# Anti-$\beta_2$-glycoprotein I autoantibodies influence thrombin generation parameters via various mechanisms

Gábor Szabó[a,b], Ildikó Beke Debreceni[a], Tünde Tarr[c], Pál Soltész[d], Bjarne Østerud[e], János Kappelmayer[a]

[a]*Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Hungary*
[b]*Kálmán Laki Doctoral School of Biomedical and Clinical Sciences, University of Debrecen, Hungary*
[c]*Division of Clinical Immunology, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, Hungary*
[d]*Division of Angiology, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, Hungary,*
[e]*Department of Medical Biology, The Arctic University of Norway, Tromsø*

*Corresponding author:*
János Kappelmayer MD PhD
Department of Laboratory Medicine,
Faculty of Medicine, University of Debrecen, Hungary
4032, Debrecen, Nagyerdei krt. 98.
email: kappelmayer@med.unideb.hu

**Abstract**

**Introduction:** Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterised by recurrent thrombotic events, pregnancy loss and thrombocytopenia and the presence of antiphospholipid antibodies (APL). The exact pathomechanism of APS is still unknown, thus we investigated the effect of anti-$\beta_2$-glycoprotein I (anti-$\beta_2$GPI) on thrombin generation in different plasma samples.

**Methods:** For the separation of anti-$\beta_2$GPI IgG, overall 12 APS patients were selected. The criteria were the existence of lupus anticoagulant, and the presence of anti-CL and anti-$\beta_2$GPI, the latter exceeding at least 25 times the upper reference limit. We purified anti-$\beta_2$GPI IgG antibodies from APS patients by affinity chromatography and added the antibodies to normal pooled, and heterozygous forms of inherited thrombophilia plasma samples (prothrombin G20210A, factor V Leiden). To further specify the mechanism of the effect, we also used factor deficient plasmas in the thrombin generation assay.

**Results:** In normal pooled plasma, the anti-$\beta_2$GPI significantly prolonged Lag Time according to the lupus anticoagulant effect, in contrast, it also elevated Peak Thrombin significantly, which suggests a procoagulant effect. The antibody was also able to exert this multi-faceted effect both in $FV_{Leiden}$ heterozygous plasma and prothrombin G20210A heterozygous polymorphism, however, the prolonging effect was more remarkable in the latter. By using factor deficient plasmas, it was found that FVII is required for the prolongation, while intrinsic factors are needed for the elevation of the Peak Thrombin.

**Conclusion:** The anti-$\beta_2$GPI autoantibodies exert their effect in both normal and thrombophilic plasmas via various mechanisms.

**Keywords:** antiphospholipid syndrome, anti-$\beta_2$-glycoprotein I, thrombin generation, Factor V Leiden, Prothrombin G20210A

## 1. Introduction

Antiphospholipid syndrome (APS) is a multisystem autoimmune disease that is characterised by recurrent thrombotic events, pregnancy loss and thrombocytopenia and by the presence of antiphospholipid antibodies (APL). The three tests that are incorporated in recent guidelines [1, 2] for APL testing are two immunological assays, the anticardiolipin (anti-CL) and the anti-$\beta_2$-glycoprotein I (anti-$\beta_2$GPI) tests as well as the lupus anticoagulant (LA) assay. APLs are heterogenous antibodies and are frequent findings in patients with autoimmune disorders, which is designated as secondary APS as they are the consequence of a pre-existing disorder. At the other end of the spectrum are those apparently healthy individuals where APLs can be detected with no underlying clinical symptom. Several of these subjects only show positivity in either or both immunological assays used for APL detection hence termed as single or double positive cases. Many of these laboratory alterations are temporary APL positivity and they are of minor pathological significance. In between these two groups are the cases where no underlying syndrome is detectable, but patients suffer from venous or arterial thrombosis, and APLs are detectable with all three assays described above including LA. The triple positive cases are usually not temporary since APLs are detectable consequently in plasma samples drawn 12 weeks apart. More recently the term tetra positivity was also coined as phosphatidyl-serine/prothrombin antibodies were found to be major contributors to the lupus anticoagulant activity [3]. The protein $\beta_2$GPI is regarded as the primary target antigen, and it can interact with several cell types, receptors and enzymes [4, 5].

Several previous reports have dealt with the prothrombotic effect of the antiphospholipid antibodies, however, in many of the reports they used the purified whole IgG from APS patients. Since in APS, several other autoantibodies may occur [6-8] in this report we used highly purified autoantibodies and their respective purified ligand to investigate the effect of anti-$\beta_2$GPI on the thrombin generation of normal and pathological plasma samples.

The aim of this study was to characterise the effect of purified anti-$\beta_2$GPI autoantibodies on parameters of thrombin generation and to investigate the haemostatic pathways that lead to the effect of APLs.

## 2. Patients and Methods

### 2.1. Patient selection and characteristics

Our research was approved by the National Scientific and Ethical Committee, Medical Research Council of Hungary (approval number: 45368-1/2017/EKU). The inclusion criteria for selecting patients for the separation of anti-$\beta_2$GPI were the existence of lupus anticoagulant, and the presence of anti-CL and anti-$\beta_2$GPI, the latter exceeding at least 25 times the upper reference limit. Overall twelve patients were selected and 4 of them had an underlying disease, while the 8 others had no underlying disorder. One patient had both obstetric complications and venous thrombosis, while two patients had both venous and arterial thrombosis and only one was devoid of clinical symptoms. At the time of sampling, two patients were not receiving any antithrombotic therapy, the rest of the patients were taking either anticoagulant or antiplatelet drug **(Table 1.).**

| | | | Laboratory Results | | | | Clinical Results | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Patients | Sex | Age (year) | anti-β2GPI IgG (U/mL) | anti-CL IgG (U/mL) | Lupus Anticoagulant | Lowest PLT (G/L) in patients' history | Arterial Thrombosis | Venous Thrombosis | Obstetric Complications | Diagnosis | Antithrombotic drug* | Underlying Autoimmune Disease |
| 1. | female | 35 | **674** | **175** | **pos** | 272 | neg | neg | **pos** | SAPS | ASA | RA |
| 2. | female | 35 | **903** | **164** | **pos** | **6** | **pos** | **pos** | neg | PAPS | ASA | none |
| 3. | female | 40 | **1,126** | **260** | **pos** | 134 | neg | **pos** | neg | PAPS | acenocoumarol | none |
| 4. | female | 28 | **1,346** | **223** | **pos** | 276 | neg | **pos** | **pos** | PAPS | apixaban | none |
| 5. | male | 20 | **2,520** | **449** | **pos** | **31** | neg | **pos** | n/a | PAPS | dabigatran | none |
| 6. | female | 36 | **2,864** | **384** | **pos** | **30** | neg | **pos** | neg | SAPS | acenocoumarol | SLE |
| 7. | female | 28 | **2,915** | **575** | **pos** | **32** | neg | **pos** | neg | PAPS | rivaroxaban | none |
| 8. | female | 51 | **3,750** | **496** | **pos** | **120** | neg | **pos** | neg | SAPS | acenocoumarol | SLE |
| 9. | female | 33 | **5,540** | **>2024** | **pos** | **78** | neg | **pos** | neg | SAPS | none | SLE |
| 10. | female | 54 | **11,526** | **2,133** | **pos** | **108** | neg | **pos** | neg | PAPS | LMWH | none |
| 11. | female | 27 | **14,639** | **>2024** | **pos** | 216 | neg | neg | neg | LA | none | none |
| 12. | female | 55 | **60,411** | **3,909** | **pos** | **45** | **pos** | **pos** | neg | PAPS | enoxaparin | none |

**Table 1. Summary of laboratory and clinical symptoms of antiphospholipid syndrome patients enrolled in our experiments.**
**anti-β2GPI**: anti-$\beta_2$-glycoprotein I, **anti-CL**: anticardiolipin, **PLT**: platelet count, **pos**: positive, **neg**: negative, **n/a**: not applicable**, PAPS**: primary antiphospholipid syndrome, **SAPS**: secondary antiphospholipid syndrome, **ASA**: acetylsalicylic acid, **LMWH**: low-molecular-weight heparin, **LA**: lupus anticoagulant positivity, **RA**: rheumatoid arthritis, **SLE**: systemic lupus erythematosus, Cut-off value (anti-$\beta_2$GPI IgG): 20 U/mL, Cut-off value (anti-CL IgG): 20 U/mL, Normal range (platelet count): 130–400 G/L (platelet counts in bold indicate thrombocytopenia)
*Antithrombotic drug taken at the time of sampling

**Note for Table 1**: 2-column fitting

The preparation was comprised of two major steps: (i) isolation of the total IgG of a patient with a Protein G affinity column, and (ii) the preparation of an in-house

$\beta_2$-glycoprotein I ($\beta_2$GPI) affinity column, which was used for the isolation of the individual anti-$\beta_2$GPI autoantibodies.

### 2.2. Purification of the ligand $\beta_2$GPI

**Chromatographic Purification** – Isolation of $\beta_2$GPI was performed by a method previously described by Artenjak et al. [9]. The purification process comprises three major steps: (i) Perchloric Acid (PCA) Precipitation, (ii) Heparin Affinity Chromatography, (iii) Ion Exchange Chromatography. Approximately 200 mL of citrated normal pooled human plasma was diluted twofold with saline, and plasma proteins were precipitated by PCA (60%) added dropwise at 285 mM final concentration under stirring conditions in 20 minutes. The precipitate was spun (3,270 *g*, 20 min, 4 °C), the supernatant was collected, and its pH was adjusted to 8.0 with 1 M NaOH. The supernatant was dialysed against Tris-HCl 0.02 M, NaCl 0.03 M, pH 8.0 at 4 °C overnight. Proteins were separated with a heparin affinity chromatography column (HiTrap Heparin [5 mL], GE Healthcare, Uppsala, Sweden), from which they were eluted with 350 mM NaCl. Fractions containing significant amount of protein were pooled and dialysed against acetate buffer 0.05 M, NaCl 0.05 M, pH 4.8. The pooled and dialysed fractions were further purified with an ion exchange chromatography column (UNOsphere S [5 mL], BioRad, USA). Proteins were eluted with 300 mM NaCl.

**Western Blot Analysis** – We took samples from the ion exchange chromatography elution fractions. Proteins were resolved on 10% SDS-polyacrylamide gel electrophoresis in reducing conditions and transferred to nitrocellulose membrane. For specific staining, anti-human $\beta_2$GPI primary antibody (raised in goat; Novus Biologicals, Littleton, CO USA), and HRP-conjugated anti-goat IgG secondary antibody (produced in rabbit, Sigma-Aldrich, USA) were used. Staining was developed with a chemiluminescent HRP substrate (Immobilon Western, Millipore, Billerica, MA USA). After developed, the membrane was stained with Coomassie brilliant blue R250 (Sigma-Aldrich) for total protein detection.

### 2.3. Purification of anti-$\beta_2$GPI IgG from APS Patients' Plasma

We involved 12 patients in our experiments altogether. Each patient showed triple positivity in their laboratory tests (anti-$\beta_2$-glycoprotein I IgG, anticardiolipin IgG, lupus anticoagulant) and all of them except one had either venous/arterial thrombosis or pregnancy complication in their history. We isolated anti-$\beta_2$GPI IgG from the patients' serum by a two-step affinity chromatography method. First, patient IgG antibodies were purified by a Protein G column (HiTrap Protein G [5 mL], GE Healthcare, Uppsala, Sweden). The eluted IgG was dialysed against phosphate buffer 20 mM, pH 7.0 and purified with an in-house $\beta_2$GPI-coupled affinity chromatography column (HiTrap NHS-activated [5 mL], GE Healthcare, Uppsala, Sweden).

**Functional analysis of the isolated autoantibody** – In preliminary experiments we monitored the isolation procedure by checking the functionality of the isolated anti-$\beta_2$GPI autoantibodies. In these experiments purified antibodies were added to normal pooled plasma and APTT was measured with a mechanical coagulometer. After having been able to prove the lupus anticoagulant activity of purified antibodies several times, we considered our purification procedure as a functioning method.

### 2.4. Thrombin Generation Assay

Anti-$\beta_2$GPI (15–500 U/mL final concentration) or $\beta_2$GPI (200–1000 μg/mL final concentration) were added to normal pooled plasma, $FV_{Leiden}$ heterozygous plasma or FVII, FIX, FXI deficient plasma (Siemens Healthcare, Marburg, Germany). In a 96-well black plate (Greiner Bio-One North America Inc., Monroe, USA) 20 μL reagent containing 1 or 5 pM tissue factor (TF) and 4 μM phospholipid (PPP-Reagent LOW or PPP-Reagent, Thrombinoscope BV, Maastricht, The Netherlands) was combined with 80 μL antibody/antigen-treated plasma. The plate with the samples and the reagent was placed into a fluorescent microplate reader. The machine incubated the reagent and the plasma for 10 min at 37 °C and automatically added the start reagent ($CaCl_2$, aminomethyl coumarin [fluorescent thrombin substrate]; FluCa-Kit, Thrombinoscope BV, Maastricht, The Netherlands) to the samples. Signals were detected for 60 min. The Lag Time (min), Peak Thrombin (nM) and Endogenous Thrombin Potential (ETP; nM×min) were evaluated with Thrombinoscope software (Diagnostica Stago). To optimise the assay conditions for measuring the effects of

anti-$\beta_2$GPI antibodies, purified antibody was added to normal plasma in 15–500 U/mL final concentration. Thrombin generation was initiated with 1, 5 or 20 pM TF and 4 μM phospholipid (for 20 pM TF: PPP-Reagent HIGH Thrombinoscope BV, Maastricht, The Netherlands). Time parameters (Lag Time, Time-to-Peak) were prolonged in accordance with the lupus anticoagulant effect at each concentration of TF, but the best signal in prolonging the time parameters was the lowest TF, thus in subsequent experiments the 1 pM TF concentration was used (**Supplementary Figure 1**).

### 2.5. Microparticle Isolation and Flow Cytometry

To assess if microparticles influence the effect of anti-$\beta_2$GPI on thrombin generation, we isolated microparticles from normal pooled plasma, preparing microparticle-depleted plasma (MDP). Whole citrated blood from five healthy donors were centrifuged at 1,500 *g*, 15 min, 20 °C. The plasma was collected and centrifuged for a second time at 10,000 *g*, 10 min, 20 °C, which resulted in a control plasma for thrombin generation (indicated as NPP). To remove microparticles from the plasma, we centrifuged the sample further at 16,000 *g*, 30 min, 20 °C (indicated as MDP). After centrifugation the supernatant was collected. The remaining 10% of the initial volume was used to confirm the removal of the microparticles with flow cytometry.

We detected the microparticles based on their size, phosphatidylserine and platelet marker (CD41) positivity. For size calibration (size range: 0.3–1.0 μm), we used calibration beads (Megamix-Plus SSC, Biocytex, France), and we carried out our flow cytometry measurements using a BD FACSCanto II flow cytometer. By dilution factors and flow rates used during the experiment, we calculated the amount of microparticles removed from the NPP.

Antibodies from Patient 8 were added to NPP and MDP in 500 U/mL final concentration. We measured thrombin generation initiated either by 4 μM or by 0.5 μM phospholipid and 1 pM TF as described above.

### 2.6. Statistical analysis

For statistical analysis, we used GraphPad Prism software. We analysed each group for normal distribution with Kolmogorv–Smirnov test, and groups showing normal distribution were compared with one-way analysis of variance (one-way ANOVA). Groups without normal distribution were compared with Kruskal–Wallis test. P values < 0.05 were considered as statistically significant.

## 3. Results

### 3.1. Effect of anti-β2GPI IgG on thrombin generation

First, we investigated the effects of the anti-β2GPI, which was added to normal pooled plasma in 125–500 U/mL final concentration. Thrombin generation was initiated with 1 pM tissue factor and 4 μM phospholipid. The antibody significantly delayed thrombin generation, and it also significantly elevated Peak Thrombin at the highest concentration applied, but at the investigated three concentration points had no significant effect on ETP (**Figure 1**).

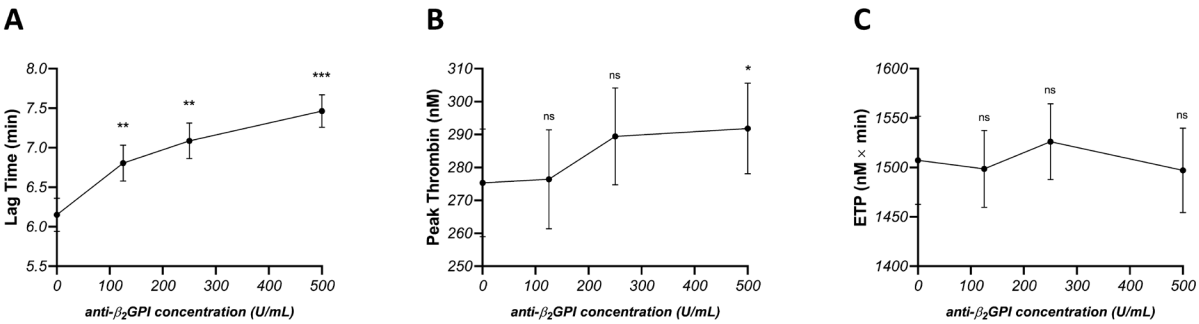


**Fig. 1. Anti-β2GPI delays thrombin generation but elevates Peak Thrombin in normal plasma.**
Anti-β2GPI was added to normal pooled plasma in 125–500 U/mL final concentration. Thrombin generation was initiated with 1 pM tissue factor and 4 μM phospholipid. The antibody delayed thrombin generation (**A**), however, Peak Thrombin (**B**) was elevated significantly from 275 to 291 nM by 500 U/mL antibody. The antibody did not have any significant effect on Endogenous Thrombin Potential (**C**) (mean + SEM, n=14, ns: no significance *: p<0.05, **: p<0.01, ***: p<0.001)

**Note for Fig. 1**: 2-column fit

### 3.2. The effect of anti-β2GPI IgG in plasma with Leiden mutation

We next determined whether the effects we observed in normal plasma are also true in

a frequent congenital thrombophilia. The heterozygous form of the coagulation factor V Leiden mutation ($FV_{Leiden}$) exerts a 6–8-fold risk for venous thrombosis. It is the most frequent form of congenital thrombophilia, in Hungary it can be observed in nearly 10% of the general population [10]. We added anti-$\beta_2$GPI to $FV_{Leiden}$ heterozygous pooled plasma at 125–500 U/mL final concentration. Thrombin generation was initiated with 1 pM tissue factor and 4 μM phospholipid.

Anti-$\beta_2$GPI delayed thrombin generation and elevated Peak Thrombin – but not Endogenous Thrombin Potential – in $FV_{Leiden}$ heterozygous plasma (**Figure 2**).

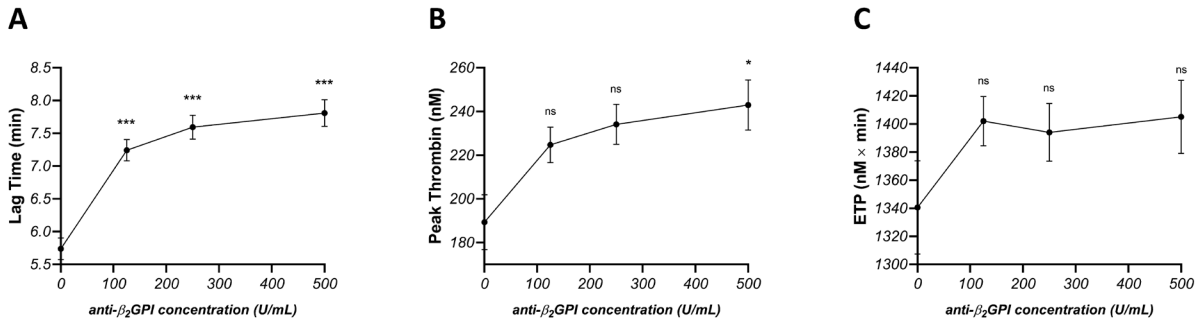


**Fig. 2. Effect of anti-$\beta_2$GPI on thrombin generation in $FV_{Leiden}$ heterozygous plasma is similar to that in normal plasma.**

Anti-$\beta_2$GPI was added to $FV_{Leiden}$ heterozygous pooled plasma in 125–500 U/mL final concentration. Thrombin generation was initiated with 1 pM tissue factor and 4 μM phospholipid. Anti-$\beta_2$GPI delayed thrombin generation (**A**) and elevated Peak Thrombin (**B**) and Endogenous Thrombin Potential (**C**) in $FV_{Leiden}$ heterozygous plasma, however, the thrombin generation of $FV_{Leiden}$ heterozygous plasma is lower than that of normal plasma. (n=5, mean + SEM, ns: no significance *: $p<0.05$, ***: $p<0.001$)

**Note for Fig. 2**: 2-column fitting

### 3.3. The effect of anti-$\beta_2$GPI IgG in plasma with prothrombin G20210A polymorphism

We also examined the effect of the antibodies in plasma samples with heterozygous prothrombin G20210A polymorphism, the second most frequent congenital thrombophilia. Anti-$\beta_2$GPI was added to prothrombin G20210A heterozygous plasma samples from five individuals at 125–500 U/mL final concentration. Thrombin generation was initiated with 1 pM tissue factor and 4 μM phospholipid. The anti-$\beta_2$GPI antibody prolonged significantly the Lag Time but had no considerable effect on the quantitative thrombin generation parameters (**Figure 3**).

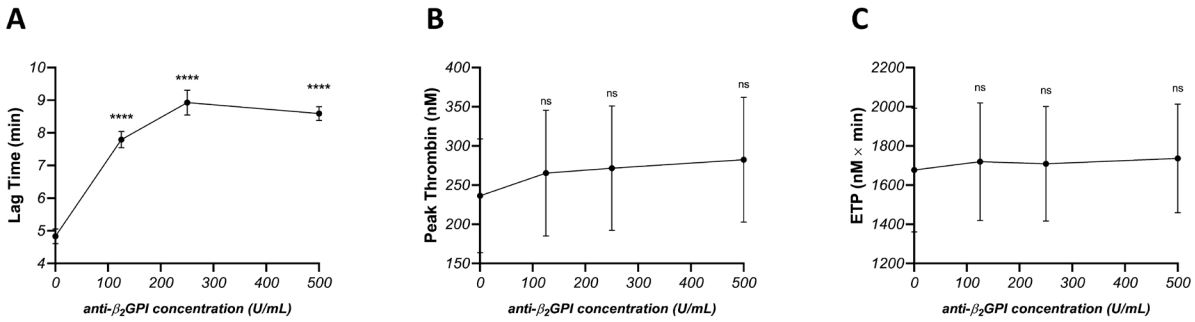


**Fig. 3. Anti-β2GPI fails to enhance thrombin generation in prothrombin G20210A heterozygous plasma.**
Anti-$\beta_2$GPI was added to prothrombin G20210A heterozygous pooled plasma in 125–500 U/mL final concentration. Thrombin generation was initiated with 1 pM tissue factor and 4 μM phospholipid. Anti-$\beta_2$GPI delayed thrombin generation (**A**) but had no effect either on Peak Thrombin (**B**) or on Endogenous Thrombin Potential (**C**) unlike in $FV_{Leiden}$ heterozygous or normal pooled plasma. (n=5, mean + SEM, ns: no significance, ***: p<0.0001)

**Note for Fig. 3**: 2-column fitting

### 3.4. The effect of low APL concentration and ligand concentration on thrombin generation

We titrated the APL concentrations down to just below the reference range and found that in normal plasma anti-β2GPI as low as 31 U/mL and 62 U/mL i.e. at 2–3 times the upper reference limit can also elicit a significant ETP (**Figure 4**).

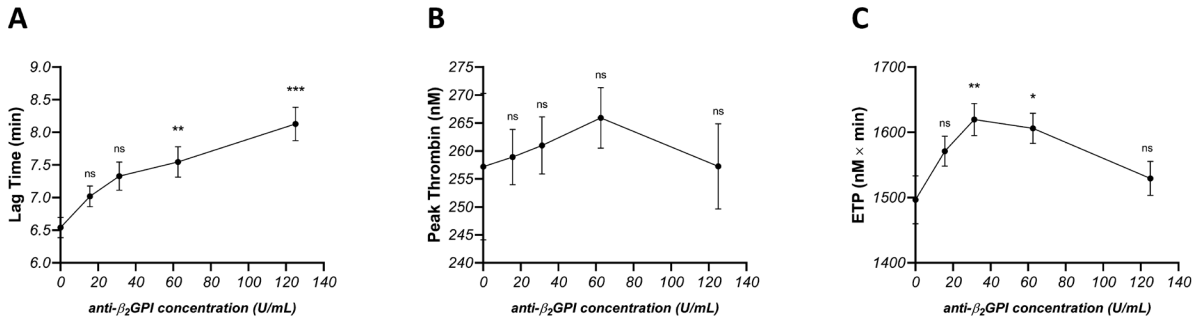


**Fig. 4. Anti-β2GPI delays thrombin generation but elevates Peak Thrombin and Endogenous Thrombin Potential in normal plasma.**
Anti-$\beta_2$GPI was added to normal pooled plasma in 15–125 U/mL final concentration. Thrombin generation was initiated with 1 pM tissue factor and 4 μM phospholipid. The antibody delayed thrombin generation (**A**), however, Peak Thrombin (**B**) and Endogenous Thrombin Potential (**C**) were also elevated, the latter significantly from 1496 to 1619 nM × min by 31 U/mL antibody. (mean + SEM, n=9, ns: no significance *: p<0.05, **: p<0.01)

**Note for Fig. 4**: 2-column fitting

Since the effect of anti-$\beta_2$GPI on cells is thought to be exerted in combination with its ligand the $\beta_2$GPI glycoprotein, we hypothesised that in plasma the ligand may modulate the effect of the anti-$\beta_2$GPI on thrombin generation. In plasma samples derived from APS patients, a highly elevated $\beta_2$GPI was described [11], thus we thought to determine the effect of varying concentrations of $\beta_2$GPI on the autoantibody-elicited effects in the thrombin generation assay. We purified anti-$\beta_2$GPI and $\beta_2$GPI in preliminary experiments and both of them were added to normal plasma in 15–125 U/mL and 200–1000 μg/mL final concentration, respectively. Thrombin generation was initiated with 1 pM tissue factor and 4 μM phospholipid. Elevating effect of anti-$\beta_2$GPI on Endogenous Thrombin Potential was found to be mitigated by the increase of $\beta_2$GPI concentration. (**Figure 5**).

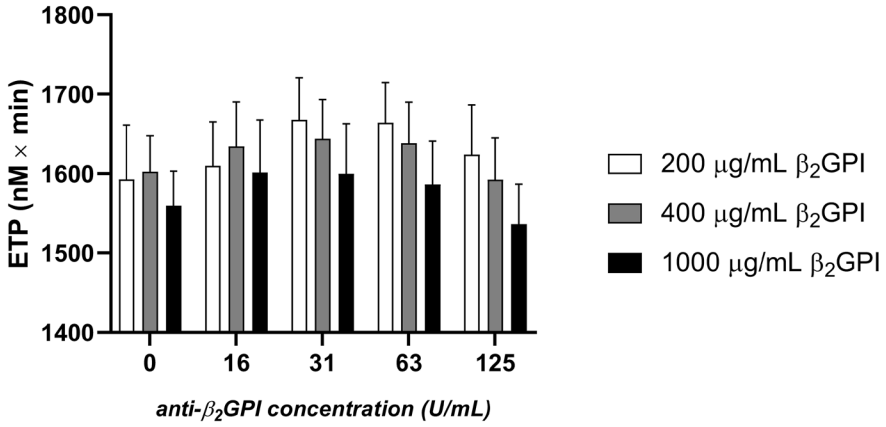


**Fig. 5. Prothrombotic effect of anti-$\beta_2$GPI is attenuated by its antigen.**
Anti-$\beta_2$GPI and $\beta_2$GPI were added to normal pooled plasma in 15–125 U/mL and 200–1000 μg/mL final concentration, respectively. Thrombin generation was initiated with 1 pM tissue factor and 4 μM phospholipid. Elevating effect of anti-$\beta_2$GPI on Endogenous Thrombin Potential was mitigated by the increase of $\beta_2$GPI concentration. (n=4, mean + SEM)

**Note for Fig. 5**: 1.5-column fitting

### 3.5. Coagulation pathways leading to altered thrombin generation

To investigate the potential mechanisms how anti-$\beta_2$GPI exerts these effects, the purified autoantibody was added to FVII, FIX or FXI deficient plasmas in addition to normal plasma at 125–500 U/mL final concentration. Since factor deficient plasmas failed to produce a reproducible thrombin generation curve at 1 pM TF concentration because of the low amount of generated thrombin here the higher TF concentration

reagent was used. After thrombin generation was initiated with 5 pM tissue factor and 4 μM phospholipid we investigated time parameters and the quantity of the formed thrombin. Lag times were similar in normal plasma and FIX and FXI deficient plasmas, but obviously it was much longer in the FVII deficient plasma. The autoantibody prolonged Lag Time in normal plasma, but it was unable to exert this effect in the absence of FVII. The absence of FIX or FXI did not influence the prolonging effect of the antibody (**Figure 6, panel A)**. In normal plasma, anti-$\beta_2$GPI showed an enhancing effect on Peak Thrombin values and this potentiating effect was highly significant. In FVII deficient plasma, obviously about fourfold less thrombin were generated, however, this low thrombin peak was significantly elevated in the presence of the autoantibody. In case of FIX and FXI deficient plasmas where also a lot less thrombin was generated compared to normal plasma, the autoantibody failed to elevate the Peak Thrombin levels (**Figure 6, panel B)**.

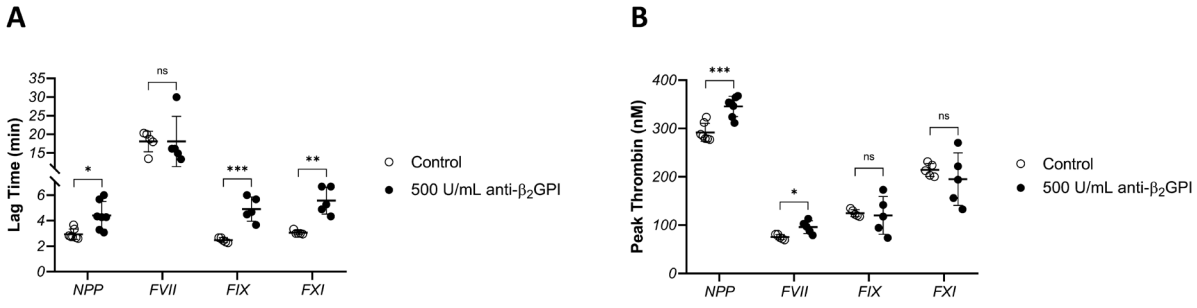


**Fig. 6. Anti-$\beta_2$GPI requires FVII to delay thrombin generation and FIX and FXI to elevate Peak Thrombin.**
Anti-$\beta_2$GPI was added to normal pooled plasma or FVII, FIX or FXI deficient plasmas in 125–500 U/mL final concentration. Thrombin generation was initiated with 5 pM tissue factor and 4 μM phospholipid. The antibody prolonged Lag Time in normal plasma, but it was unable to exert this effect in the absence of FVII. The absence of FIX or FXI did not influence the prolonging effect of the antibody (**A**). In normal plasma, anti-$\beta_2$GPI shows a prothrombotic effect, but when FIX or FXI were missing from the plasma, the antibody ceased to elevate Peak Thrombin (**B**). (**NPP**: normal pooled plasma, **FVII**: FVII deficient plasma, **FIX**: FIX deficient plasma, **FXI**: FXI deficient plasma; mean + SD, ns: no significance, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$)

**Note for Fig. 6**: 2-column fitting

### 3.6. Influence of phospholipid concentration on the effect of anti-$\beta_2$GPI

We removed microparticles from NPP, which was verified by flow cytometry. (**Figure 7, Panels A–D**). The number of microparticles removed from the NPP was 1287 microparticle/μL plasma. When 4 μM phospholipid was used in TGT, the Lag

Time was prolonged (by 11%) and Peak Thrombin (by 27%) and ETP (by 19%) were elevated similarly to the previous experiments. When 0.5 μM phospholipid was used, the effect of the antibody became more dependent on the phospholipid content of the plasma, and the antibody ceased to prolong Lag Time – which decreased by around 10% –, but the Peak Thrombin elevation (by 73%) became more marked. The removal of the microparticles in itself did not have a noticeable effect on Lag Time, however, at 0.5 μM phospholipid concentration, the marked Peak Thrombin-elevating effect (by 6%) dropped and there was a similar tendency in case of ETP but with less sharp contrast.

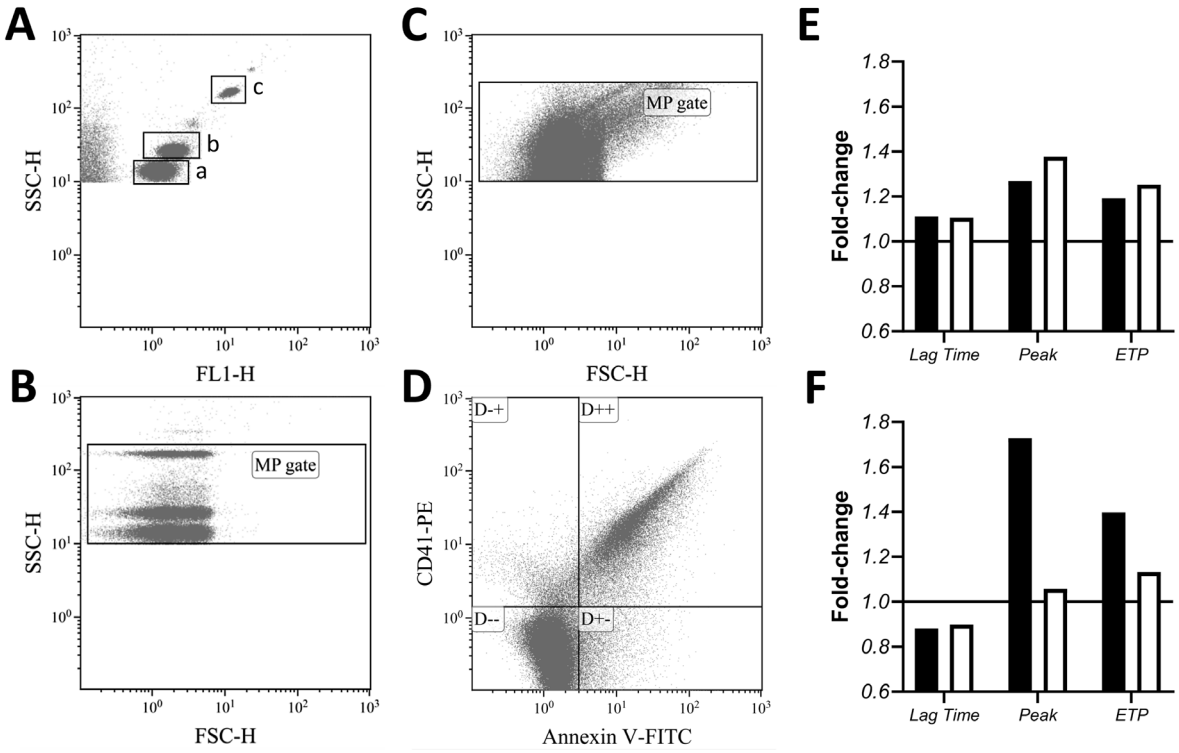


**Fig. 7. The effect of anti-β$_2$GPI is dependent on the microparticle and phospholipid concentration**
Microparticles were isolated from normal pooled plasma. Microparticle size range (panel **A**, a–c: 0.3–1.0 μm) was determined with calibration beads (panels **A** and **B**) and microparticles were identified by size (panel **C**) and CD41 and annexin V double positivity (panel **D**, gate D++). Anti-β$_2$GPI was added to normal pooled plasma (panels **E** and **F**, filled columns) and microparticle-depleted plasma (empty columns) in 500 U/mL final concentration. Thrombin generation was initiated with 1 pM tissue factor and either with 4 μM phospholipid (panel **E**) or 0.5 μM phospholipid (panel **F**). The effect of the antibody is plotted as fold-change of mean of a triplicate measurement. The absence of microparticles did not markedly alter the effect of the antibodies when the 4 μM phospholipid were added to the plasma, but the decreasing the phospholipid concentration to 0.5 μM, we got a considerable drop in Peak Thrombin and ETP in the absence of microparticles. (n=1)

**Note for Fig. 7**: 2-column fitting

## 4. Discussion

Increased thrombin generation in APS has long been observed in clinical studies, where elevated levels of prothrombin fragments (F1+2) have been described along with increased fibrinopeptide A concentrations in anticardiolipin positive APS patients [12], however, the causal relationship between the antiphospholipid antibody and the generation of the prothrombotic state was not evident from such studies. Nevertheless, later in experimental models [13], it was clearly shown that the anti-$\beta_2$GPI autoantibodies from patients with APS were sufficient to dose-dependently potentiate arterial thrombus formation in a laser-induced injury model in mice. The prothrombotic activity of APLs has been confirmed in numerous other studies, and these autoantibodies were shown to elicit cellular activation in both platelets [14-16] and endothelial cells [17].

In our experiments, we intended to determine the effect of APLs in plasma and to outline mechanisms that are involved in this effect. Thus, we used highly purified anti-$\beta_2$GPI IgG from numerous APS patients and measured thrombin generation in plasma under the effect of these autoantibodies. We used a test system with only 1 pM tissue factor (TF) concentration as thrombin generation is known to be more sensitive when lower concentrations of TF are used. In these circumstances, we could confirm the intense prolonging effect on time parameters, which was described in previous publications [18], but we also observed a dose-dependent thrombin enhancement effect of the isolated APLs. The effects of the isolated anti-$\beta_2$GPI autoantibodies were compared according to the clinical parameters of the patients from whom these autoantibodies were isolated. No significant differences were observed in the prolongation of Lag Time or elevation of Peak Thrombin levels either when anti-$\beta_2$GPI derived from the patient group with low (<100 G/L) or normal platelet count was added to NPP or when autoantibodies derived from patients with very high anti-$\beta_2$GPI titres (>5000 U/mL) or lower titres were added to NPP.

It was described earlier that the ETP of asymptomatic $FV_{Leiden}$ carriers is not significantly different from that of controls [19]. We found that high autoantibody concentration also elevated the peak concentration of generated thrombin in normal plasma as well as in asymptomatic carriers of factor V Leiden heterozygous plasmas. This means that in this frequent congenital thrombophilia, the presence of APLs can

further aggravate the thrombotic risk. The APLs also prolonged the time parameters in another frequently occurring congenital thrombophilic state the prothrombin G20210A polymorphism. Although there was a tendency for augmented thrombin peak at all antibody concentrations, even at the highest level (500 U/mL), it failed to result in significant elevation of both Peak Thrombin and Endogenous Thrombin Potential. This is plausible since the Lag Time here is the shortest, and the antibody intensely prolonged the Lag Time for thrombin generation by 79% compared to what we found in $FV_{Leiden}$ heterozygous plasma or normal plasma, which was 34% and 21% prolongation, respectively.

It is well known that prothrombin G20210A results in an elevated factor II level, which does not seem to be further boosted by the APLs. Indeed, in our plasma samples the ETP of the plasmas with G20210A polymorphism tended to be higher compared to $FV_{Leiden}$ heterozygous or normal plasmas. Since the thrombin generation assay integrates the pro- and antithrombotic effects, we further investigated the lower range of the pathological APL concentrations in normal plasmas and observed the time-prolonging effect already at low anti-$\beta_2$GPI IgG concentration, but also found that the ETP became significantly elevated just at moderately elevated anti-$\beta_2$GPI IgG levels, exceeding only at 2–3 times the upper reference limit. This would imply a potential prothrombotic effect of even subtle elevations in anti-$\beta_2$GPI concentrations. As for the mechanism of enhanced thrombin generation the most widely investigated phenomenon is that APLs cause an acquired activated protein C resistance [20-22]. More recently this mechanism has been more delicately analysed by advanced computational analysis of thrombin generation curves where it was established that the haemostatic balance shifts towards the more prothrombotic phenotype in APS due to elevated prothrombin conversion but unchanged thrombin inactivation rates [18]. Nevertheless several other mechanisms have been described like increased resistance to annexin A5 [23], impairment of fibrinolysis [24], and a decrease in tissue factor pathway inhibitor (TFPI) activity [25].

Coagulation pathways contributing to the effect of anti-$\beta_2$GPI IgG were investigated by normal and factor deficient plasmas where we could confirm the significant prolongation in Lag Times and the elevation in Peak Thrombin concentrations. Naturally, the observed Peak Thrombin levels for normal plasmas were considerably higher in this series of measurements than in the preceding experiments due to the

higher concentration of TF in the activation reagent. We found that Lag Times were the longest and the Peak Thrombin was the smallest in case of FVII deficient plasmas. Lag Times of FIX and FXI deficient plasmas were comparable to normal plasma, which shows that this parameter is mostly dependent on FVII. In case of the Peak Thrombin parameter, understandably, values provided by the FXI deficient plasma were the closest to those of normal plasma, followed by FIX and FVII deficient plasmas. After the addition of anti-$\beta_2$GPI IgG, it was found that the Lag Time was prolonged in FIX and FXI deficient plasmas, but regarding the Peak Thrombin parameter, only the FVII deficient plasma resulted in significant elevation. These observations may refer to the varying inhibitory effects of anti-$\beta_2$GPI IgG on APC and TFPI.

The ligand of the investigated APL, the $\beta_2$GPI, is an abundant plasma protein with no known physiological function. However, its conformation is known to affect coagulation processes and its interaction with the autoantibody [26, 27]. Here, we found that, at higher concentrations, the $\beta_2$GPI ligand can mitigate the prothrombotic effect of the anti-$\beta_2$GPI autoantibody possibly by scavenging the activating IgG.

Our test system during the experiments was platelet poor plasma as we intended to study the effects of APLs devoid of cells. Nevertheless, it is not unlikely that microparticles that are present in normal plasma contribute to the observed changes. This question was approached by removing microparticles from plasma and investigating the changes in Lag Time and Peak Thrombin parameters. Using the standard 1 pM TF and 4 μM phospholipid concentrations, we obtained no difference in these parameters. However, when the phospholipid content was lowered to 0.5 μM, the Peak Thrombin parameter became sensitive to the presence of microparticles providing a 70% higher value in their presence. This experiment showed that there may be two distinct effects of the antibody (the pro- and the anticoagulant) exerted at the same time, and the outcome depends on the balance of the two. When the phospholipid concentration was low, the anticoagulant effect could not suppress the procoagulant effect, consequently the Peak Thrombin value managed to shoot up.

Our studies confirm and extend previous observations in APS. In clinical studies, increased thrombin generation may be a result of direct as well as numerous indirect effects of the autoantibodies [28]. Most of these reports rely on the two-hit hypothesis for thrombotic risk assessment [29]. In our series of experiments the prothrombotic

effect of APLs was determined in plasmas of normal as well as congenital thrombophilic subjects by purified anti-$\beta_2$GPI IgG with no additional activation. Furthermore, the modulatory effect of the $\beta_2$GPI ligand, platelet microparticles and various coagulation pathways were measured on the prothrombotic effect of anti-$\beta_2$GPI IgG in appropriately modified test systems. We think that such studies may advance the field by shedding light on previously undescribed associations during the pathogenesis of thrombotic processes in APS.

**Acknowledgements**

The authors would like to acknowledge the help of Anikó Veszprémi Györfi and Erzsébet Nagy (Haemostais Unit at the Department of Laboratory Medicine) with collecting clinical samples. This work was supported by Data Intensive and Open Science School (EFOP-3.6.1-16-2016-00022).

**Funding**

This research was supported by the grant OTKA K16 120725 and GINOP-2.3.2-15-2016-00043.

## References

[1] K.M. Devreese, S.S. Pierangeli, B. de Laat, A. Tripodi, T. Atsumi, T.L. Ortel, Testing for antiphospholipid antibodies with solid phase assays: guidance from the SSC of the ISTH, J Thromb Haemost 12(5) (2014) 792-5.
[2] K.M.J. Devreese, T.L. Ortel, V. Pengo, B. de Laat, Laboratory criteria for antiphospholipid syndrome: communication from the SSC of the ISTH, J Thromb Haemost 16(4) (2018) 809-813.
[3] M.G. Cattini, E. Bison, E. Pontara, C. Cheng, G. Denas, V. Pengo, Tetra positive thrombotic antiphospholipid syndrome: Major contribution of anti-phosphatidyl-serine/prothrombin antibodies to lupus anticoagulant activity, J Thromb Haemost 18(5) (2020) 1124-1132.
[4] T. Shi, G.M. Iverson, J.C. Qi, K.A. Cockerill, M.D. Linnik, P. Konecny, S.A. Krilis, Beta 2-Glycoprotein I binds factor XI and inhibits its activation by thrombin and factor XIIa: loss of inhibition by clipped beta 2-glycoprotein I, Proc Natl Acad Sci U S A 101(11) (2004) 3939-44.
[5] T. McDonnell, C. Wincup, I. Buchholz, C. Pericleous, I. Giles, V. Ripoll, H. Cohen, M. Delcea, A. Rahman, The role of beta-2-glycoprotein I in health and disease associating structure with function: More than just APS, Blood Rev 39 (2020) 100610.
[6] J.F. de Carvalho, M.T. Caleiro, Primary antiphospholipid syndrome and thyroid involvement, J Clin Rheumatol 16(4) (2010) 164-7.
[7] N.Y. Kim, H.J. Cho, H.Y. Kim, K.M. Yang, H.K. Ahn, S. Thornton, J.C. Park, K. Beaman, A. Gilman-Sachs, J. Kwak-Kim, Thyroid autoimmunity and its association with cellular and humoral immunity in women with reproductive failures, Am J Reprod Immunol 65(1) (2011) 78-87.
[8] R. Promberger, K. Walch, R. Seemann, S. Pils, J. Ott, A Retrospective Study on the Association between Thyroid Autoantibodies with β2-glycoprotein and Cardiolipin Antibodies in Recurrent Miscarriage, Iran J Allergy Asthma Immunol 16(1) (2017) 72-76.
[9] A. Artenjak, A. Leonardi, I. Križaj, A. Ambrožič, S. Sodin-Semrl, B. Božič, S. Cučnik, Optimization of unnicked β2-glycoprotein I and high avidity anti-β2-glycoprotein I antibodies isolation, J Immunol Res 2014 (2014) 195687.
[10] I. Balogh, R. Póka, G. Pfliegler, M. Dékány, Z. Boda, L. Muszbek, High prevalence of factor V Leiden mutation and 20210A prothrombin variant in Hungary, Thromb Haemost 81(4) (1999) 660-1.
[11] A. Banzato, V. Pengo, Clinical relevance of $\beta_2$-glycoprotein-I plasma levels in antiphospholipid syndrome (APS), Curr Rheumatol Rep 16(6) (2014) 424.
[12] J.S. Ginsberg, C. Demers, P. Brill-Edwards, M. Johnston, R. Bona, R.F. Burrows, J. Weitz, J.A. Denburg, Increased thrombin generation and activity in patients with systemic lupus erythematosus and anticardiolipin antibodies: evidence for a prothrombotic state, Blood 81(11) (1993) 2958-63.
[13] A. Arad, V. Proulle, R.A. Furie, B.C. Furie, B. Furie, $\beta_2$-Glycoprotein-1 autoantibodies from patients with antiphospholipid syndrome are sufficient to potentiate arterial thrombus formation in a mouse model, Blood 117(12) (2011) 3453-9.
[14] W. Jy, M. Tiede, C.J. Bidot, L.L. Horstman, J.J. Jimenez, J. Chirinos, Y.S. Ahn, Platelet activation rather than endothelial injury identifies risk of thrombosis in subjects positive for antiphospholipid antibodies, Thromb Res 121(3) (2007) 319-25.
[15] T. Shi, B. Giannakopoulos, X. Yan, P. Yu, M.C. Berndt, R.K. Andrews, J. Rivera, G.M. Iverson, K.A. Cockerill, M.D. Linnik, S.A. Krilis, Anti-beta2-glycoprotein I antibodies in complex with beta2-glycoprotein I can activate platelets in a

dysregulated manner via glycoprotein Ib-IX-V, Arthritis Rheum 54(8) (2006) 2558-67.
[16] R.T. Urbanus, M.T. Pennings, R.H. Derksen, P.G. de Groot, Platelet activation by dimeric beta2-glycoprotein I requires signaling via both glycoprotein Ibalpha and apolipoprotein E receptor 2', J Thromb Haemost 6(8) (2008) 1405-12.
[17] N. Clemens, K. Frauenknecht, A. Katzav, C. Sommer, P. von Landenberg, In vitro effects of antiphospholipid syndrome-IgG fractions and human monoclonal antiphospholipid IgG antibody on human umbilical vein endothelial cells and monocytes, Ann N Y Acad Sci 1173 (2009) 805-13.
[18] R.M.W. Kremers, S. Zuily, H. Kelchtermans, T.C. Peters, S. Bloemen, V. Regnault, H.C. Hemker, P.G. de Groot, D. Wahl, B. de Laat, Prothrombin conversion is accelerated in the antiphospholipid syndrome and insensitive to thrombomodulin, Blood Adv 2(11) (2018) 1315-1324.
[19] P. Billoir, T. Duflot, M. Fresel, M.H. Chrétien, V. Barbay, V. Le Cam Duchez, Thrombin generation profile in non-thrombotic factor V Leiden carriers, J Thromb Thrombolysis 47(3) (2019) 473-477.
[20] B. de Laat, C.M. Eckmann, M. van Schagen, A.B. Meijer, K. Mertens, J.A. van Mourik, Correlation between the potency of a beta2-glycoprotein I-dependent lupus anticoagulant and the level of resistance to activated protein C, Blood Coagul Fibrinolysis 19(8) (2008) 757-64.
[21] M. Galli, G.M. Willems, J. Rosing, R.M. Janssen, J.W. Govers-Riemslag, P. Comfurius, T. Barbui, R.F. Zwaal, E.M. Bevers, Anti-prothrombin IgG from patients with anti-phospholipid antibodies inhibits the inactivation of factor Va by activated protein C, Br J Haematol 129(2) (2005) 240-7.
[22] S. Zuily, K. Ait Aissa, A. Membre, V. Regnault, T. Lecompte, D. Wahl, Thrombin generation in antiphospholipid syndrome, Lupus 21(7) (2012) 758-60.
[23] B. de Laat, X.X. Wu, M. van Lummel, R.H. Derksen, P.G. de Groot, J.H. Rand, Correlation between antiphospholipid antibodies that recognize domain I of beta2-glycoprotein I and a reduction in the anticoagulant activity of annexin A5, Blood 109(4) (2007) 1490-4.
[24] J. Gombás, A. Tanka-Salamon, J. Skopál, Z. Nagy, R. Machovich, K. Kolev, Modulation of fibrinolysis by the combined action of phospholipids and immunoglobulins, Blood Coagul Fibrinolysis 19(1) (2008) 82-8.
[25] S.Y. Lean, P. Ellery, L. Ivey, J. Thom, R. Oostryck, M. Leahy, R. Baker, M. Adams, The effects of tissue factor pathway inhibitor and anti-beta-2-glycoprotein-I IgG on thrombin generation, Haematologica 91(10) (2006) 1360-6.
[26] M. Ninivaggi, H. Kelchtermans, T. Lindhout, B. de Laat, Conformation of beta2glycoprotein I and its effect on coagulation, Thromb Res 130 Suppl 1 (2012) S33-6.
[27] G. Szabó, K. Pénzes, B. Torner, M. Fagyas, T. Tarr, P. Soltész, G. Kis, M. Antal, J. Kappelmayer, Distinct and overlapping effects of β(2)-glycoprotein I conformational variants in ligand interactions and functional assays, J Immunol Methods (2020) 112877.
[28] A. Membre, D. Wahl, V. Latger-Cannard, J.P. Max, P. Lacolley, T. Lecompte, V. Regnault, The effect of platelet activation on the hypercoagulability induced by murine monoclonal antiphospholipid antibodies, Haematologica 93(4) (2008) 566-73.
[29] S. Sciascia, S. Baldovino, K. Schreiber, L. Solfietti, M. Radin, M.J. Cuadrado, E. Menegatti, D. Erkan, D. Roccatello, Thrombotic risk assessment in antiphospholipid syndrome: the role of new antibody specificities and thrombin generation assay, Clin Mol Allergy 14 (2016) 6.