

# Chapter 2

## Natural Proteins Involved in Antiphospholipid Syndrome

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### Introduction

The antiphospholipid syndrome (APS) is characterized by antiphospholipid antibodies (aPL) in the plasma or serum of patients with thrombosis or pregnancy complications [1]. The APS is a misnomer, because the so-called aPL are directed not against phospholipids but against plasma proteins with affinity for anionic phospholipids. Autoantibodies against many different plasma proteins have been described. In this chapter we will enumerate these proteins, discuss the arguments why they are linked to the syndrome and discuss why these proteins become prothrombotic in the presence of autoantibodies.

### Plasma Proteins Involved in Antiphospholipid Syndrome

#### *$\beta_2$ -Glycoprotein I*

$\beta_2$ -glycoprotein I ( $\beta_2$ GPI) is a 50 kDa plasma protein with increasing evidence that it has important roles in innate immunity and coagulation [2–4]. Many studies show that anti- $\beta_2$ GPI antibodies (a $\beta_2$ GPI), either mouse monoclonal or patient derived, induce a prothrombotic phenotype in mice that have been primed either with lipopolysaccharide (LPS) or an injury to the vessel wall [5, 6]. Studies that separate a $\beta_2$ GPI-associated aPL from those in which a $\beta_2$ GPI are removed by affinity chromatography show that the prothrombotic effect of aPL is present only in the a $\beta_2$ GPI containing fraction [7].

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Further studies, characterizing correlations of the individual domains of  $\beta_2$ GPI with thrombosis and fetal loss, show correlation for both manifestations only with anti-domain I antibodies [8, 9]. Studies using  $\alpha\beta_2$ GPI depleted of anti-domain I antibodies further demonstrate that domain I antibodies are pathogenic, while antibodies against the other domains are not [10]. The major epitope for the autoantibodies is located within the region of amino acids arginine 39 and arginine 43 and a minor epitope involving lysine19 [11]. Exogenous human domain I can inhibit the prothrombotic phenotype in a mouse model of APS [12]. When arginine 39 is replaced by serine, the inhibitory potential of domain I is lost. These experiments in mouse models show conclusively that  $\beta_2$ GPI, in particular its first domain, is central to the pathogenesis of APS.

### ***Prothrombin***

Antiprothrombin antibodies are commonly found in patients with APS. Prothrombin is one of the major coagulation factors in blood. However, antiprothrombin antibodies do not correlate with thrombosis. A recently developed assay that measures autoantibodies against prothrombin complexed with phosphatidylserine (PS) shows better correlation of these antibodies with thrombosis [13]. Two studies show that antiprothrombin antibodies are prothrombotic in mouse models of APS [14, 15]. The thrombotic response to an induced vascular injury was much stronger with antiprothrombin than with control antibody. However, the antibodies used in these studies were not well characterized. In rare cases antiprothrombin antibodies can cause bleeding due to decreased levels of prothrombin because, in contrast to autoantibodies against  $\beta_2$ GPI, antiprothrombin antibodies enhance clearance of prothrombin from the circulation [16].

### ***Annexin A2***

The annexins constitute a family of highly conserved,  $\text{Ca}^{2+}$ -regulated, phospholipid-binding proteins that have many functions related to membrane-mediated processes [17]. Annexin A2 influences haemostasis as it is a receptor for tissue plasminogen activator (tPA) on endothelial cells and for  $\beta_2$ GPI. Annexin A2 knockout ( $-/-$ ) mice show deposition of fibrin in the lungs, spleen, liver, and kidney as they age consistent with decreased fibrinolysis [18]. Annexin A2 autoantibodies develop in patients with APS; high titers of anti-annexin A2 autoantibodies correlate with thrombosis [19]. Annexin A2 ( $-/-$ ) mice, in a model of APS, reduce the prothrombotic effect of injected aPL, suggesting that the mechanism by which annexin A2 is involved in APS is due to disruption of its fibrinolytic function by the autoantibodies [20].

## ***Annexin A5***

Annexin A5 is another member of the annexin family of calcium-dependent phospholipid-binding proteins. The anticoagulant properties of this protein result from its rapidly forming two-dimensional crystal arrays over the polar heads of the anionically charged membrane phospholipids [21]. Anionic phospholipids are required cofactors for the four critical phospholipid-dependent coagulation reactions: the IXa-mediated tenase reaction, the tissue factor-VIIa-mediated tenase, the tissue factor-VIIa-mediated IXase reactions, and the prothrombinase reaction. Assembly of the annexin A5 array shields phospholipids from contributing to the enzymatic reactions. Annexin A5 knockout mice have increased placental thrombosis and infarction but no increased propensity for systemic thrombosis [22].

Several studies explore the possibility that anti-annexin A5 antibodies correlate with clinical manifestations of APS [23]. IgG anti-annexin A5 antibodies occur in patients with pregnancy complications but not in those with venous or arterial thrombosis.

An alternative research path asks whether aPL antibody-mediated disruption of annexin A5 crystallization, on activated platelets and on phospholipid vesicles, leading to reduction of anticoagulant activity (called “A5 resistance”) correlates with adverse clinical outcomes. Recent data suggest that A5 resistance does correlate with increased risk of thrombosis and pregnancy complications. A recent paper [24] correlates A5 resistance with increased prevalence of thrombosis in a “real-world” retrospective population and in a group of prospectively observed asymptomatic patients. In both the retrospective and prospective groups, A5 resistance correlates with positivity for multiple criteria-based aPL assays.

In summary, A5 resistance correlates with an increased risk for thrombosis. The resistance is specifically mediated by anti-domain one of  $\beta_2$ GPI [25] and potentially other aPL cofactor proteins, but it is not mediated by antibodies to annexin A5. To date, although anti-annexin A5 antibody assays may be associated with an APS process, it is not clear that they have a causal relationship to the disease.

## ***Platelet Factor 4 (CXCL4) and Other Platelet-Derived Chemokines***

Platelet factor 4 (PF4) or CXCL4 is an 8kD molecule belonging to the CXC chemokine family. It circulates as a tetramer. It was first recognized to play a role in APS when platelet membrane protein extracts, from three healthy donors and seven APS patients, were passed through a  $\beta_2$ GPI affinity column and analysed by mass spectrometry [26]. Experiments using in silico molecular docking models indicated that a tetramer of PF4 act as a scaffold to which two molecules of  $\beta_2$ GPI bound. According to this model, domain I of  $\beta_2$ GPI became accessible for recognition by a $\beta_2$ GPI, while domain V was available to interact with other proteins on the platelet membrane [26]. The multimeric

complex  $(\text{PF4})_4/(\beta_2\text{GPI})_2/\alpha\beta_2\text{GPI}$  can exist in solution. Furthermore, platelets from healthy individuals, primed with very small amounts of thrombin, were activated only when  $\text{PF4}$ ,  $\beta_2\text{GPI}$  and  $\alpha\beta_2\text{GPI}$  were present and were associated with the phosphorylation of p38 MAP kinase. Natural dimerization of  $\beta_2\text{GPI}$  is necessary for more effective recognition by  $\alpha\beta_2\text{GPI}$ , the whole complex being a powerful platelet activator [27]. The interaction of  $\beta_2\text{GPI}$  with  $\text{PF4}$  induces  $\beta_2\text{GPI}$  dimers in a completely natural way and facilitates antibody binding and platelet activation, which itself is important for enhanced activation of endothelium and fibrinogen in a mouse thrombosis model. Plasma levels of platelet-derived chemokines such as  $\text{PF4}$ ,  $\text{PF4var}$  ( $\text{PF4}$  variant, also known as  $\text{CXCL4L1}$ ),  $\text{CXCL7}$ , and  $\text{CCL5}$  are elevated in patients with APS but not in patients with systemic lupus erythematosus (SLE), coronary artery disease (CAD), or healthy controls [28], indicating marked platelet activation in APS patients. These data support the notions that platelet activation in APS is induced by the complex  $(\text{PF4})_4/(\beta_2\text{GPI})_2/\alpha\beta_2\text{GPI}$ .

### ***Other Proteins***

A recent study suggests that true anticardiolipin antibodies (those that recognize cardiolipin without the support of a plasma protein) may also induce a prothrombotic state in mice [28]. It is difficult to prove that these antibodies function in the absence of a natural protein because, in *in vivo* models, many candidate molecules are obligatorily present. Anticardiolipin antibodies, as measured with currently available assays, correlate weakly with thrombosis compared to lupus anticoagulant (LA); moreover, syphilis and leprosy patients have these autoantibodies without a clear increased risk of thrombosis. Cardiolipin is likely too small to elicit an immune response on its own without a carrier protein.

Autoantibodies to a number of other coagulation-relevant proteins, such as protein S, protein C, tissue factor pathway inhibitor, factor X, XI and XII, are found in a small subgroup of patients with APS [29]. Some correlate with clinical manifestations, and mechanisms regarding how these autoantibodies might induce a prothrombotic state have been proposed, but none of the autoantibodies have been tested in *in vivo* models. There is no convincing evidence that they play a role in thrombosis or pregnancy complications in APS.

### **Why Do Plasma Proteins Become Prothrombotic in the Presence of Autoantibodies?**

Based on animal models of APS, three natural proteins,  $\beta_2\text{GPI}$ , prothrombin, and annexin A2, are identified as important antigens in APS. Although inhibition of annexin A2 can inhibit fibrinolysis, the absence of plasminogen does not cause high risk for thrombosis in humans, suggesting a minor role of annexin A2.

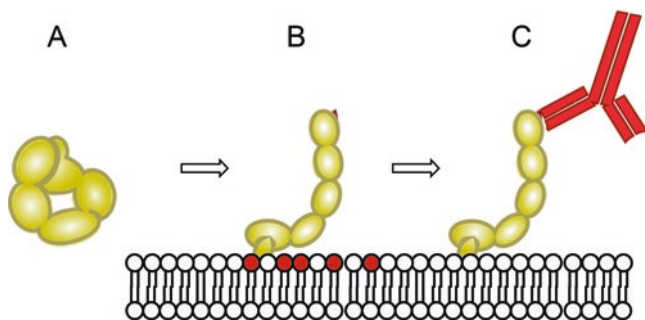
Autoantibodies against prothrombin differ from autoantibodies against a prothrombin- phosphatidylserine (PS) complex. There are autoantibodies that recognize prothrombin only when it is bound to an anionic phospholipid, suggesting that prothrombin undergoes a conformational change when it is bound to PS, exposing a cryptic epitope. Based on human studies, antibodies directed against this cryptic epitope correlates better with thrombosis than do antibodies against the rest of the prothrombin molecule. Further analysis of the specific antigenic epitope will help us understand how these autoantibodies might be prothrombotic.

It is not immediately clear why antibodies against  $\beta_2$ GPI or prothrombin could induce thrombosis. Inhibition of prothrombin would result in bleeding, and no physiological function has been described for  $\beta_2$ GPI to explain its role in a prothrombotic risk. To become prothrombotic, the autoantibodies should induce a new property in their target proteins. Here are a few possible mechanisms: (a) increased affinity due to dimerization by antibodies [27], (b) conformational changes and expression of a hidden epitopes [30], and (c) reshuffling of disulphide bridges within proteins [31].

### *Conformational Changes of $\beta_2$ -Glycoprotein I*

The first demonstration that a plasma cofactor was required for aPL to bind cardiolipin was made by McNeil et al. in 1989 [32]. In the following year, this cofactor was identified as  $\beta_2$ GPI by peptide sequencing which itself was later identified as the major autoantigen for aPL [33, 34].  $\beta_2$ GPI consists of 326 amino acid residues organized in five CCP (complement control protein) domains [35] (DI-DV), which function as protein-protein interaction modules in many proteins. DI-DIV have evolutionary conserved sequences; DV contains a six-residue insertion, a 19-residue C-terminal extension and an additional disulphide bond that includes a C-terminal cysteine residue. DV also harbours a large, positively charged patch that determines affinity for anionic phospholipids. The crystal structure of  $\beta_2$ GPI, solved in 1999, [36, 37] suggests a stretched arrangement of the DI-IV, with DV lying at a right angle to the other domains, in the shape of a J. The phospholipid-binding site is located at the bottom of DV and consists of 14 charged amino acid residues and a flexible and hydrophobic loop. This crystal structure predicts that, when  $\beta_2$ GPI is bound to a lipid layer, DI to IV point away from the lipid layer and that the potential binding site for  $\alpha\beta_2$ GPI autoantibodies in DI is fully exposed [38].

There are no circulating nor tissue deposition of  $\beta_2$ GPI-antibody immune complexes in patients with APS. A logic interpretation of this observation is that the antibodies directed against  $\beta_2$ GPI do not recognize  $\beta_2$ GPI in the circulation. The antibodies recognize a cryptic epitope in the molecule. It has been shown that  $\beta_2$ GPI expose the autoantibody binding site when it binds to anionic phospholipids [39].



**Fig. 2.1** Conformational change within  $\beta_2$ GPI. (A)  $\beta_2$ GPI as it circulates in plasma. (B) Binding to negatively charged phospholipids opens up  $\beta_2$ GPI. (C) Binding of autoantibodies stabilizes  $\beta_2$ GPI in its stretched conformation

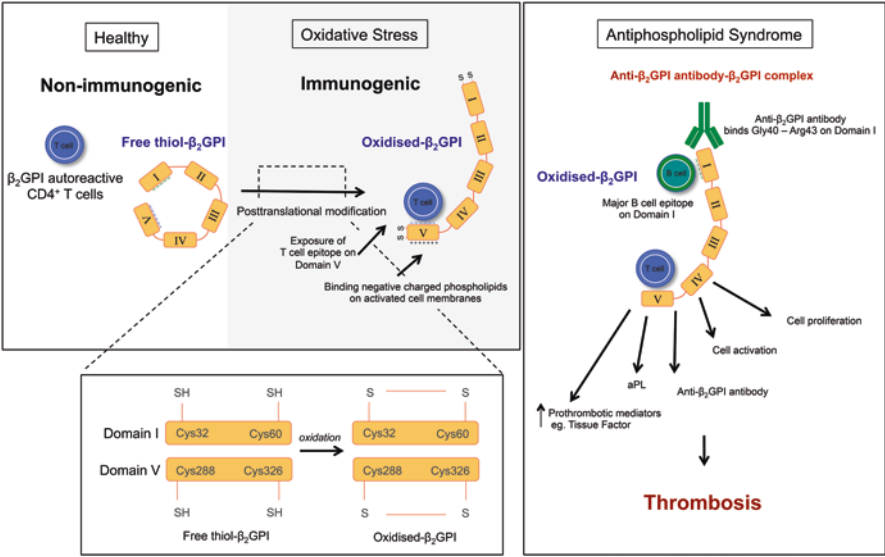
Electron microscopy studies show that  $\beta_2$ GPI exists in two different conformations (Fig. 2.1). In plasma, it is present as a circular protein in which DI interacts with DV. On binding to anionic surfaces, the protein opens up and expresses the #hockey-stick conformation of the crystal structure [40]. The circular conformation predicts shielded epitopes within DI and DV [40], and, indeed, autoantibodies against DI recognize  $\beta_2$ GPI only when it is bound to anionic surfaces, not when it is present in the circulation. Since  $\beta_2$ GPI binds to cell receptors via its DV, and since binding is enhanced by autoantibodies, it must be mediated by a cryptic epitope in DV that is expressed after the molecule has opened.

Small-angle X-ray scattering (SAXS) experiments suggested that in solution,  $\beta_2$ GPI adopts an S-shaped conformation with an additional buckle between DII and DIII [41]. Additional SAXS experiments show that  $\beta_2$ GPI adopts different conformations, depending on pH, ionic strength, and certain cations.  $\beta_2$ GPI turns out to be a flexible molecule, not constrained to a single, specific conformation; its conformation depends on interactions with its surroundings. Apparently  $\beta_2$ GPI can adapt a number of different structural conformations that in vitro can coexist in a dynamic equilibrium. Factor H, a complement factor built up of 20 CCP domains, also adopts different domain orientations in solution with consequences for its functional activity [42, 43]. Proteins consisting of CCP domains vary their conformations, depending not only on the length and flexibility of the linker sequences between domains but also, predominantly, on interactions with their surroundings.

Redox Balance

Antiphospholipid syndrome is characterized by oxidative stress and systemic inflammation [44, 45]. The overproduction of reactive oxygen species (ROS) results in an oxidative microenvironment that exacerbates inflammation, inducing cell death and tissue damage, compromising antioxidant defence mechanisms [46]. Patients with APS have high levels of circulating pro-inflammatory cytokines interleukin-2 (IL-2), interleukin-6 (IL-6), and tumour necrosis factor (TNF), together with markers of oxidative stress and inflammation such as serum amyloid A (SAA), C-reactive protein (CRP), 8-isoprostane, and prostaglandin E2 (PGE2) [47, 48].

In vivo and under normal physiological conditions,  $\beta_2$ GPI is produced in the liver and exists predominately in circulation in its free thiol form, which is less immunogenic than the oxidized form (Fig. 2.2). The precise role of  $\beta_2$ GPI and its different forms are complex [49]; it is thought to act as a natural anticoagulant that mediates a range of functions including the clearance of liposomes, apoptotic bodies and microparticles [49–52].



**Fig. 2.2** How does oxidized  $\beta_2$ GPI participate in the formation of thrombotic APS? During oxidative stress, free thiol  $\beta_2$ GPI can undergo post-translational modification to form the immunogenic form, oxidized  $\beta_2$ GPI after binding phospholipids.  $\beta_2$ GPI autoreactive  $CD4^+$  T cells recognize newly exposed epitopes located on Domain V but not on free thiol  $\beta_2$ GPI. A complex is formed between a $\beta_2$ GPI, autoreactive  $CD4^+$  T cells and oxidized  $\beta_2$ GPI triggering the production of aPL, specifically a $\beta_2$ GPI, cell proliferation and the release of pro-inflammatory cytokines which are key events in the pathophysiology of thrombotic APS

## ***Quantitation of Oxidized $\beta_2$ GPI as a Biomarker for Antiphospholipid Syndrome***

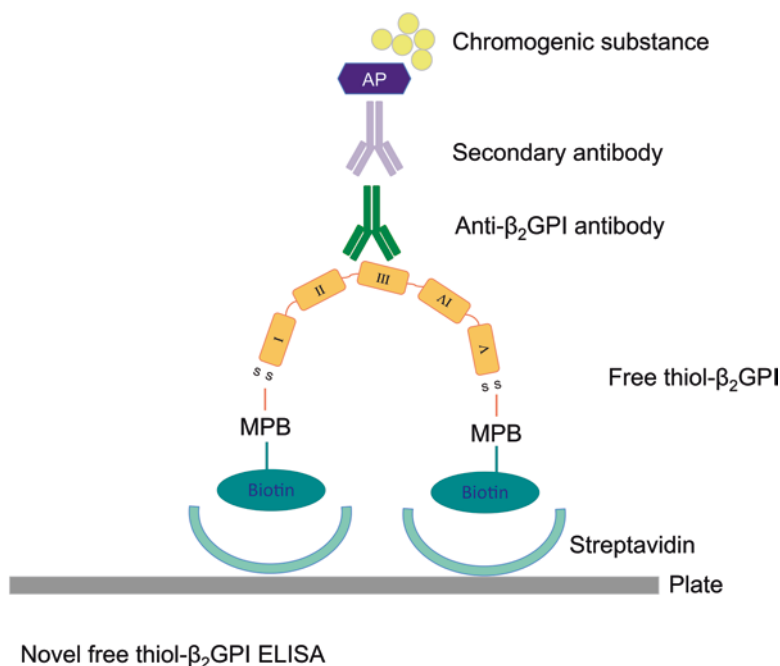
Oxidized  $\beta_2$ GPI level in APS has been proposed as a biomarker of thrombotic risk for APS. Levels of oxidized  $\beta_2$ GPI in patients with APS are higher than in healthy subjects and patients with other autoimmune disease or non-aPL disease controls with thrombosis [53]. Free thiol  $\beta_2$ GPI may play a protective role in APS, since free thiol  $\beta_2$ GPI protects human umbilical vein endothelial cells (HUVEC) against hydrogen peroxide-induced cell injury [54]. Decreased plasma free thiol  $\beta_2$ GPI may thus lower the physiological buffer against oxidative stress-induced injury. Free thiol  $\beta_2$ GPI also protects human retinal pigment epithelium and the subretinal endothelial cell against oxidative, hydrogen peroxide stress-induced, cell death [55].

A multicentre, cross-sectional, international study using prospectively acquired samples has demonstrated that the redox status of  $\beta_2$ GPI differs between healthy individuals and patients with thrombotic APS [53]. In the former it exists predominately with free thiols; APS patients have higher levels of total and oxidized  $\beta_2$ GPI compared to both healthy subjects and patients with other autoimmune disease [53, 56].

## ***Diagnostic and Prognostic Implications of the Oxidized $\beta_2$ GPI ELISA***

Anticardiolipin antibodies,  $a\beta_2$ GPI, and LA test serve as diagnostic markers in APS. The predominant isotypes of aPL in APS patients are IgG aCL and IgG  $a\beta_2$ GPI [57, 58]. Although, non-criteria or non-classical aPL such as antiphosphatidylserine, antiphosphatidylethanolamine, and antiphosphatidylglycerol have been reported, only the three classical aPL tests are used in diagnosis of APS [59]. The LA assay identifies autoantibodies against either prothrombin and/or  $\beta_2$ GPI, whereas the aCL assay detects the aCL and/or  $a\beta_2$ GPI antibodies. The  $a\beta_2$ GPI assay detects only antibodies against  $\beta_2$ GPI. Concomitant triple positivity for aCL,  $\beta_2$ GPI and LA may indicate severe APS and high recurrence risk [60], a point that is still controversial [61]. Lupus anticoagulant correlates much better with the clinical manifestations of APS than the detection of the autoantibodies with an ELISA [62, 63], and a positive LA assay due to  $a\beta_2$ GPI has a stronger correlation for thrombotic risk than due to antiprothrombin autoantibodies [64].

In a clinical setting, it is important to stratify risk for development of clinical events in APS and in asymptomatic, aPL-positive individuals. Delayed or inadequate treatment can result in damage and impaired quality of life [65, 66]. Although  $\beta_2$ GPI levels are not routinely measured in patients with APS, considering the specificity of high levels of oxidized  $\beta_2$ GPI, measuring their levels may assist in diagnosis and prognosis. The level of oxidized  $\beta_2$ GPI is calculated by subtracting the concentration of free thiol from total  $\beta_2$ GPI. Using an ELISA to measure post-translational redox modifications of  $\beta_2$ GPI (including total and free thiol  $\beta_2$ GPI) [53] (Fig. 2.3) and  $\beta_2$ GPI



**Fig. 2.3** Schema of enzyme-linked immunosorbent assay (ELISA) to measure free thiol  $\beta_2$  glycoprotein I ( $\beta_2$ GPI). Free thiol  $\beta_2$ GPI bind streptavidin via biotin-conjugated maleimidyl-propionyl biocytin (MPB) and become immobilized. Acetone precipitation removes unbound MPB, and bound free thiol  $\beta_2$ GPI is quantified with  $\alpha\beta_2$ GPI and detected using a secondary antibody, alkaline phosphatase (AP) and chromogenic substance para-nitrophenylphosphate (PnPP)

plasma levels in 359 patients (identified through an international multicentre initiative) who had either APS or other autoimmune diseases or non-APS vascular thrombosis, Ioannou et al. found that the redox state of  $\beta_2$ GPI and its concentration in APS patients had a profile distinct from that in the various control groups.

## Group Conclusion

Evidence from both clinical and animal studies supports the concept that  $\beta_2$ GPI is the main autoantigen in APS. Understanding of the pathophysiology of APS and the involvement of  $\beta_2$ GPI and its post-translational modified forms remains incomplete; understanding the relevance of oxidized  $\beta_2$ GPI in APS will be important. Although  $\#aPL$  are a defining, hallmark feature of APS, their presence does not exclusively indicate APS nor do they stratify individuals for risk of thrombosis.

Current methods for diagnosing APS patients do not incorporate quantitation of total and free thiol  $\beta_2$ GPI. Specific ELISAs for quantifying these parameters may enhance our diagnostic and prognostic capabilities. Prospective studies may validate

measurement of oxidatively modified forms of  $\beta_2$ GPI as biomarkers. The AntiPhospholipid Syndrome Alliance For Clinical Trials and InternatiOnal Networking (APS ACTION), an international research network that collects patient samples from 25 centres around the world [67], may allow a longitudinal study that measures oxidized  $\beta_2$ GPI.

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