (50  $\mu$ L/well) followed by 30 mmol/L CaCl<sub>2</sub> (50  $\mu$ L/well). The final reaction volume was 200  $\mu$ L. Using a Multiskan Ascent microtiter plate spectrophotometer the plate was read at 405nm immediately and every two minutes until peak absorbance was reached. Background absorbance was accounted for each sample by substituting thrombin chromogenic substrate with 0.9% NaCl.

The rate of thrombin generation in a test sample was expressed as a percentage of the absorbance reached by the test sample compared to the reference control (containing NPP only) when the reference control reached half maximum absorbance. Thrombin generation in NPP was designated at 100%.

### Anti-TFPI activity

Anti-TFPI activity was determined as previously described.<sup>14</sup> Equal volumes of NPP and 25 µg/mL IgG or antibody were incubated for 30 minutes at 37°C and then assayed in the TFPI activity assay.<sup>21</sup> Anti-TFPI activity was the difference of TFPI activity between the IgG/NPP mixture and the 1U/mL control (buffer/NPP).

### Immunoblotting

Immunoblotting (dot blot) involved immobilization of proteins on to a synthetic membrane support and recognition of the proteins' native epitopes by test antibodies. A polyvinylidene fluoride (PVDF) membrane (Hybond™-P, Amersham Biosciences, Buckinghamshire, UK) was prepared by immersion in 100% methanol and then tris-buffered saline (TBS) pH 8.0. Antigens (TFPI, β2GPI and prothrombin) (5 µg/mL) were blotted (0.5 µL per dot) onto the PVDF membrane and allowed to dry. Blocking solution (5% skim milk in TBS buffer containing 0.05% Tween pH 8.0) was added and incubated with gentle agitation at room temperature for 30 minutes. The membrane was sequentially incubated with gentle agitation for 1 hour at room temperature or overnight at 4°C, in primary antibody (0.5-10 µg/mL antibody in blocking solution) and washed three times in TBS buffer containing 0.05% Tween pH 8.0 at 10-minute intervals. Secondary antibody (0.5 µg/mL alkaline phosphatase-conjugated antibody in blocking solution) was added and incubated with gentle agitation for 1 hour at room temperature or overnight at 4°C, followed by three washes in TBS buffer containing 0.05% Tween pH 8.0 at 10-minute intervals. Color development was generated using an alkaline phosphatase-conjugate chromogenic substrate (Biorad, California, USA). Deionized water stopped the reaction and the membrane was dried at 37°C.

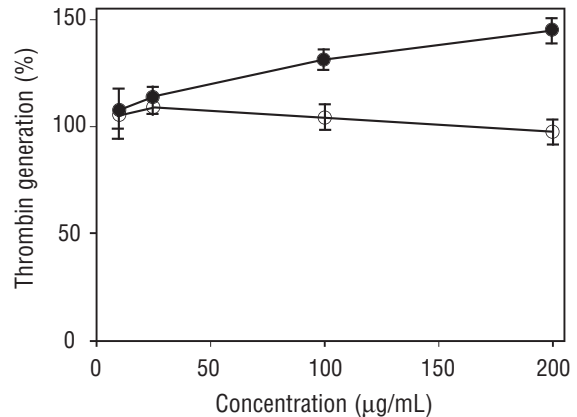
### Statistical analysis

All experiments were performed in duplicate. Statistical analysis was performed using Superior Performance Statistical Software® (SPSS) for Mac® OS X (version 11.0.2.). Results for the effect of IgG on thrombin generation were expressed as the median (interquartile range) as data was not normally distributed. Wilcoxon's signed rank test was used to test for the differences between groups. Correlation between the effect of IgG on thrombin generation and anti-TFPI activity was derived using Spearman's rank correlation test (rs). All other results are reported as mean±standard deviation. Statistical significance was defined at *p* values of <0.05.

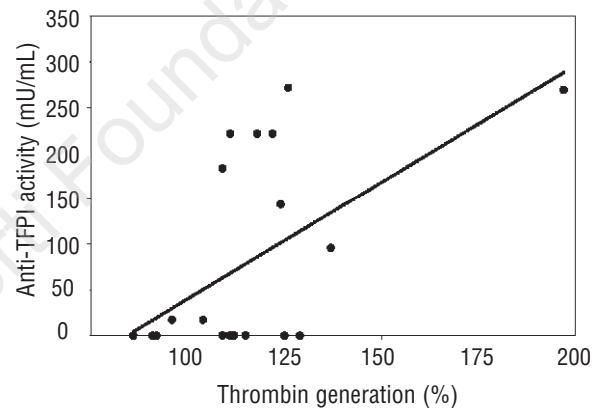
## Results

### Thrombin generation and anti-TFPI activity

Sheep anti-human TFPI antibody induced a dose-dependent increase in thrombin generation and normal control IgG had no significant effect on thrombin gener-



**Figure 1.** Effect of sheep anti-human TFPI control antibody (●) and normal control IgG (○) on thrombin generation in NPP. Anti-human TFPI antibody induced a dose-dependent increase in thrombin generation and normal control IgG had no significant effect on thrombin generation.

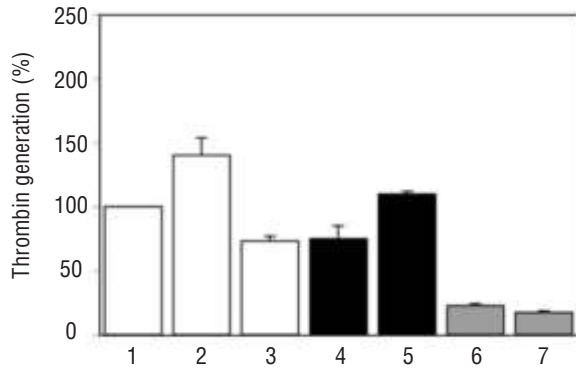


**Figure 2.** Thrombin generation and anti-TFPI activity in IgG fractions of patients with aPL. The effect of patients' IgG on thrombin generation was significantly correlated with anti-TFPI activity (rs=0.452; *p*=0.039).

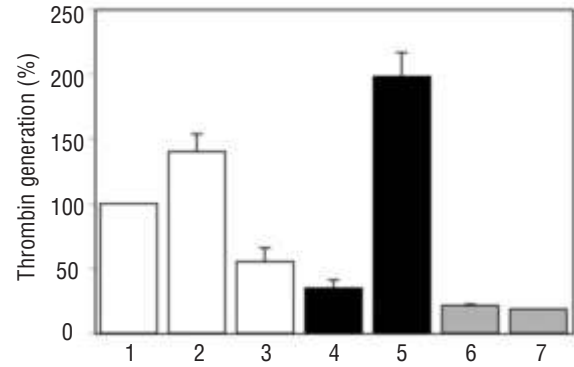
ation (Figure 1). The effect of IgG on thrombin generation was significantly greater in patients [112.0 (104.0-124.0)%] than in controls [89.9 (85.7-100.9)%; *p*<0.001]. Anti-TFPI activity was confirmed in 10/21 (47.6 %) of patients and was significantly correlated with the effect of IgG on thrombin generation (rs=0.452; *p*=0.039) (Figure 2). This relationship was reproducible on two separate occasions.

### Interaction of purified protein and antibody markers

Thrombin generation in NPP (100%) was individually reduced by rTFPI (73.1±3.8%), anti-β2GPI antibodies (74.9±10.1%) and anti-prothrombin antibodies (22.7±1.7%) (Figure 3). In the presence of additional TFPI, thrombin generation in NPP was increased by anti-β2GPI (110.0±2.1%) and decreased by anti-prothrombin (17.3±1.2%) (Figure 3). These results were



**Figure 3.** Effect of anti-β<sub>2</sub>GPI (100 μg/mL) and anti-prothrombin (100 μg/mL) antibodies on thrombin generation in NPP in the presence and absence of rTFPI (100 ng/mL). Thrombin generation was enhanced by anti-β<sub>2</sub>GPI, but not anti-prothrombin, in NPP spiked with rTFPI. 1: NPP; 2: TDP; 3: NPP+TFPI; 4: NPP+anti-β<sub>2</sub>GPI; 5: NPP+anti-β<sub>2</sub>GPI+TFPI; 6: NPP+anti-prothrombin; 7: NPP+anti-prothrombin + TFPI.



**Figure 4.** Effect of anti-β<sub>2</sub>GPI (100 μg/mL) and anti-prothrombin (100 μg/mL) antibodies on thrombin generation in TDP in the presence and absence of rTFPI (100 ng/mL). Thrombin generation was enhanced by anti-β<sub>2</sub>GPI, but not anti-prothrombin, in the presence of rTFPI in TDP. 1: NPP; 2: TDP; 3: TDP+TFPI; 4: TDP+anti-β<sub>2</sub>GPI; 5: TDP+anti-β<sub>2</sub>GPI+TFPI; 6: TDP+anti-prothrombin; 7: TDP+anti-prothrombin+TFPI.

reproducible and enhanced in TDP. Compared to NPP, TDP had higher thrombin generation (139.9±13.5%), which was reduced with TFPI (55.3±10.6%), anti-β<sub>2</sub>GPI (34.9±6.0%) and anti-prothrombin (21.3±0.9%) (Figure 4). Thrombin generation in TDP spiked with TFPI was markedly increased by anti-β<sub>2</sub>GPI (198.0±18.5%) and decreased by anti-prothrombin (18.5±0.2%) (Figure 4).

Purified antibodies (anti-TFPI, anti-β<sub>2</sub>GPI and anti-prothrombin) demonstrated specificity for their target antigen with no cross-reactivity (Figure 5).

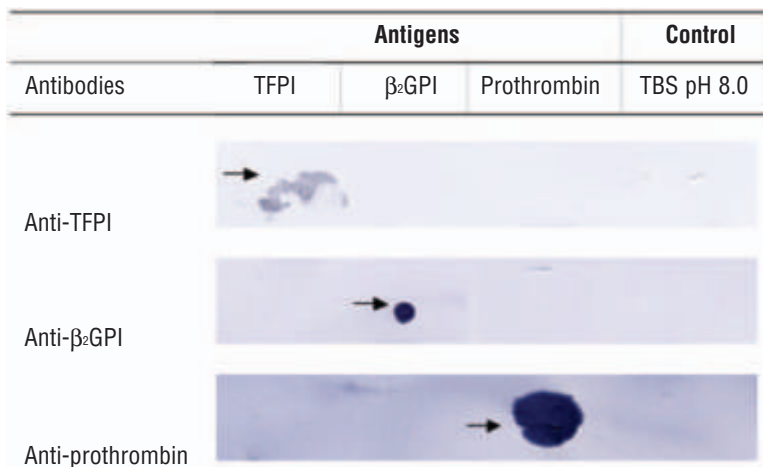
#### Interaction of patient IgG with purified protein and antibody markers

IgG fractions from ten patients with aPL were divided into two groups (five patients with and five without anti-TFPI activity) and assessed for their effect on thrombin generation in TDP, in the presence and

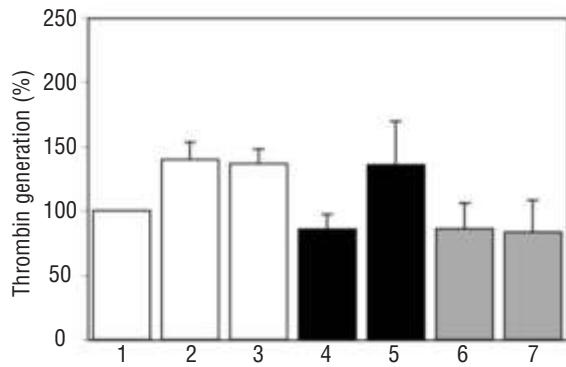
absence of TFPI. Thrombin generation in TDP was similarly decreased with IgG from patients with anti-TFPI activity (85.7±11.4%) and without anti-TFPI activity (85.9±19.9%) (Figure 6). Thrombin generation in TDP spiked with TFPI was significantly increased by IgG from patients with anti-TFPI activity (135.8±33.7%) compared to IgG from patients without anti-TFPI activity (83.3±25.0%) ( $p=0.005$ ) (Figure 6).

#### Discussion

Despite its discovery more than two decades ago,<sup>22</sup> aPS remains an enigma. Laboratory diagnosis and treatment of aPS is often difficult due to limited understanding of its pathophysiology. To date, no single pathogenic mechanism sufficiently explains how aPL contribute



**Figure 5.** Antigen specificity of purified antibodies by immunoblotting. No cross-reactivity was demonstrated between antigens and antibodies. Arrows indicate positive reactions. TBS pH 8.0 was a negative control.



**Figure 6.** Mean effect of IgG (25  $\mu\text{g}/\text{mL}$ ) from patients with ( $n=5$ ) and without ( $n=5$ ) anti-TFPI activity on thrombin generation in TDP, in the presence and absence of rTFPI (50  $\text{ng}/\text{mL}$ ). Mean thrombin generation was significantly enhanced by IgG from patients with anti-TFPI activity, but not with IgG from patients without anti-TFPI activity, in the presence of rTFPI, in TDP ( $p=0.005$ ). 1: NPP; 2: TDP; 3: TDP+TFPI; 4: TDP+patient IgGs with anti-TFPI activity; 5: TDP+patient IgGs with anti-TFPI activity+TFPI; 6: TDP+patient IgGs without anti-TFPI activity; 7: TDP+patient IgGs without anti-TFPI activity TFPI.

to the development of thrombosis in aPS and account for all clinical manifestations observed in patients. It may be that an array of pathogenic mechanisms contributes to an ongoing hypercoagulable state. Recently, interference to the control of the TF pathway of blood coagulation has been implicated in the pathogenesis of thrombotic aPS.<sup>12-17,23,24</sup> Therefore, the aim of the present study was to determine the influence of anti- $\beta_2\text{GPI}$  and anti-prothrombin aPL on the TF pathway in the presence and absence of TFPI. It was clearly demonstrated that anti- $\beta_2\text{GPI}$ , but not anti-prothrombin IgG antibodies interfere with TFPI-dependent inhibition of TF-induced thrombin generation.

Anti- $\beta_2\text{GPI}$  antibodies, anti-prothrombin antibodies and rTFPI reduced thrombin generation in both NPP (Figure 3) and TDP (Figure 4). TFPI is a natural anticoagulant and inhibits thrombin generation through its inhibitory activity against FXa and the TF: FVIIa complex. In contrast, anti- $\beta_2\text{GPI}$  and anti-prothrombin are acquired antibodies with an anticoagulant effect on *in vitro* TF-induced thrombin generation.<sup>20,25</sup> Their anticoagulant activities may be mediated by binding with their co-factors as it has been demonstrated that the anti- $\beta_2\text{GPI}$ :  $\beta_2\text{GPI}$  complex<sup>7,9,26</sup> and anti-prothrombin: prothrombin complex<sup>8,27</sup> have increased affinities for anionic phospholipids and thereby may compete with other coagulation factors for the binding to anionic phospholipids. Thus, *in vitro* thrombin generation may be inhibited by anti- $\beta_2\text{GPI}$  and anti-prothrombin antibodies due to decreased catalytic phospholipid surfaces for assembly and activation of coagulation factors. Despite the demonstrated reduction of *in vitro* thrombin generation, anti- $\beta_2\text{GPI}$  and anti-prothrombin antibodies are associated with thrombotic complications in aPS.

The reason for this paradox is presently unclear.

Thrombin generation in both NPP and TDP spiked with rTFPI was increased by anti- $\beta_2\text{GPI}$  antibodies and decreased by anti-prothrombin antibodies (Figures 3 and 4). This suggests that anti- $\beta_2\text{GPI}$ , but not anti-prothrombin antibodies have inhibitory activity towards TFPI. Anti- $\beta_2\text{GPI}$  anti-TFPI-like activity has been previously reported.<sup>16</sup> Using a FXa generation assay initiated with low concentrations of recombinant TF (rTF), phosphatidylserine (anionic phospholipids) and phosphatidylcholine (neutral phospholipids), it was demonstrated that anti- $\beta_2\text{GPI}$  IgG antibodies isolated from aPS patients required TFPI and  $\beta_2\text{GPI}$  to enhance FXa generation. The authors of this study subsequently hypothesized that the anti- $\beta_2\text{GPI}$ :  $\beta_2\text{GPI}$  complex interfered with TFPI dependent inhibition of FXa by competing with the TFPI: FXa complex for the same phospholipid binding sites to therefore increase FXa generation. Although this hypothesis was not tested, it could have been supported if the stimulatory effect of anti- $\beta_2\text{GPI}$  IgG antibodies on FXa generation was neutralized in presence of high concentrations of phospholipids. In the present study, the stimulatory effect of anti- $\beta_2\text{GPI}$  and TFPI was demonstrated by increased thrombin generation with the reaction initiated by high concentrations of thromboplastin (rTF and synthetic phospholipids). Therefore, the mechanism by which anti- $\beta_2\text{GPI}$  inhibits TFPI activity is probably not related to the competition of proteins for binding to catalytic phospholipid surfaces. Instead, the current study proposes a novel mechanism whereby anti- $\beta_2\text{GPI}$  binds directly or indirectly to TFPI. This binding may then completely or partially abolish TFPI-dependent inhibition of blood coagulation resulting in accelerated thrombin generation.

Immunoblotting was used to determine whether anti- $\beta_2\text{GPI}$  IgG antibody binds directly to TFPI. The interaction between aPL and their target proteins is currently not well understood, with two models of interaction proposed in the literature. The first model proposed that aPL may recognize and bind epitopes on the conformationally altered protein structure resulting from interactions with an oxidized surface or anionic phospholipids.<sup>28</sup> The other model describes aPL as low-affinity antibodies that require their target antigen in high densities to form divalent complexes.<sup>26,29</sup> No cross-reactivity was demonstrated between anti- $\beta_2\text{GPI}$  IgG and TFPI (Figure 5). Other possible mechanisms may include anti- $\beta_2\text{GPI}$  antibody binding a neo-epitope on TFPI when bound to FXa or the anti- $\beta_2\text{GPI}$ :  $\beta_2\text{GPI}$  complex blocking TFPI binding to FXa.

Anti- $\beta_2\text{GPI}$  in the presence of TFPI has inhibitory activity against the regulation of TF-induced blood coagulation. Additions of anti- $\beta_2\text{GPI}$  and TFPI in TDP resulted in elevated thrombin generation, compared to TDP alone (Figure 4). This inhibitory component may be another natural anticoagulant e.g. protein C,

antithrombin, or  $\beta_2$ GPI. Although the physiological role of  $\beta_2$ GPI has not been established, it has been reported to have procoagulant and anticoagulant effects in blood coagulation.<sup>30</sup> In keeping with the mechanisms suggested earlier between anti- $\beta_2$ GPI and TFPI, it is possible that this inhibitory component is  $\beta_2$ GPI. Anti- $\beta_2$ GPI may preferentially bind to TFPI in the presence of increased concentrations of TFPI compared to  $\beta_2$ GPI, thus simultaneously inhibiting the anticoagulant functions of TFPI and  $\beta_2$ GPI, resulting in enhanced thrombin generation.

Thrombin generation in TDP was reduced in groups of patients with and without anti-TFPI activity (Figure 6), reflecting anticoagulant activities of anti- $\beta_2$ GPI and anti-prothrombin IgG antibodies. In comparison, thrombin generation in TDP spiked with TFPI was significantly enhanced by IgG fractions from the group with anti-TFPI activity and essentially unchanged by the group without anti-TFPI activity (Figure 6). These findings demonstrate that the entity in IgG fractions from patients with aPL responsible for the increase in thrombin generation compared to IgG fractions from normal controls, has anti-TFPI activity. It is probable that this entity is an anti-TFPI IgG and/or anti- $\beta_2$ GPI IgG antibody. Isolation of these entities from IgG fractions will help elucidate the antibodies involved.

This study represents the first work to provide evidence that anti-TFPI-like activity expressed by both anti-TFPI and anti- $\beta_2$ GPI IgG antibodies is associated with increased *in vitro* TF-induced thrombin generation. The precise relationship and/or interactions between anti- $\beta_2$ GPI antibodies, TFPI and potentially other as yet unidentified entities, remains to be determined. The findings from this study will provide better understanding of how interactions within the TF pathway may contribute to thrombotic complications in aPS. Additionally, these results may have implications for the use of rTFPI as an antithrombotic agent in patients with aPL.

*SYL: laboratory work, writing and review of manuscript; PE: laboratory work, writing and review of manuscript; LI: sample collection, laboratory work, writing and review of manuscript; JT: sample collection, laboratory work, writing and review of manuscript; RO: project design, writing and review of manuscript; ML: sample collection, writing and review of manuscript; RB: sample collection, writing and review of manuscript; MA: study design, laboratory work, sample collection, writing and review of manuscript.*

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