SHORT THESES FOR THE DEGREE OF DOCTOR OF PHILOSOPHY(PHD)

Role of anti-β2-glycoprotein-I antibody in blood coagulation and its interaction with its ligand

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1. Introduction

Antiphospholipid syndrome is perhaps one of the most enigmatic diseases in medicine. The syndrome can affect any vascularised tissue and therefore can be linked to almost every field of clinical medicine. If a patient is suspected to have thrombosis, antiphospholipid syndrome should always be considered.

1.1. Definition of the Antiphospholipid Syndrome

Antiphospholipid syndrome is an autoimmune disease in which thrombosis affecting the arteries, veins or small vessels or pregnancy complications may develop, and the symptoms are pathologically related to circulating antiphospholipid autoantibodies (aPLs). The diagnosis of APS is based on the Sapporo criteria, which was amended in Sydney in 2006. APS is defined as having at least one clinical symptom and at least one laboratory abnormality, the latter confirmed at least once after at least 12 weeks—to exclude the transient positivity that otherwise often occurs after infections and lasts only a few weeks.

When APS occurs alone, in the absence of autoimmune comorbidity(s), it is referred to as primary antiphospholipid syndrome (PAPS). APS is called secondary antiphospholipid syndrome (SAPS) when it occurs in association with another autoimmune disease, the most common comorbidity of APS is SLE. It can also be associated with rheumatoid arthritis, Sjogren's syndrome, scleroderma, polymyositis, dermatomyositis, and other autoimmune diseases. The clinical presentations of primary and secondary APS are identical, but clear differences in their genetic background have been described between the two, which further demonstrates that the PAPS is a separate entity.

1.2. Clinical Features of the Antiphospholipid Syndrome

Based on its clinical characteristics, APS can be divided into two main groups: thrombotic and obstetric APS.

Thrombotic APS—The most common clinical manifestation of APS is deep vein thrombosis. In general, venous thrombosis is more common in APS than arterial thrombosis. In APS, clotting can occur in veins, arteries, and small vessels, so the disease can affect any vascularised tissue or organ and cause symptoms related to the function of the organ.

According to the Sapporo criteria, thrombosis must be confirmed beyond reasonable doubt by imaging or histology. The management of thrombotic symptoms in APS is not different from that of any other thrombotic cases, but in all cases the patient will subsequently require chronic anticoagulation.

Obstetric APS—Obstetric complications of APS may affect only the mother or both the mother and the foetus. The most common forms affecting the mother only are pre-eclampsia and eclampsia, with a prevalence of 9.5% and 4.4% respectively. The most common pregnancy manifestation affecting the foetus, and therefore the mother, is habitual miscarriage caused by death of the foetus before the 10th week of pregnancy. The live birth rate in APS is 47.7%.

1.3. Laboratory Abnormalities in the Antiphospholipid Syndrome

The laboratory investigation of autoimmune diseases most often involves qualitative and quantitative testing for autoantibodies. APS is unique in this respect, as the antibody test is complemented by a third, functional test.

According to the Sapporo criteria for laboratory abnormalities in APS, at least one of the following tests must give a positive result:

- IgG or IgM isotype anti-β2-glycoprotein-I (anti-β2GPI)
- IgG or IgM isotype anticardiolipin (anti-CL)
- lupus anticoagulant (LA)

The above tests should be repeated at least once after at least 12 weeks to distinguish transient APL positivity—which often occurs after infections—from true APS.

Today, the ELISA (enzyme-linked immunosorbent assay) is the most commonly used method for the detection of classical aPLs (anti-β_2GPI and anti-CL IgG and IgM isotype), but its role is being replaced by an automated method based on electrochemiluminescence (ECL).

Lupus anticoagulant is a paradoxical phenomenon, named for its anticoagulant effect in vitro, but in vivo, on the contrary, it induces a prothrombotic effect. In the LA assay, we need to prove two things: (i) the plasma clotting time is prolonged, (ii) this prolongation is phospholipid-dependent.

Although not a criterion symptom for APS, thrombocytopenia is still a characteristic laboratory abnormality, with a relatively high prevalence of 30–40% in APS, which varies in different sources. Less frequently (~10%) it may be < 50 G/l or even recurrent. The prothrombotic immunological abnormalities and haemorrhagic haematological abnormalities may often occur simultaneously.

1.4. Classification of the Antiphospholipid Syndrome

APS can be divided into two types based on its association with other autoimmune diseases. When APS occurs alone, in the absence of an autoimmune comorbidity, it is referred to as primary antiphospholipid syndrome (PAPS). APS is called secondary antiphospholipid syndrome (SAPS) when it occurs in association with another autoimmune disease, the most common comorbidity of APS is SLE. It may also be associated with rheumatoid arthritis, Sjogren's syndrome, scleroderma, polymyositis, dermatomyositis and other autoimmune diseases. The clinical presentations of primary and secondary APS are identical, but clear differences in their genetic background have been described between the two, which further demonstrates that the PAPS is a separate entity.

1.5. Possible Pathomechanisms of Thrombosis Caused by the Antiphospholipid Syndrome

Since the description of the antiphospholipid syndrome, it has been debated by which mechanism aPLs cause the contradictory clinical prothrombotic symptoms and laboratory anticoagulant abnormalities. The events leading to the development of symptoms are described by the 'two-hit' theory, according to which the presence of aPLs (first hit) represents a subclinical prothrombotic state, a latent threat. By inducing a procoagulant state, aPLs prepare the ground for a possible prothrombotic stimulus (second hit), which is only supposed to 'pull the trigger'. The most common second-hit stimuli include infections, surgery (transient bacteraemia), wound healing or any process that forces the immune system to defend itself.

There is evidence of effects of aPLs on both the cellular (platelets, endothelium, monocytes, neutrophil granulocytes) and humoral (coagulation cascade, fibrinolytic system, complement system) components of haemostasis. Animal platelet activation experiments have also provided evidence of the two-hit hypothesis that the presence of aPLs alone does not cause thrombosis in vivo, but platelet-rich thrombi are formed in blood vessels after the combined intraperitoneal administration of a prothrombotic agent (e.g., lipopolysaccharide) and intravenous administration of aPLs. APLs induce increased production of monocyte chemoattractant protein 1 (MCP 1) in endothelial cells, MCP 1 directly enhances tissue factor expression in monocytes, which ultimately shifts the haemostatic balance into a procoagulant state. In APS, MCP-1 production by endothelial cells may enhance tissue factor production in monocytes, but it has been demonstrated in several experiments that aPLs also directly enhance tissue factor expression on the surface of monocytes, which directly contributes to

the initiation of coagulation. A new coagulation-triggering function of aPLs has recently been discovered, namely the formation of neutrophil extracellular traps (NET), also known as NETosis. In APS, the effect of aPLs—especially anti-β2GPI—in inducing NETosis has also been observed. However, the longest known effect of aPLs on coagulation is the lupus anticoagulant effect, which is an in vitro prolongation of coagulation, but this is known to be a paradoxical phenomenon, and lupus anticoagulant positivity in vivo is also indicative of an increased risk of thrombosis. The question of the exact molecular mechanism is not yet clear, although recently published results by Noordermeer et al. suggest that two independent mechanisms may explain the LA effect: anti-β2GPI inhibits activation of coagulation factor V (FV) by active factor X (FXa), or anti-prothrombin (anti-PT) competes with FXa for phospholipid surfaces. The latter also explains the principle of the LA laboratory test (low and high phospholipid concentrations). It has also been known for a long time and confirmed by numerous experiments that aPLs cause activated protein C (APC) resistance, also observed in the Leiden mutation of FV, the mechanism of which is still unknown, but a close correlation between the presence of antibodies produced against the β2GPI domain I (anti-β2GPI-DI) and APC resistance has been demonstrated. Antiphospholipid antibodies do not leave the extrinsic coagulation pathway unimpaired, they inhibit the function of the tissue factor pathway inhibitor (TFPI), and consequently an important factor limiting the function of the extrinsic pathway is eliminated, and coagulation is enhanced.

1.6. The Main Autoantigen of the Antiphospholipid Syndrome, β2-glycoprotein-I

One of the major autoantigens of APS is β2-glycoprotein-I (β2GPI), also known as apolipoprotein H (apoH). β2GPI is a highly glycosylated serum protein of 45–50 kDa (depending on the method of determination) and is present in the circulation at approximately 200 mg/l concentration. The molecule is composed of five consecutive bead-like domains, designated by Roman numerals (I–V). Domains I–IV belong to the CCP (complement control protein) superfamily and contain 60-amino acid peptide sequence repeats. Domain V has a special structure, containing a peptide loop that extends from the surface of the molecule and acts as an anchor, and a positively charged lysine-rich region in the immediate vicinity of the loop. These tools enable β2GPI to anchor stably to negatively charged phospholipid surfaces.

Three conformations of the molecule have been described that can transform into one another, these are a closed or circular, an open or "J" shaped and an "S" shaped form representing a transition between the first two. The closed form is the most common in the

circulation. Normally, aPLs bind to the open form or promote the opening of the molecule, so the open form can be considered the active form.

The physiological role of a protein, gene or vitamin is usually recognised in a state of deficiency or damage through loss of function or abnormal function. Although the genetic deficiency of β2GPI is very rare, in a Japanese study, a frameshift mutation in the *APOH* gene encoding β2GPI in 6.3% of 222 healthy individuals caused a heterozygous deficiency of the protein, resulting in a decrease in serum levels of β2GPI from an average of 243 mg/l to 86 mg/l in the study. In the same study, two families with homozygous β2GPI deficiency were identified. Examining the lipid profile and coagulation status of the individuals, no abnormalities—in either heterozygous or homozygous forms of β2GPI deficiency—were detected that would have suggested a loss of function of the protein.

The known functions of β2GPI:

- Inhibits—mainly ADP-induced (adenosine diphosphate)—platelet aggregation by various mechanisms, which is suspended by anti-β2GPI antibodies, thus enhancing platelet aggregation.
- Binds to coagulation factor XI and inhibits its activation by factor XII.
- Inhibits the procoagulant function of thrombin.
- Protects thrombin from inactivation by heparin and its cofactor.
- Binds to complement factor C3, where it induces a conformational change, and promotes its degradation by factors H and I, thus reducing the activation capacity of the complement system.

There is growing evidence in the literature on the physiological function of β2GPI, but no central role has yet been assigned to the molecule analogous with vitamin C deficiency—scurvy, iron deficiency—anaemia or *CFTR* gene mutation—cystic fibrosis.

2. Objectives

The main objective of our work was to study the molecular pathophysiology of the antiphospholipid syndrome by investigating the physiological and pathophysiological role of the central antigen of the disease, β2-glycoprotein-I, and its reactive antibody, anti-β2-glycoprotein I, by molecular biological and functional studies.

The following objectives were set:

1. Based on data available in the literature, we hypothesized that the closed and open conformation of β2GPI, independently of its antibody, has a different physiological function and that this difference is reflected in its effect on blood coagulation. Therefore, we aimed to investigate the effect of β2GPI with closed and open conformations on clotting times in extrinsic and intrinsic coagulation pathways and on thrombin generation as a global characterization of coagulation.
2. It is known that anti-β2GPI binds to the open form of β2GPI and promotes its conversion to the open conformation. Our hypothesis was that surface plasmon resonance would reveal a different anti-β2GPI–ligand interaction with closed/open conformation β2GPI.
3. It is known that the effects of anti-β2GPI are almost invariably β2GPI-dependent, and that the lupus anticoagulant effect is known to be phospholipid-dependent. Our aim was to investigate the effect of anti-β2GPI on thrombin generation by varying β2GPI and phospholipid concentrations in control plasma and its effect on the extrinsic and intrinsic pathways in factor VII, IX and XI deficient plasmas.
4. Furthermore, as an extension of the ‘two-hit’ theory, we aimed to investigate the effect of anti-β2GPI as an acquired prothrombotic agent on thrombin generation in patients with the two most common congenital prothrombotic diseases, the heterozygous Leiden mutation of coagulation factor V and the heterozygous polymorphism of the prothrombin gene G20210A.
5. Anti-β2GPI has been shown to affect cellular players in coagulation, and we have therefore extended the ‘two-hit’ theory to cellular experiments. We hypothesized that anti-β2GPI has a platelet-activating effect, but at least a sensitising effect in response to platelet activation stimuli.

3. Patients and Methods

3.1. Patient Inclusion Criteria, Clinical Characterisation of Patients

For our study, sample collection was performed with the approval of the National Scientific and Ethical Committee, Medical Research Council of Hungary (approval number 45368-1/2017/EKU). Patients included in our experiments were divided into two groups: (i) antiphospholipid syndrome patients, from whose serum samples we purified IgG anti-β2GPI antibody, (ii) individuals carrying the Leiden mutation of coagulation factor V in heterozygous form (FV_{Leiden}) or the G20210A polymorphism of the prothrombin gene ($FII_{G20210A}$) in heterozygous form, whose plasma was used in our thrombin generation assays.

For purification of IgG anti-β2GPI antibodies, the inclusion criteria for APS patients were LA positivity, anti-CL positivity and anti-β2GPI positivity—the latter showing 25 times higher level than the reference limit (20 U/ml).

For TG measurements, citrated plasma was collected from patients in a haemostasis diagnostic laboratory who were confirmed by molecular genetics to carry either the Leiden mutation of FV or the G20210A polymorphism of the prothrombin gene in heterozygous form, but with a wild-type genotype for the other.

3.2. Purification of β2GPI Ligand and Anti-β2GPI Antibody

3.2.1. Purification of Human β2GPI from Plasma

The purification of β2GPI was carried out following a method having been described by Artenjak et al. which was complemented by a step of conformation conversion of the molecule into open or close. The protein purification process was carried out from citrated plasma from healthy donors and consisted of three steps: (i) protein precipitation with perchloric acid, (ii) heparin affinity chromatography, (iii) cation exchange chromatography.

3.2.2. Purity Analysis of the β2GPI Preparation by Western Blot and MALDI–TOF

Western blot—The final step of the β2GPI purification method, a sample was taken from the eluate yielded during cation exchange chromatography. The proteins were separated on a 10% sodium dodecyl sulphate–polyacrylamide gel under reducing conditions and transferred to a nitrocellulose membrane. The membrane was blocked with 3% gelatine (BioRad, USA) dissolved in TBS (Tris-buffered saline; 20 mM Tris/HCl, 500 mM NaCl) for 1 h. Membrane-bound proteins were labelled with anti-human β2GPI (goat-derived; Novus

Biologicals, USA, Colorado, Littleton) primary antibody and HRP (horseradish peroxidase) conjugated anti-rabbit IgG (rabbit-derived; Sigma-Aldrich, USA) secondary antibody. Staining was developed with a chemiluminescent substrate of HRP (Immobilon Western, Millipore, USA, Billerica, Massachusetts). Subsequently, the membrane was stained with Coomassie brilliant blue R250 (Sigma-Aldrich) stain for nonspecific protein detection.

MALDI–TOF—The β2GPI solution was concentrated for mass spectrometry analysis using a modified C18 chromatography pipette tip (Pierce Biotechnology, Thermo Scietific, USA, Illinois, Rockford). Mass spectrometry was performed on a MicroFlex LT MALDI–TOF mass spectrometer (Bruker Daltonics GmbH) using a 96-well polished steel plate. Sinapic acid (trans-3,5-dimethoxy-4-hydroxycinnamic acid dissolved in ethanol; Merck) was used as matrix. First the sample, then the matrix was applied to the plate in a volume of 1-1 μl.

3.2.3. Conformation Conversion of β2GPI to Open and Closed

It has been described previously in the literature that the conformation of β2GPI can be changed from closed to open, and vice versa, by varying the pH and NaCl concentration of the solvent in different directions and to different extents. Approximately 2-2 mg of purified β2GPI was dialysed against buffer of appropriate Na^+ concentration and pH for 48 h. In all cases, dialysis was performed at 4–8 °C. The efficacy and efficiency of the conformational conversion was verified structurally by transmission electron microscopy with negative contrast and functionally by activated partial thromboplastin time at 100× dilution and prothrombin time at 500× dilution.

3.2.4. Purification of IgG Isotype Anti-β2GPI Antibody from Serum

IgG anti-β2GPI antibody was purified from blood serum of patients with antiphospholipid syndrome by a two-step affinity chromatography. In the first step, total IgG was extracted from patient serum using an affinity chromatography column (HiTrap Protein G [5 ml], GE Healthcare, Sweden, Uppsala) containing Protein G as ligand. The IgG eluate was further purified using a column containing β2GPI as ligand, and specific anti-β2GPI bound by the column was eluted.

3.3. Functional Studies of Anti-β2GPI and β2GPI

3.3.1. Trombin Generation Test

In all the thrombin generation assays, platelet-poor plasma was used, to which β2GPI and/or anti-β2GPI were added at various final concentrations in 10% of the plasma volume, and the reagents used for the assay contained 1 or 5 pM TF (tissue factor) and 4 μM PL (phospholipid). Seven different plasmas were used in the thrombin generation assays: (i) normal pooled plasma (NPP) from healthy individuals, (ii) pooled plasma from individuals carrying the Leiden mutation of coagulation factor V in heterozygous form (FV_{Leiden}), (iii) individual plasma from patients heterozygous for the G20210A polymorphism of the prothrombin gene ($FII_{G20210A}$), (iv) FVII, (v) FIX, (vi) FXI deficient plasma (Siemens Healthcare, Germany, Marburg) and (vii) microparticle-depleted plasma (MDP) from healthy individuals.

The results were analysed using the Lag Time (time between the addition of the start reagent and the onset of thrombin generation [min]), the Peak Thrombin (the highest amount of thrombin measured at a single time point [nM]) and the parameter corresponding to the area under the curve, the endogenous thrombin potential (ETP; [nM × min]).

3.3.2. Study of the Interaction Between Anti-β2GPI and Its Ligand by Surface Plasmon Resonance

Surface plasmon resonance (SPR) was performed on a Biacore X kit (GE Healthcare, Sweden, Uppsala). Five anti-β2GPI preparations from patients with antiphospholipid syndrome were selected and individually immobilised onto SPR sensor chips (500 nm, medium density carboxymethyldextran hydrogel-coated chip, XanTec bioanalytics GmbH, Düsseldorf) via primary amino groups following the manufacturer's instructions. To study the β2GPI–heparin interaction, a heparin-coated SPR chip (Heparin, 50 nm hydrogel-coated chip, XanTec bioanalytics GmbH, Düsseldorf) was used. Open and closed conformation β2GPI at six different concentrations (50, 100, 150, 300, 500 and 750 mM) were injected into the microflow cell of the instrument. The system flow rate was 10 μl/min and the temperature was 37 °C throughout the experiment. The Langmuir 1:1 binding model was used for curve fitting. Equilibrium dissociation constant (Kd) and association rate constant (ka) were calculated and analysed using BIAevaluation software (GE Healthcare, Sweden, Uppsala).

3.3.3. Influence of Microparticles on the Effect of Anti-β2GPI on Thrombin Generation

To investigate the effect of microparticles (MPs) on blood coagulation in relation to the effect of anti-β2GPI on thrombin generation, microparticle-depleted plasma (MDP) was prepared by separating MPs from normal pooled plasma. For this purpose, MPs were removed from citrated whole blood from five healthy donors by high-speed centrifugation (16,000 g, 30 min, 20 °C), which was confirmed by flow cytometric assay. MPs were identified by their size, phosphatidylserine and CD41 positivity (platelet-specific cell surface molecule). The amount of MPs separated from NPP was calculated based on the dilution ratios used during sample preparation and the volumetric flow rate used during the measurement.

Anti-β2GPI purified from the serum of an APS patient was added to both NPP and MDP at a final concentration of 500 U/ml. For thrombin generation, we used start reagents containing low (4 μM PL + 0.5 pM TF) and high (4 μM PL + 1 pM TF) TF.

3.3.4. Effect of Anti-β2GPI on Platelet Activation Detected by Direct and Indirect Cell Activation Markers by Flow Cytometry

The flow cytometric method we used to directly detect platelet activation is the surface P-selectin expression assay (PSE), and the indirect method is the detection of platelet–leukocyte heterotypic aggregates formation. Samples were measured on a FC500 Beckman Coulter flow cytometer.

For the P-selectin expression assay, platelet-rich plasma (PRP) was obtained from citrated whole blood from healthy individuals by low-speed centrifugation (170 g, 15 min, 20 °C) with platelet count adjusted to 250 G/l. To the PRP was added TRAP (thrombin receptor-activating peptide) at a final concentration of 10 μM and anti-β2GPI at a final concentration of 5 μg/ml and 10 μg/ml, respectively. The platelet cell surface marker, glycoprotein IX (CD42a) and platelet activation marker, P-selectin (CD62P) were labelled with the corresponding fluorescein isothiocyanate- and phycoerythrin-conjugated antibody (anti-CD42a FITC, anti-CD62P PE). Platelets were incubated in a water bath at 37 °C for 5 min. In these measurements, cell populations were gated according to two criteria: (i) size and granularity and (ii) CD42a and CD62P positivity. By gating based on size–granularity, we were able to determine the proportion of platelet-derived microparticles, and by gating based on the positivity of cell surface proteins, we were able to determine the proportion of activated platelets.

In platelet–leukocyte heterotypic aggregates platelet–monocyte and platelet–granulocyte aggregates were identified. For this purpose, citrated whole blood from healthy individuals was used. TRAP at a final concentration of 10 µM and anti-β2GPI at a final concentration of 5 µg/ml and 10 µg/ml were added to 100 µl of whole blood. Simultaneously with the activation, the platelet surface marker (CD42a) and the monocyte marker (CD14) were labelled with the corresponding antibodies (anti-CD42a FITC, anti-CD14 PE). Samples were incubated in a water bath at 37 °C for 15 min. For the identification of platelet–leukocyte aggregates, monocytes were identified based on CD14 positivity and granulocytes were reliably gated by size and granularity. The copresence of white blood cells and platelets within each white blood cell population was assessed by the ratio of CD42a positivity of events.

3.4. Statistics

Statistical analysis was performed using GraphPad Prism software. The normal distribution of each group was tested using Kolmogorov–Smirnov test. Groups with a normal distribution were compared using one-way ANOVA and groups without a normal distribution were compared using the Kruskal–Wallis test. In the SPR tests, results of open/closed β2GPI were compared using paired t-test. Statistical differences between groups were considered significant at $P < 0.05$.

4. Results

4.1. Physiological and Pathophysiological Role of β2GPI in Blood Coagulation

4.1.1. Preparation of Open/Closed Conformation β2GPI

Qualitative study of the β2GPI preparation—To investigate the effect of β2GPI of different conformations on the coagulation cascade, native β2GPI was purified from human plasma. Western blot total protein staining revealed a single band on the membrane at a molecular mass of approximately 55 kDa, which corresponded to the specific protein detected by antibody labelling. MALDI–TOF results also showed that only one molecule of ~45.2 kDa was present in our sample. Although the Western blot and MALDI–TOF results may seem contradictory, separately they are both in agreement with the data reported in the literature. The difference between the two can be explained by the highly glycosylated nature of β2GPI (sugar side chains interact with the polyacrylamide gel, which reduces the electrophoretic velocity of molecules). Our control experiments with Western blot and MALDI–TOF confirmed that the extracted molecule was β2GPI and that the preparation did not contain significant impurities.

Conformational conversion of β2GPI—We visualised the molecule with closed and open conformation by negative staining, which allowed us to demonstrate the effectiveness of the closing and opening treatment. From electron micrographs, we were able to clearly identify the ring-shaped closed and the linear open conformation of β2GPI. To quantify the treatment efficacy, we counted the number of closed molecules in 3-3 fields of view in the $β2GPI_{closed}$ and $β2GPI_{open}$ samples. A total of 103 β2GPI molecules with closed conformation were found in the $β2GPI_{closed}$ sample and 3 in the $β2GPI_{open}$ sample, thus this method was considered highly effective.

4.1.2. β2GPI Delays Fibrin Formation in Both the Intrinsic and Extrinsic Pathways

The clotting time assays performed in our experiments had a dual role: firstly, to test the effect of the two conformations of β2GPI on fibrin formation, and secondly, to serve as a confirmatory test of the efficacy of the conformational transformation. Two coagulation screening assays were used: aPTT is a commonly used coagulation screening assay in which a contact activator, phospholipids and $Ca2^{+}$ are used to induce coagulation, while PT is a screening assay in which fibrin formation is induced by tissue factor instead of contact

activator; accordingly, the intrinsic coagulation pathway is investigated with aPTT, whereas the extrinsic coagulation pathway is investigated with PT.

In the dilute aPTT assay, $\beta 2GPI_{closed}$ increased fibrin formation by 6.6% ($P < 0.001$) and $\beta 2GPI_{open}$ increased it even further, by a total of 18.5% ($P < 0.001$) compared to control. In the PT test, $\beta 2GPI_{closed}$ had a negligible effect, whereas $\beta 2GPI_{open}$ had a very strong effect: it increased PT by 105% ($P < 0.001$) from 55.7 s to 114.1 s. Our results suggest that the open form of β2GPI had an approximately 20-fold stronger effect on the extrinsic coagulation pathway than the closed form of β2GPI.

4.1.3. β2GPI Has Negligible Effect on Thrombin Generation

In contrast to the clotting time assays, TGT has the advantage of being able to measure the amount of thrombin generated during the coagulation process, which gives a more global picture of the coagulation status due to the pleiotropic effect of the thrombin in blood coagulation.

Similar to the effect seen in the coagulation assays, β2GPI—both conformations at both concentrations—had a slight delaying effect: β2GPI at an open concentration of 30 µg/ml prolonged the Lag Time by 4.1%, although this change was not statistically significant. ETP, the total thrombin measured during TGT, also showed a slight increase, but this was not statistically significant either compared to control values.

4.1.4. Binding Characteristics of β2GPI to Its Autoantibody and Heparin

In our assays, we first bound anti-β2GPI to the surface of SPR chip and injected $\beta 2GPI_{closed}$ and $\beta 2GPI_{open}$ into the microflow cell at six different concentrations.

We obtained similar dissociation equilibrium constants for the open and closed forms: although both $\beta 2GPI_{closed}$ and $\beta 2GPI_{open}$ showed strong affinity for anti-β2GPI (Kd values of 5.17×10^{-8} M and 7.18×10^{-8} M, respectively), there was no statistically significant difference between the two results.

To investigate the interaction between β2GPI and heparin, an SPR chip with heparin on its surface was used, and $\beta 2GPI_{closed}$ and $\beta 2GPI_{open}$ were injected into the microflow cell at 6-6 different concentrations. As expected, β2GPI showed a significant affinity for heparin and, although without statistically significant difference, we found an order of magnitude stronger affinity ($Kd = 2.98 \times 10^{-7}$ M) for the open conformation compared to the closed ($Kd = 3.50 \times 10^{-6}$ M).

4.2. Pathophysiological Role of IgG anti-β2GPI in blood coagulation

4.2.1. Anti-β2GPI Has Both Anti- and Procoagulant Effects on Thrombin Generation in Normal Plasma

The effect of anti-β2GPI on thrombin generation in normal pooled plasma was investigated in both low (15–125 U/ml) and high (125–500 U/ml) concentration ranges. The reaction was initiated with 1 pM TF and 4 µM PL.

In the low concentration range, consistent with the anticoagulant clotting time prolonging effect of lupus anticoagulant, anti-β2GPI significantly prolonged the Lag Time already at 62 U/ml (from 6.54 min to 7.55 min; $P < 0.01$), which was further enhanced at 125 U/ml (8.13 min; $P < 0.001$). In contrast, however, the antibody caused an increase in quantitative parameters: although we observed some increase in Peak Thrombin, 31 U/ml anti-β2GPI significantly increased the total amount of thrombin generated during the whole reaction from 1496 nM × min to 1619 nM × min ($P < 0.01$).

In the experiment with the higher concentration range, similar results were obtained for the effect on the time parameter: 125 U/ml anti-β2GPI already significantly prolonged the Lag Time ($P < 0.01$), an effect that was even stronger at 500 U/ml ($P < 0.001$). The effect of anti-β2GPI in increasing quantitative parameters was also demonstrated at higher concentrations, but here the antibody had no effect on ETP, but significantly increased the peak value at 500 U/ml ($P < 0.05$).

4.2.2. Effect of Anti-β2GPI on Thrombin Generation Depends on the Phospholipid Content and β2GPI Levels of the Plasma

MPs were removed from normal pooled plasma by multiple centrifugation and confirmed by flow cytometric assay. In the experiment, first, 4 µM PL was used, so that, as before, anti-β2GPI prolonged the time parameter by 11% and increased the peak and ETP by 27% and 19%, respectively. In the second part of the experiment, the PL content of the reagent was reduced to 0.5 µM to make the effect of the antibody dependent on the PL and MP content of the system. These conditions suspended the effect of the antibody on the Lag Time (Lag Time shortened by approximately 10% compared to the control), but the Peak Thrombin showed a more pronounced increase of 73%. The removal of MPs alone had no effect on Lag Time at either 4 or 0.5 M PL concentrations, but with the removal of MPs, the marked Peak Thrombin-elevating effect of anti-β2GPI observed at 4 µM PL concentrations disappeared. The same phenomenon, although with minor differences, was observed for ETP.

We investigated how different concentrations of β2GPI influences its effect on thrombin generation. β2GPI was added to control plasma at a final concentration of 200–1000 μg/ml and anti-β2GPI at a final concentration of 15–125 U/ml, and thrombin generation was induced with 1 pM TF and 4 μM PL.

The most influential effect of β2GPI was detected in its effect on ETP. Similar to previous results, the antibody increased ETP, which was most pronounced at 31 U/ml, however, with a simultaneous increase of β2GPI concentration, the ETP-enhancing effect of anti-β2GPI seemed to decrease, at 1000 μg/ml for all antibody concentrations.

4.2.3. Anti-β2GPI Has Different Effects on Thrombin Generation in FV_{Leiden} and $FII_{G20210A}$ Plasmas

We investigated whether anti-β2GPI can exert its paradoxical effect observed in the control plasma in the two most common inherited thrombophilia, further enhancing the prothrombotic state in the heterozygous form of the Leiden mutation of coagulation factor V and the G20210A polymorphism of the prothrombin gene. Anti-β2GPI at a final concentration of 125–500 U/ml was added to FV_{Leiden} and $FII_{G20210A}$ plasmas, and thrombin generation was induced with 1 pM TF and 4 μM PL.

The LA effect of anti-β2GPI in FV_{Leiden} plasma was similar to that observed in the control plasma, with significant Lag Time prolonging even at the lowest concentration ($P < 0.001$). The concentration of anti-β2GPI 500 U/ml in the FV_{Leiden} plasma was also able to enhance the Peak Thrombin of the quantitative parameters from 189 nM to 243 nM ($P < 0.05$), but this effect was not more significant than that observed in the control plasma.

When the experiment was also performed with the $FII_{G20210A}$ plasma, we obtained that anti-β2GPI increased the Lag Time even more ($P < 0.0001$), but did not increase the quantitative parameters (Peak Thrombin, ETP) compared to those observed in previous experiments with $FII_{G20210A}$.

4.2.4. Anti-β2GPI Exerts Its Anti- and Procoagulant Effects Through Different Coagulation Pathways

We also investigated the mechanism(s) responsible for the effects described above using FVII , FIX and FXI deficient plasma. Anti-β2GPI was added to the plasmas at a final concentration of 500 U/ml. The thrombin generation of factor deficient plasmas is significantly poorer compared to healthy plasma, no reproducible thrombogram was obtained

with 1 pM TF, therefore in this series of experiments thrombin generation was induced with a higher concentration of 5 pM TF and 4 μM PL.

The Lag Times in NPP, FIX and FXI deficient plasma were similar to controls, but in FVII deficient plasma, understandably (since we induce the reaction with tissue factor), it took a markedly longer time to initiate thrombin generation. The absence of FIX and FXI did not affect the Lag Time-prolonging effect of anti-β2GPI, but the prolongation was completely absent in FVII deficient plasma.

The Peak Thrombin in all three factor-deficient plasmas was significantly lower compared to NPP. Anti-β2GPI caused a pronounced increase in Peak Thrombin in NPP, which is also partly seen in FVII deficient plasma, but in the absence of intrinsic factors, this Peak Thrombin-enhancing effect was absent and even a slight decrease was observed.

4.2.5. Anti-β2GPI Enhances Platelet Activation

Direct and indirect assay methods were used to study anti-β2GPI-induced platelet activation. In support of the 'two-hit' hypothesis, platelets were incubated simultaneously with anti-β2GPI and a known activating agent, TRAP, which is a potent activation stimulus to platelets via the thrombin receptor. TRAP was applied at a (supraminimal) concentration that exceeds the threshold concentration of activation, but induces the lowest possible platelet activation, which was found to be 10 μM in preliminary experiments.

When platelets were incubated with anti-β2GPI alone, P-selectin expression was very similar to the control value (1.3%), similarly the proportion of platelets heterotypic aggregates formed with monocytes (5%) and granulocytes (9%) showed insignificant differences compared to the control value. Supraminimal TRAP concentration, as expected, slightly increased the proportion of P-selectin positive platelets (7.9%), similar to the proportion of platelet–monocyte aggregates (16%). Despite the fact that the antibody alone did not induce activation, the effect of TRAP seems to be facilitated by increasing the concentration of anti-β2GPI, with an increase in TRAP effect in both P-selectin expression (5 μg/ml anti-β2GPI: 20%, 10 μg/ml anti-β2GPI: 25%) and in platelet–monocyte (5 μg/ml anti-β2GPI: 35%, 10 μg/ml anti-β2GPI: 46%) and platelet–granulocyte aggregate (5 μg/ml anti-β2GPI: 16%, 10 μg/ml anti-β2GPI: 24%) formation.

5. Discussion

β2-glycoprotein-I has been known for 60 years and its association with antiphospholipid syndrome for more than 30 years. The physiological versatility of the protein is demonstrated by its anti- and procoagulant properties, such as inhibiting the activation of coagulation factor XI or inhibiting the degradation of thrombin. The presence of β2GPI is also required for the pathogenic effects of its autoantibody. Its genetic deficiency is very rare, making its physiological role difficult to investigate, and since no functional defect in either blood coagulation or lipid metabolism has been described in partial or complete β2GPI deficiency, the effects of β2GPI may either be minor in its physiology or more complex than those so far identified.

To understand how β2GPI works, it is also important to consider that two extreme conformations of β2GPI have been described: the closed (circular) and the open ('J' shaped) forms. Since the closed form is predominantly found in the circulation and anti-β2GPI contributes to the opening of the molecule, the open form is considered to be the more biologically functional. In our experiments, we purified β2GPI, which was found to have a molecular size of 41.5 kDa by MALDI–TOF, and converted it into a closed and open conformation by varying the buffer conditions. Using electron microscopy, we were able to identify the closed and open conformation molecules. The open conformation provided a significant prolongation of the phospholipid-dependent coagulation times. In the dPT assay, the open form caused spectacular prolongation, while the closed one gave very similar results to the control, suggesting that the dPT assay is suitable for distinguishing the biologically active and inactive forms. We also tested the effect of β2GPI in a thrombin generation assay using the default composition of the start reagent (5 pM tissue factor and 4 μM phospholipid) as recommended by the manufacturer, but in this study, we did not obtain significant results even with the open conformation β2GPI. These results are in agreement with those found in the literature, Ninivaggi et al. obtained equivalent results under similar measurement conditions, β2GPI had no effect on thrombin generation and the inhibitory effect was only observed at low concentrations of phospholipid, similar to our results in the dPT assay.

The domain V of β2GPI is unique in that it has phospholipid binding sites on its surface, but the molecule can bind not only to phospholipids but also to heparin. The versatility of β2GPI is exemplified by its ability to bind and protect thrombin from inhibition by heparin cofactor II. Examining the interaction between β2GPI and heparin by SPR, we

obtained that although both conformations of β2GPI bound to heparin, the open one showed an order of magnitude higher affinity for heparin. Kolyada et al. showed that although heparin binds to β2GPI, the binding is not through the phospholipid-binding lysine side-chains, so their function is not affected. It is conceivable that this circumstance may influence the efficacy of heparin or LMWH therapy in APS and thus their optimal therapeutic dose. However, this issue requires further investigation, as heparin does not prevent β2GPI from competing with coagulation factors for phospholipid surfaces and thus exerting its anticoagulant effect, whereas β2GPI exerts a prothrombotic effect by preventing thrombin degradation, so it would be difficult to predict which effect would be more pronounced by varying the dose of heparin.

We examined the strength of binding of two conformations of β2GPI to autoantibodies by SPR. The experiment was also performed with irrelevant antibodies to exclude nonspecific binding, no significant sensorgrams were obtained with these antibodies, which suggests that nonspecific binding is negligible. Both conformations bound to their antibodies, with the observed binding affinity falling in the 10^{-8} M range for both conformations, which was confirmed by a reverse orientation experiment. Not all anti-β2GPI antibodies induce a pathological state, the strongest correlation is attributed to anti-β2GPI produced against the domain I of β2GPI, which requires the open conformation of the antigen for binding. Since the same amount of ligand (anti-β2GPI) was present on the surface of the SPR chips for all assays and the same concentration of analyte (closed/open β2GPI) was injected into the microflow cells, it can be concluded from the RU values of the sensorgrams that the antibodies were able to bind a higher amount of open β2GPI than closed. Our results could have been further refined by determining the anti-β2GPI DI ratio in our samples, however, these results may help us to understand the details of β2GPI function in more detail.

Although there are still many questions to be clarified about the physiological function of β2GPI, most is known about its pathophysiological role in the antiphospholipid syndrome, since the pathophysiological processes of APS are linked to, among other things, the production of and reactions to autoantibodies against it and their effects. Even before the description of APS in 1983, the self-contradictory phenomenon was known that if a patient's plasma shows an uncorrectable, phospholipid-dependent elongation in coagulation tests, then in that patient one would not expect haemorrhage, but thrombosis. The anticoagulant phenomenon of lupus is caused by antiphospholipid antibodies, but since they form a heterogeneous group, it is not clear which antibody(s) and which molecular mechanism(s) are responsible for this complex effect. Several clinical and experimental results suggest that

anti-β2GPI reacting with the domain I of β2GPI is the pathogenic antibody and may be held responsible for the increased risk of thrombosis.

In APS, increased thrombin generation has long been observed to be characteristic of the disease, and a concomitant increase in prothrombin F_{1+2} fragment and fibrinopeptide A levels has been described in patients with anti-CL positive APS. However, these clinical findings were not conclusive because increased thrombin generation could only occasionally be detected in APS. However, under experimental conditions, it has been demonstrated that anti-β2GPI from APS patients can dose-dependently enhance laser-induced arterial thrombosis in a mouse model. The in vivo effect of aPLs has also been demonstrated in other animal studies, where they have also been described to enhance thrombosis. In our experiments, we investigated the effect of anti-β2GPI on coagulation to better understand the mechanism of their thrombotic effect. To this end, we purified IgG isotype anti-β2GPI from the serum of APS patients and measured the thrombin generation in healthy plasma after the addition of antibodies. Low amount of tissue factor (1 pM) was used in the assay because the thrombin generation assay is known to be more sensitive with lower amounts of tissue factor. Under these conditions we could better test the well-known in vitro prolonging effect of anti-β2GPI. In addition to the LA effect, we also observed the thrombin generation enhancing effect of anti-β2GPI.

A retrospective study showed that in carriers of the Leiden mutation of coagulation factor V (FV_{Leiden}), ETP in asymptomatic individuals was not different from that in healthy individuals. In our study, we found that high anti-β2GPI in heterozygous FV_{Leiden} plasma also increased Peak Thrombin concentrations similar to those found in NPP, which suggests that the presence of aPLs in the most common inherited thrombotic disease may further increase the risk of thrombosis. Anti-β2GPI was also found to delay thrombin generation in another common inherited thrombotic disease, the G20210A polymorphism of the gene encoding prothrombin ($FII_{G20210A}$). Although we observed that Peak Thrombin and ETP increased in response to anti-β2GPI, no statistically significant effect was detected even at the highest antibody concentration (500 U/ml). This may be explained by the fact that we found the strongest prolonging effect in the $FII_{G20210A}$ plasma (79%), which counteracts the procoagulant effect, compared to the 34% and 21% prolongation found in FV_{Leiden} and NPP, respectively.

The aPL levels and the associated clinical picture in APS are highly variable, thrombotic complications can occur at antibody levels as low as 100 U/ml, and patients can be asymptomatic at extremely high levels. For this reason, we investigated whether

anti-β2GPI can exert the effects seen so far at lower concentration ranges. We found that aPL values only 2-3 times above the reference limit (20 U/ml) were able to significantly delay thrombin generation and even significantly increase ETP, which is in agreement with what was observed in vivo, that aPL concentrations are not necessarily related to the severity of the clinical condition.

Several theories and experimental results have been developed to explain the effect of aPLs on the coagulation system. One of the oldest known mechanisms is APC resistance. However, under cell-free conditions we used, this was presumably not an issue—unless activated protein C or thrombomodulin, which promotes its activation, would have been artificially added to the system—since the change in substrate specificity of thrombin and thus the anticoagulant action is promoted by thrombomodulin expressed on the endothelial cell surface. Other mechanisms that may be involved in a cell-free environment involve coagulation factors and their regulatory systems, such as annexin A5 resistance, attenuation of fibrinolysis or inhibition of the function of tissue factor pathway inhibitor (TFPI). In our studies, we sought to answer the question through which pathways anti-β2GPI exerts the pro- and anticoagulant effects observed in our TG experiments using NPP and factor-deficient plasma. Due to factor deficiency, in many cases no valuable thrombogram was obtained with a lower TF concentration (1 pM), and therefore in these experiments we initiated the reaction with a higher tissue factor concentration (5 pM), in contrast to previous experiments. Consequently, the Peak Thrombin of the NPP was also significantly higher. Factor deficiencies alone also strongly affected the parameters of the TG. From the composition of the start reagent (TF + PL), it can be understood that the FVII deficient plasma had the longest Lag Time and the lowest Peak Thrombin value. The Lag Time in FIX and FXI deficient plasmas were similar to those measured in NPP. Peak values increased away from FVII, with the highest close to that of NPP in FXI, followed by FIX, and the lowest in FVII deficiency. In the deficient plasmas, the effect of anti-β2GPI IgG antibody on time parameters and quantitative parameters appeared to be distinct according to whether the extrinsic or intrinsic pathway remained intact. When the extrinsic pathway was impaired, anti-β2GPI was able to increase Peak but could not prolong Lag Time, unlike when the intrinsic pathway was impaired, where the antibody was able to delay TG but the increase in quantitative parameters was lost. This result suggests that the paradox of the lupus anticoagulant effect exerted by anti-β2GPI is the result of independent parallel processes occurring at different points in the coagulation cascade, which are probably in constant 'tug-of-war' with each

other, and the effect that finally induces thrombosis (second hit) pulls this taut rope in a 'pro' direction.

It has been demonstrated by several experiments that the ligand itself, β2GPI, is required for the anti-β2GPI effect to develop. Banzato and Pengo have shown that in triple positive (LA, anti-CL IgG, anti-β2GPI IgG) APS patients, β2GPI levels are significantly higher. Based on these results, we designed our experiments to investigate the influence of increasing levels of β2GPI on the effect exerted by anti-β2GPI in TGT. We found that higher concentrations of β2GPI are able to attenuate the effect of anti-β2GPI, which is thought to be due to a 'dilution' mechanism, whereby all antibodies are bound to β2GPI, and a high proportion of β2GPI is present to which no antibody is bound, so that competition for a role in coagulation occurs between free β2GPI and the β2GPI/anti-β2GPI complex.

Although our thrombin generation assay was cell-free, the TGT sample preparation was such that microparticles may have remained in the plasma, which may also have contributed to the observed effects. To investigate the contribution of MPs to the anti-β2GPI effect, microparticles were removed from the NPP and anti-β2GPI was added to the microparticle-depleted plasma. The reagent formulation was the same as that used in NPP (1 pM TF, 4 µM PL), but then no difference was obtained between the anti-β2GPI effects observed in NPP and MDP. To make the system more sensitive to PL concentration and the presence of MPs, we reduced the PL concentration of the reagent to 0.5 µM. At this point, the relatively high phospholipid content of the reagent did not mask the difference between the phospholipid concentrations of NPP and MDP, and anti-β2GPI elicited a much higher increase in Peak Thrombin in the presence of MPs than in their absence. This experiment also confirms the idea that anti-β2GPI exerts both pro- and anticoagulant effects independently; at higher PL concentrations the anticoagulant effect suppresses the procoagulant, but at low PL concentrations the procoagulant can prevail and the peak value in TGT increases dramatically.

The largest amount of microparticles in the circulation comes from cellular activation, mainly platelet activation. The effect of aPLs on haemostasis and the influence of MPs seem to be two intertwined processes, as the cell activation capacity of aPLs has been demonstrated directly and indirectly in vivo and in vitro in several publications. In our studies, we have also attempted to demonstrate the anti-β2GPI platelet-activating effect and the 'two hit' hypothesis that the presence of aPLs alone is only a permissive factor for increased thrombosis risk. Both in the P-selectin expression assay, which directly detects platelet activation, and in the assay that indirectly determines activation by platelet–leukocyte

aggregates, we found that the presence of anti-β2GPI alone was not a sufficient stimulus for cell activation but was able to enhance the effect of a direct activating agent, TRAP, in a concentration-dependent manner. Results from animal experiments, in vivo, also support the 'two hit' hypothesis that intravenous injection of aPLs is not a direct trigger but an enhancing factor of thrombosis.

Our experiments have demonstrated and validated the different physiological effects of β2GPI in blood coagulation. We were able to demonstrate the paradoxical effect of aPLs being both anti- and procoagulant, which was reflected in the timing and amount of thrombin formation. We were also able to show this dual effect separately, attributing the two different functions to separate coagulation pathways. With these results, a new piece of the puzzle of the physiological effects of β2-glycoprotein-I and the pathophysiology of antiphospholipid syndrome has been depicted. Even today, many fundamental questions about antiphospholipid syndrome remain unanswered. According to the PubMed database, more than 14,400 publications have been published under the search term antiphospholipid syndrome between 1977 and 2021, which implies a considerable amount of knowledge. However, this is probably just the tip of the iceberg, as illustrated by the fact that at the Lupus Anticoagulant Discussion Forum at the 2018 ISTH SSC conference in Dublin, Vittoro Pengo, a renowned researcher on antiphospholipid syndrome, told his audience, 'If I were to ask you now what is lupus anticoagulant, you would not be able to answer because nobody knows today!'

6. Summary

The exact molecular processes/players underlying the pathophysiology of antiphospholipid syndrome are still unclear. It is known that in antiphospholipid syndrome, arterial and/or venous thromboses and pregnancy morbidity may develop. These pathological processes are proved to be related to the presence of autoantibodies detectable in antiphospholipid syndrome and to a paradox effect—exerted by these autoantibodies—the lupus anticoagulant. LA (lupus anticoagulant) positivity entails *in vitro* anticoagulant effect and *in vivo* prothrombotic events, however, still there is no clear explanation for the molecular background of this contradictory effect.

Our objective was to investigate the LA phenomenon, the pathophysiology of anti-β2GPI (anti-β2-glycoprotein-I) and the physiological function of β2GPI, the central molecule of the antiphospholipid syndrome. To address this, we purified antibody and its ligand and investigated the effect they exert on normal pooled, hereditary prothrombotic and artificial factor deficient plasmas.

Our results demonstrated the role of anti-β2GPI in the development of LA effect, of which both the anticoagulant and the prothrombotic components were detectable by thrombin generation assay. Our thrombin generation test results suggest that the two opposing effects are exerted via two different coagulation pathways. We were able to relate the effect of β2GPI to its already known conformational changes, which suggests that the open conformation can be considered the biologically active form.

7. New Results and Clinical Significance of the Thesis

New results on the physiological and pathophysiological effect of β2-glycoprotein-I:

- The open conformation of β2GPI statistically significantly prolongs phospholipid-dependent clotting times, of which prothrombin time is suitable to distinguish between biologically active (open) and inactive (closed) conformations.
- β2GPI binds to heparin independently of its conformation, but the open conformation shows an order of magnitude higher affinity for heparin.
- Both conformations of β2GPI bind to its autoantibodies (anti-β2GPI), which are able to bind larger amounts of open β2GPI.

New results on the pathophysiological effects of anti- β2-glycoprotein-I:

- Anti-β2GPI plays a role in the paradoxical effect of lupus anticoagulant. The antibody exerts both a thrombin-delaying and a thrombin-enhancing effect.
- Anti-β2GPI exerts its procoagulant effect via the intrinsic pathway and its anticoagulant effect via the extrinsic pathway.
- In addition to healthy plasma, anti-β2GPI can also exert its pro- and antithrombotic effects in plasma carrying the Leiden mutation of coagulation factor V in heterozygous form and in plasma carrying the G20210A polymorphism of the prothrombin gene in heterozygous form, thereby further worsen thrombophilia.
- The effect of anti-β2GPI is influenced by the phospholipid concentration in plasma. At low phospholipid concentrations, the prothrombotic effect of anti-β2GPI is more pronounced.
- The platelet activation effect exerted by anti-β2GPI is consistent with the ‘two hit’ hypothesis that the presence of an antibody is only a permissive factor for increased platelet activation, and that some direct activating agent is required for increased activation to occur.

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List of publications related to the dissertation

1. **Szabó, G.**, Bekéné Debreceni, I., Tarr, T., Soltész, P., Osterud, B., Kappelmayer, J.: Anti-[beta]2-glycoprotein I autoantibodies influence thrombin generation parameters via various mechanisms.
   Thromb. Res. 197, 124-131, 2021.
   DOI: http://dx.doi.org/10.1016/j.thromres.2020.10.032
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2. **Szabó, G.**, Antal-Szalmás, P., Kerényi, A., Pénzes-Daku, K., Bécsi, B., Kappelmayer, J.: Laboratory Approaches to Test the Function of Antiphospholipid Antibodies.
   Semin. Thromb. Hemost. "Accepted by Publisher", 2021.
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3. **Szabó, G.**, Pénzes-Daku, K., Torner, B., Fagyas, M., Tarr, T., Soltész, P., Kis, G., Antal, M., Kappelmayer, J.: Distinct and overlapping effects of [beta]2-glycoprotein I conformational variants in ligand interactions and functional assays.
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