Looking into a paradox –

Lupus anticoagulant and its relation to thrombosis in the antiphospholipid syndrome

Jessica Eline Molhoek

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About the cover: Zoomed display of an ELISA wherein beta2-glycoprotein I is catched by an anti-beta2-glycoprotein I antibody.

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Kijkend naar een paradox –
Lupus anticoagulant en de relatie tot trombose in het antifosfolipiden syndroom
(met een samenvatting in het Nederlands)

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“Remember you’re loved
and you always will be.
This melody will bring you right
back home.”

-Linkin Park, The messenger

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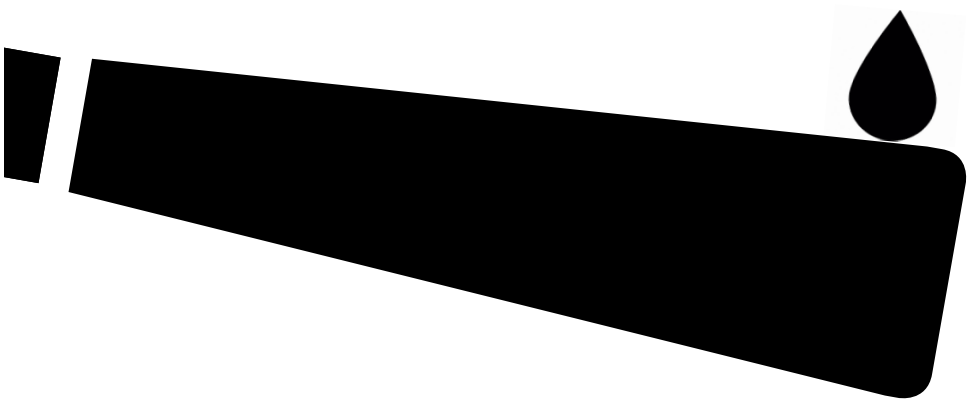
General introduction and thesis outline

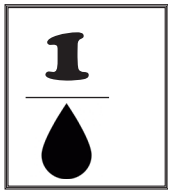


The integrity of the vasculature in our body is tightly regulated. After an injury, collagen and tissue factor become exposed to blood. The exposure of collagen leads to binding and activation of platelets resulting in the attraction of new platelets that will aggregate. The aggregated platelets form a weak plug that temporarily stops bleeding, a process that is known as primary haemostasis. If this weak plug is not strengthened via the formation of a fibrin scaffold, it will dissolve within a hour[1].

Plug stability – secondary haemostasis

The stability of the plug is effectuated by fibrin fibers. These fibers are the end product of the coagulation cascade, which is known as secondary haemostasis. The coagulation cascade is initiated by tissue factor (TF). TF is present in the sub-endothelium and is exposed after a vessel is damaged. A complex consisting of TF and factor VIIa is formed on a phospholipid surface, this complex initiates the activation of factors IX and X[1]. Activated factors IX (IXa) and X (Xa) enable two downstream pathways in the coagulation cascade: (1) Factor IXa forms the intrinsic tenase complex with cofactor VIIIa to activate factor X (Xa) and (2) Factor Xa forms the prothrombinase complex with cofactor Va to activate prothrombin[1-4]. TF concentrations determine the route in the coagulation cascade. At low TF concentrations, the route in the coagulation cascade will be via the intrinsic tenase complex, whereas the route will be via the prothrombinase complex at high TF concentrations. The prothrombinase complex converts prothrombin to thrombin, and thrombin converts soluble fibrinogen to insoluble fibrin fibers for stable plugs. The coagulation cascade continues via a feedback loop with the activation of factor XI by thrombin. Thrombin generated via the feedback loop is necessary to stabilize the clot (figure 1). A stable clot is generated by activation of thrombin-activatable fibrinolysis inhibitor (TAFI) and factor XIII[1,2].

Inhibitory proteins such as protein C, protein S, tissue factor pathway inhibitor (TFPI), antithrombin and C1 inhibitor regulate the coagulation cascade (figure 1). Protein C is activated by thrombin bound to thrombomodulin on endothelial cells. Activated protein C inactivates factor Va and VIIIa mediating an antithrombotic effect. TFPI binds protein S and factor Xa, thereby inhibiting the extrinsic tenase complex consisting of factor VIIa and TF. Antithrombin blocks activated coagulation factors such as thrombin, factor Xa, factor IXa and factor XIa. Thrombin in solution is inhibited by antithrombin, although thrombin bound to fibrin is protected by antithrombin. C1 inhibitor is the most important inhibitor of factor XIa, factor XIIa and kallikrein[1,2].



An imbalance of the haemostatic system will lead to bleeding or thrombosis, as seen in the antiphospholipid syndrome[1]. This syndrome is an autoimmune disease leading to thrombosis and pregnancy morbidity[5,6]. Thrombosis occurs in the deep veins in the lower limbs (venous thrombosis) and in the cerebral arterial circulation (arterial thrombosis)[5,7]. An important player in the antiphospholipid syndrome is identified as β2-glycoprotein I (β2GPI)[5,6]. β2GPI consists of five identical domains except for domain V. Domain V contains an extra C-terminal loop and a positively charged patch to bind phospholipids and the surface of platelets, monocytes and endothelial cells[8,9]. The antiphospholipid syndrome and β2GPI are discussed in **chapter 2**.

Regulation of secondary haemostasis – activated protein C

Activated protein C degrades coagulation factors VIIIa and Va[10]. Protein S acts as a cofactor and is important in the anticoagulant function of activated protein C. Also factor V acts as cofactor for activated protein C in the degradation of factor VIIIa[11-14]. Incorporation of factor VIIIa in the intrinsic tenase complex and factor Va in the prothrombinase complex protects the cofactors from activated protein C degradation[10]. The presence of anionic phospholipids is essential for activated protein C to dock for its function[12].

Factor V is highly homologous to factor VIII. Activated protein C cleaves activated factor V (Va) at Arg306, Arg506 and Arg679. Arg506 is the kinetic favourable cleavage site[12,13]. However, in the presence of protein S, the favourable cleavage site is Arg306. Protein S leads to a 20-fold faster cleavage resulting in complete inactivation[13,15]. Protein S abrogates the protection of factor Va by factor Xa and prothrombin in the prothrombinase complex for activated protein C[12].

A mutation at Arg506 in factor V (factor V Leiden) causes activated protein C resistance. Due to this mutation, activated protein C is not able to efficiently inactivate factor Va[1,15]. Activated protein C resistance is frequently seen in patients with venous thrombosis or with the antiphospholipid syndrome[15].

Fibrinolysis

A thrombus needs to be degraded in time. TAFI becomes activated by thrombin formed in the coagulation cascade. Activated TAFI removes C-terminal lysine residues from the clot that are essential in binding of fibrinolytic factors. Factor XIIIa helps in the strengthening of the fibrin network by cross-linking fibrin fibers and incorporation of (active) TAFI and α2-antiplasmin. Fibrinolysis starts with the release of tissue plasminogen activator (tPA) from endothelial cells. tPA activates plasminogen to plasmin[1,2]. Both tPA and plasminogen bind to the lysine residues on partially degraded fibrin and enable the degradation of the clot.

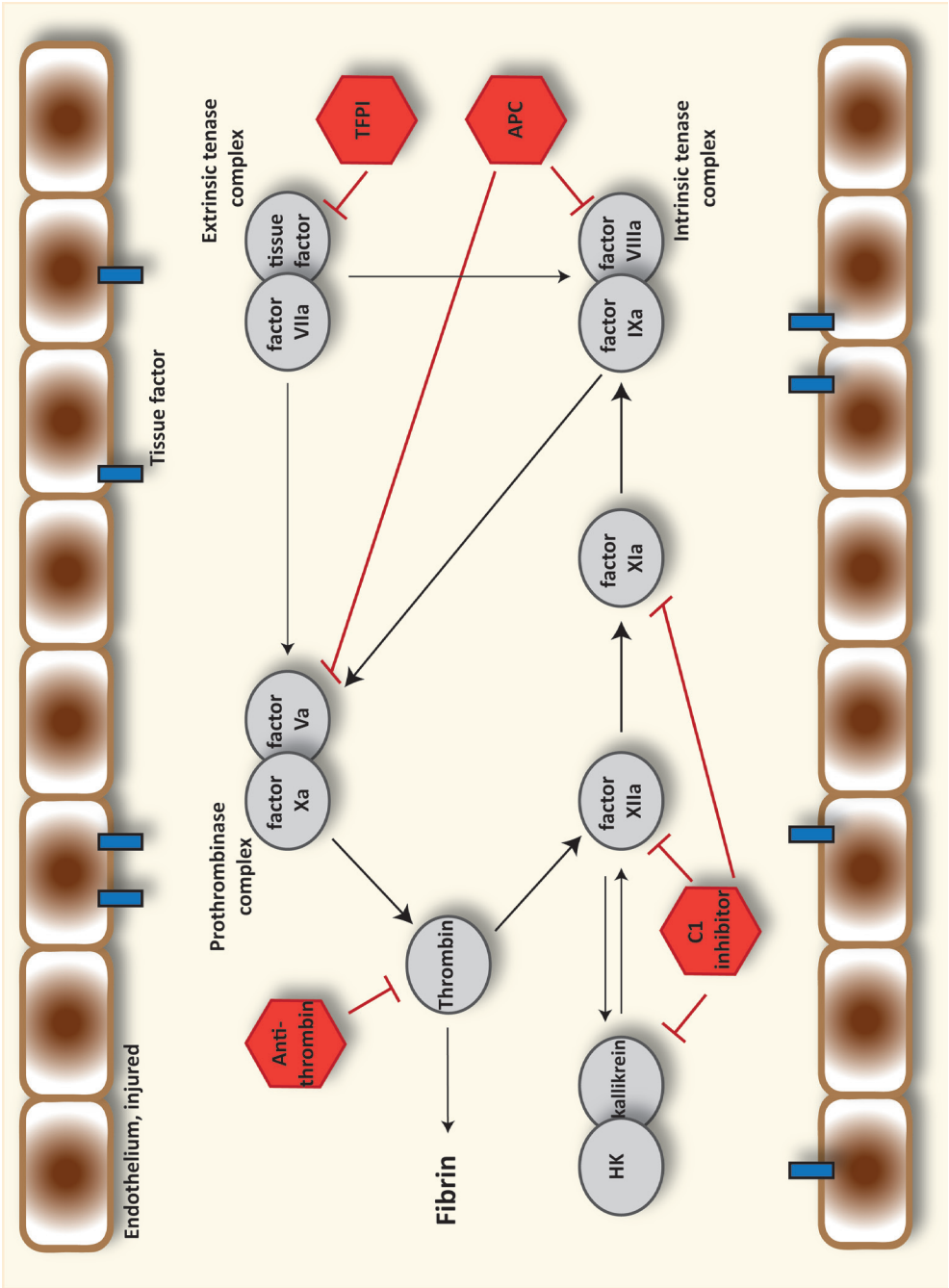

Endothelium, injured
Tissue factor
Extrinsic tenase complex
tissue factor
factor VIIa
TFPI
APC
Intrinsic tenase complex
factor VIIIa
factor IXa
Prothrombinase complex
factor Va
factor Xa
Anti-thrombin
Thrombin
Fibrin
factor XIa
factor XIIa
C1 inhibitor
HK
kallikrein

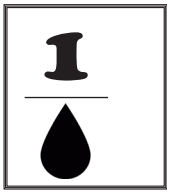
Figure 1. **Overview of the coagulation cascade.** Injured endothelium exposes tissue factor (TF) and activates the coagulation cascade via the extrinsic tenase complex (factor VIIa and TF). The extrinsic tenase complex continues the cascade via the intrinsic tenase complex (factor VIIIa and IXa) and the prothrombinase complex (factor Va and Xa). The prothrombinase complex can activate prothrombin to thrombin. Thrombin can activate factor XI resulting in a feedback loop, represented with the larger arrows and is necessary to stabilize the clot. Thrombin is also able to generate fibrin fibers and strengthen the clot via activation of factor XIIIa and inhibition of the fibrinolytic system. The coagulation cascade can also be activated via the contact pathway. Reciprocal activation of this pathway occurs via factor XIIa that subsequently generates kallikrein and factor XIa. Inhibitory proteins counteract the activation in the coagulation cascade. Activated protein C inactivates factor Va and VIIIa mediating an antithrombotic effect. TFPI binds protein S and factor Xa, thereby inhibiting the extrinsic tenase complex consisting of factor VIIa and TF. Antithrombin blocks activated coagulation factors such as thrombin, factor Xa, factor IXa and factor XIa. Thrombin in solution is inhibited by antithrombin. HK=high molecular weight kininogen; APC=activated protein C; TFPI=tissue factor pathway inhibitor.

Binding of tPA and plasminogen to lysine residues is regulated by activated TAFI[1]. α2-antiplasmin inactivates plasmin, however plasmin bound to fibrin fibers protected from inactivation. Finally, plasmin dissolves the clot[1,2].

Content of this thesis

One of the diagnostic hallmarks of the antiphospholipid syndrome associated with thrombotic risk is a prolonged clotting time. A prolonged clotting time is normally suggestive for bleeding tendency indicating a paradox in the antiphospholipid syndrome. Patients with antiphospholipid syndrome have persistent so-called anti-phospholipid antibodies. A prolonged clotting time due to an inhibitor is known as lupus anticoagulant. The anti-phospholipid antibodies do not bind to phospholipids, but to plasma proteins that are bound to phospholipids. Antiphospholipid antibodies are directed to an antigen known as β2GPI. Understanding the antiphospholipid paradox is difficult as no physiological function related to thrombosis is described for β2GPI. The aim of this thesis is to understand lupus anticoagulant and its relation to thrombosis in the scope of the antiphospholipid syndrome. The paradox between prolonged coagulation times and thrombosis is discussed in **chapter 2**. In **chapter 3** we study the clinical consequence of lupus anticoagulant in stroke in a case-control study in Tanzania. In this case-control study we found a high correlation between lupus anticoagulant and the risk for stroke. Several studies identified the presence of anti-β2GPI antibodies upon an infection. Our group previously showed that

injection of surface protein H of *Streptococcus pyogenes*, a subset of *Streptococcus*, induces anti-β2GPI antibodies in mice. However, this study did not investigate the effect of β2GPI on thrombus formation. In **chapter 4**, we describe that anti-β2GPI antibodies against domain I that were generated upon protein H injection enhances thrombus formation. In the antiphospholipid syndrome the main protein is β2GPI, although its physiological function is under debate. Previous studies showed at least two different conformations of β2GPI. We developed a nanobody to distinguish between these conformations and describe the results in **chapter 5**. The important sequences for a possible function for β2GPI are the cryptic epitopes in domain I and V. These epitopes are often involved in antibody and receptor/negative surfaces binding. Our nanobody recognizes an epitope when anti-β2GPI antibodies bind to and change the conformation of β2GPI. In **chapter 6** we describe the ability of β2GPI-anti-β2GPI antibody complexes to inhibit the activation of factor V by factor Xa. This inhibition partly explains lupus anticoagulant in antiphospholipid syndrome patients. β2GPI-anti-β2GPI antibody complexes are able to influence the protein C pathway via the inactivation of factor VIIIa. **Chapter 7** summarizes the research described in this thesis, discusses the research outcomes and gives future perspectives in β2GPI/antiphospholipid syndrome research.



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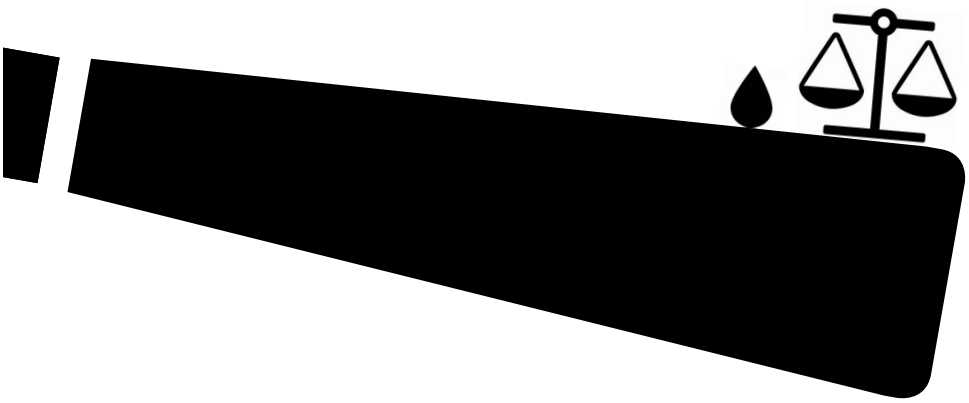
The lupus anticoagulant paradox



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Abstract

Lupus anticoagulant represents the most enigmatic antibody population in patients with antiphospholipid syndrome and represents a paradox that is still unsolved. This class of antiphospholipid antibody causes a phospholipid-dependent prolongation of the clotting time, but is associated with an increased risk of thrombosis and pregnancy morbidity. In this review, we will provide an overview of the different antibodies that have been associated with lupus anticoagulant activity, their importance based on clinical studies, and address the question why this prolongation of the clotting time is associated with thrombosis rather than a bleeding tendency.

Introduction

Antiphospholipid antibodies (aPL) are acquired risk factors for thrombosis and adverse pregnancy outcome. When patients present with thrombosis or pregnancy-related morbidity and persistent aPL, they are diagnosed with the autoimmune disorder antiphospholipid syndrome (APS). The term 'aPL' refers to a heterogeneous group of auto-antibodies directed against a wide variety of proteins with affinity for negatively charged phospholipids, including several coagulation factors and other plasma proteins. Although the list with antigens for aPL is long, the latest official classification criteria for APS only recognize three distinct aPL, with partially overlapping specificity[1]. Two of these are named for their antigens: antibodies against the plasma glycoprotein β2-glycoprotein I (β2GPI), or anti-β2GPI antibodies, and antibodies against the combination of the phospholipid cardiolipin (aCL) and the protein β2GPI, or aCL. The definition of the third antibody population is based on their functional activity rather than their antigen: this antibody population causes a prolonged clotting time *in vitro* and is known as lupus anticoagulant (LA). LA can be caused by anti-β2GPI or anti-prothrombin antibodies, but is also frequently encountered in the absence of these antibodies. Although it has been over 50 years since the first description of the LA phenomenon in the plasma from patients with systemic lupus erythematosus (SLE), the identity of the responsible antigen or antigens largely remains unclear[2].

Out of the three aPL populations described in the classification criteria for APS, LA correlates best with the occurrence of thrombosis or adverse pregnancy outcome[3,4]. This represents a paradox, as a prolonged *in vitro* clotting time, as is evident with LA, is otherwise indicative of a bleeding tendency. Since patients with APS suffer from thrombotic complications, we can only assume that LA, as an *in vitro* phenomenon, does not necessarily reflect the *in vivo* mechanism that causes thrombosis or pregnancy complications. In this review, we will provide an overview of the current knowledge of the cause of the LA phenomenon, as well as the laboratory procedures to detect LA and will address the question why this prolongation of the clotting time is associated with thrombosis rather than a bleeding tendency.

Detection of lupus anticoagulant

A sample is considered positive for LA when the clotting time is prolonged in a phospholipid-dependent manner. Hence, procedures for detection of LA should always include a comparison of the clotting time obtained with a "screening" reagent, containing a limiting phospholipid concentration, and a clotting time obtained with a "confirmatory" reagent, containing an excess of phospholipid and (preferably) the same activator. When clotting times are prolonged with the screening reagent

and are corrected with the confirmatory reagent, a phospholipid-dependent prolongation is deemed to be proven. To exclude coagulation factor deficiencies as the cause for phospholipid-dependent prolongation of clotting, mixing tests, in which index plasma is mixed with an equal volume of normal plasma, should be performed. If clotting times with screening tests are still prolonged in the mixing test, coagulation factor deficiency is excluded as the cause of the prolongation and the presence of an inhibitor is therefore assumed. It should be noted that the mixing test does not prove the inhibitor is an antibody. Whether or not mixing tests are required for accurate detection of LA, and whether they should be performed before or after the confirmatory test, remains a matter of ongoing debate[5-10].

In principle, all coagulation tests that investigate phospholipid-dependent coagulation reactions are suitable for detection of LA, provided the phospholipid concentration in the reagent is low enough to be sensitive for LA. These include dilute prothrombin time (dPT), activated partial thromboplastin time (APTT) variants and tests that involve snake venom coagulation activators, such as the Textarin Time,[11] the Taipan Snake Venom Time (TSVT)[12] or the dilute Russell's Viper Venom Time (dRVVT)[13]. The Ecarin Time, based on the activation of prothrombin by the snake venom protease ecarin, is not phospholipid-dependent and therefore insensitive for LA, and may therefore be used as a separate confirmatory test. Antibodies that cause LA are heterogeneous, and no single LA-test has proven to be 100% sensitive for LA. For this reason, guidelines from the Scientific Standardization Subcommittee on LA of the International Society on Thrombosis and Hemostasis (ISTH) suggest that assessment of LA should always include two tests of a different principle: preferably a dRVVT, a robust test that investigates the common pathway of coagulation, and an LA-sensitive APTT[14]. The use of more than two different tests for detection of LA is not advised by the SSC, as this is considered to lead to an unacceptable increase in false positive test results. However, the British guidelines and the guidelines from the CLSI do not mandate a maximum of two tests[9,10].

Lupus anticoagulant and the risk of thrombosis

LA is undoubtedly the least well-defined antibody population found in patients with APS: the identity of the antibody population(s) that causes the *in vitro* prolongation of clotting is largely unknown and the mechanism(s) responsible for the phospholipid-dependent prolongation of clotting is similarly unclear. Moreover, whether there is a causal relationship between the *in vitro* prolongation of clotting seen with laboratory testing and the occurrence of vascular disease remains to be determined. Nevertheless, data from prospective clinical studies indicate that the presence of LA correlates better with thrombotic events and adverse pregnancy outcome than the presence of anti-β2GPI and aCL antibodies[3,4].

Patients with persistent aPL, i.e. LA, anti-β2GPI antibodies or aCL antibodies, confirmed to be present on at least two occasions tested at least 12 weeks apart, are currently considered to be at risk for the recurrence of thrombotic events. As a result, the duration of anticoagulant treatment in patients with aPL and thrombosis is often longer than would be indicated in other patients, and is frequently prescribed life-long. This would seem justified considering the reported thrombotic risk in APS, but based on available evidence, the adoption of aPL as biomarkers for recurrent disease is probably premature. There are good arguments that support the utility of LA as a biomarker for a first thrombotic event, but currently available evidence is not as strong for the other aPL subtypes. First of all, a good biomarker occurs in patients at risk for disease, but is not found in healthy persons. Indeed, case-control studies and studies amongst cross-sectional patient cohorts indicate that LA, but not the other aPL, is consistently more prevalent in patients with thrombosis than in control groups and associated with an increased risk of thrombosis[15-17]. However, anti-β2GPI and aCL antibodies frequently occur in the general population and are often observed after infections (see ref 18 for an overview). Second, prognostic value, a key characteristic of a good biomarker, has been shown for LA in a prospective cohort study amongst carriers of aPL[3], in which carriers of LA had a 3.9-fold increased chance of developing venous thromboembolism (VTE) than persons without LA. The same study indicated that the other aPL are not associated with the occurrence of a first thrombotic event. Similar data have been obtained in a prospective study amongst pregnant carriers of aPL[4,19], in which LA was associated with adverse pregnancy outcome, but the presence of the other aPL subtypes did not associate with adverse events.

Good predictors for a first thrombotic event are not necessarily associated with an increased risk of a second thrombotic episode. This is well illustrated by the coagulation factor V R506Q (Factor V (FV) Leiden) variant. Whereas this genetic variant is associated with a strongly increased risk of venous thrombosis in asymptomatic carriers, it is not associated with a substantially increased risk of recurrence, which is why screening for FV Leiden in patients with venous thrombosis is now considered clinically irrelevant[20]. Data on the risk of recurrence in patients with aPL is mostly obtained from studies that were subject to methodological flaws and the combined evidence from these studies is weak; moreover, a systematic review on the risk of recurrent venous thromboembolism in carriers of aPL, indicated that aPL are not associated with an increased risk of recurrence[21]. This represents a challenge, because aPL are used in every day clinical practice as biomarkers for recurrent disease. There is an urgent need for methodologically sound prospective studies to establish the clinical utility of aPL as risk factors for recurrent disease.

This will become even more important in coming years due to the introduction of direct oral anticoagulants, which are not compatible with LA testing using most of the currently available diagnostic tools[22].

Antibodies against prothrombin cause lupus anticoagulant

The plasma protein prothrombin is one of the antigens to which aPL with LA activity are directed, although anti-prothrombin antibodies are not included in the official classification criteria of APS. This 72 kDa glycoprotein is synthesized by hepatocytes in the liver. It is the pro-enzyme of the central enzyme of the coagulation system, thrombin, and circulates at a plasma concentration of approximately 1.4 µM. Prothrombin consists of a N-terminal phospholipid-binding domain known as Fragment 1.2, which is cleaved off by the prothrombinase complex during coagulation reactions, and a C-terminal serine protease domain. The first 10 glutamate residues in the Fragment 1.2 domain have undergone posttranslational γ-carboxylation, resulting in a Ca^{2+} dependent high affinity binding site for the negatively charged phospholipid phosphatidylserine (PS). In the absence of Ca^{2+}, the affinity of prothrombin for PS is much lower[23].

Prothrombin has been suggested as a cofactor for antibodies with LA activity as early as 1959[24]. Since then, it has been shown that antibodies from patients with LA can be adsorbed on prothrombin, leading to a reduction in anticoagulant activity in supernatant. Moreover, the adsorbed antibodies retained their LA activity after elution from solid phase prothrombin,[25] providing proof that anti-prothrombin antibodies can have LA activity.

Some patients with antibodies against prothrombin and LA have hypoprothrombinaemia, leading to a bleeding tendency rather than a thrombotic tendency. This acquired prothrombin deficiency is probably caused by high affinity anti-prothrombin antibodies that bind prothrombin in solution, leading to clearance of antibody-prothrombin immune complexes[26]. The majority of patients with LA and anti-prothrombin antibodies, however, have normal or slightly reduced prothrombin levels and no clinical evidence of a bleeding tendency,[25] which is why this subgroup of anti-prothrombin antibodies is referred to as non-neutralizing. Why some patients develop an acquired prothrombin deficiency and others do not, is not well understood. It is hypothesized that patients with persistent anti-prothrombin antibodies and normal prothrombin levels have low-affinity antibodies, or antibodies that specifically recognize phospholipid-associated prothrombin. The latter hypothesis is supported by data obtained with purified human IgG with LA activity, which showed that the conversion of prothrombin to thrombin by the prothrombinase complex is only inhibited in the presence, but not in the absence

of phospholipids[27].

The association between anti-prothrombin antibodies and thrombosis is currently unclear: Several studies report an association with thrombosis,[28-32] which is contradicted by others[17,28,33,34]. There are several possible reasons for this discrepancy, including differences in study design, patient population, control group and method used to detect the anti-prothrombin antibodies (and thus, probable heterogeneity in anti-prothrombin antibodies themselves). While some studies focused on venous thromboembolism, others investigated arterial thrombosis, which likely represent two different pathophysiologic mechanisms, with differences in the involved cell type, fluid shear stress and the contribution of the coagulation system to thrombus formation.

β2GPI-dependent vs. β2GPI–independent lupus anticoagulant

With the discovery of β2GPI as the antigen for aCL,[35,36] it soon became apparent that anti-β2GPI antibodies can have LA activity[37,38]. Similar to prothrombin, the plasma glycoprotein β2GPI is synthesized by hepatocytes. Despite its high plasma concentration (5 μM), its physiological function is currently unclear. Based on its phospholipid binding characteristics and the reported interaction with members of the low density lipoprotein receptor family,[39] a class of receptors involved in clearance processes, a role for β2GPI in the clearance of microparticles or apoptotic bodies from the circulation seems likely[40]. However, individuals with a complete deficiency of β2GPI do not have a clinical phenotype and mice with a null mutation in β2GPI do not display a clear phenotype either[41,42].

β2GPI has an elongated structure and consists of five highly homologous complement type repeats, or sushi-domains, designated domain (D)I to V. Each domain contains two internal disulphide bonds, with the exception of DV, which contains three and has a slightly aberrant conformation. Both the first and the fifth sushi domain of β2GPI have been reported to interact with phospholipids[43,44]. The crystal structure of β2GPI indicates that the fifth sushi domain contains a putative phospholipid binding site, consisting of a hydrophobic loop that is flanked by a large positively charged cluster of lysine residues, which aid in the interaction with anionic phospholipid[45]. In contrast to the effect of Ca^{2+} on prothrombin, the presence of Ca^{2+} ions reduces the affinity of β2GPI for phospholipids considerably[46].

The observation that adsorption of either anti-β2GPI (aCL antibodies),[47] or anti-prothrombin antibodies from a sample that contains all three types of antibodies does not always completely neutralize LA activity in plasma samples with these aPL[48]. This indicates that these antibody subtypes can be present at the same time and that both can contribute separately to the strength of the LA in a single sample.

Several attempts have been made to discriminate between β2GPI-dependent and β2GPI-independent LA. For instance, it was shown that aCL antibodies adsorb on cardiolipin vesicles, leading to a reduction in clotting time in samples with a β2GPI-dependent LA[49]. This principle was used to develop a simple APTT-based clotting test, in which plasma was first incubated with cardiolipin vesicles to adsorb the anti-β2GPI antibodies, followed by incubation with a contact activator (silica) and the initiation of coagulation with $CaCl_2$[50]. Addition of cardiolipin neutralized the LA activity of monoclonal anti-β2GPI antibodies in human plasma, whereas it caused an increase in clotting time when LA was induced with monoclonal anti-prothrombin antibodies. LA that can be neutralized with cardiolipin, i.e. β2GPI-dependent LA, was subsequently shown to correlate well with thrombosis in a retrospective cross-sectional study amongst patients with SLE, primary APS and secondary APS, whereas β2GPI-independent LA was not associated with thrombosis[51].
An alternative method to discriminate between β2GPI-dependent and β2GPI-independent LA uses the inhibitory effect of Ca^{2+}-ions on the interaction between β2GPI and phospholipids[52,53]. By lowering the Ca^{2+} concentration in a dPT or dRVVT-based clotting assay, the inhibitory effect of anti-β2GPI on clotting increases. Unfortunately, this assay has never been used in clinical studies to investigate the contribution of β2GPI-dependent LA to thrombotic risk.

A third means of discriminating between β2GPI-dependent and β2GPI-independent LA is based on the phospholipid composition in the confirmatory reagent. It was shown that the inhibitory effect of LA can be neutralized by incubating a plasma sample with hexagonal II phase phosphatidylethanolamine (PE) prior to initiation of coagulation[54,55]. In an aqueous environment, phospholipids normally orient themselves with their polar head groups towards the aqueous layer and the hydrophobic fatty acid tails pointed in the other direction. While this can result in vesicles of any size, including small micelles, slightly larger unilamellar vesicles and large multilamellar vesicles, phospholipids can also arrange themselves into tubule-like structures. Depending on the size of the head group and the angle between the head group and the fatty acid moieties, these tubules either orient with their head group outward (hexagonal I phase), or with their head group directed inward (hexagonal II phase), resulting in an aqueous pore inside the tubule. Both PE and cardiolipin can form these hexagonal II phase structures[56]. One elegant study reported that purified human IgG with LA activity can only be neutralized with hexagonal II phase phospholipid when prothrombin is present. Addition of either β2GPI or annexin A5 to hexagonal II phase PE had no effect on the LA activity of the purified immunoglobulins,[57] indicating that the hexagonal II phase neutralization is specific for prothrombin-dependent LA. However, a study in which

correlation between solid phase aCL, anti-β2GPI, anti-prothrombin IgG and LA was investigated in 110 patients, reported that neutralization of LA with hexagonal II phase phospholipids also occurs when LA coincides with aCL or anti-β2GPI IgG in the same sample[58]. This study did not provide evidence that LA neutralized with hexagonal II phase PE was caused by either anti-prothrombin antibodies or anti-β2GPI antibodies.

The tools listed above provide a means to differentiate between β2GPI-dependent LA and LA caused by other aPL. Unfortunately, they have not been used in studies other than the ones already mentioned. Instead, it is now common practice to consider a LA as being β2GPI-dependent when the patient also has anti-β2GPI or aCL antibodies. This is an attractive assumption, because evidence that patients with a "triple positive" antibody profile, i.e. anti-β2GPI antibodies, aCL and LA, have a higher risk of thrombosis than patients with isolated LA positivity is accumulating[59-61]. However, we should always keep in mind that LA can be caused by antibodies other than those against β2GPI. In fact, a large proportion of patients with LA do not have concomitant anti-β2GPI or anti-prothrombin antibodies[62]. Data obtained from large population-based case-control studies and prospective cohort studies amongst aPL carriers support the prothrombotic properties of isolated LA[3,17,34,61]. Prospective data indicate that thrombotic complications occur 3- to 4-fold more often in these patients than is expected in people of the same age[3,61].

The lupus anticoagulant paradox

Before we can provide tailored treatment or primary prophylaxis to patients with LA, we need to understand the thrombotic risk associated with LA. To do this, we first need to understand the mechanism behind the LA phenomenon. It is clear that patients with LA do not have a bleeding tendency, indicating that LA is an *in vitro* laboratory phenomenon and does not represent the actual haemostatic response of the patient. The current consensus is that aPL with LA activity compete with coagulation factors for binding sites on anionic phospholipids, thereby preventing formation of the enzymatic complexes that drive coagulation (figure 1). Indeed, both anti-prothrombin antibodies and anti-β2GPI antibodies have been shown to enhance the avidity of the interaction between these proteins and the phospholipid surface[46,63-66]. However, competition with coagulation factors has only been shown for anti-prothrombin antibodies[67].

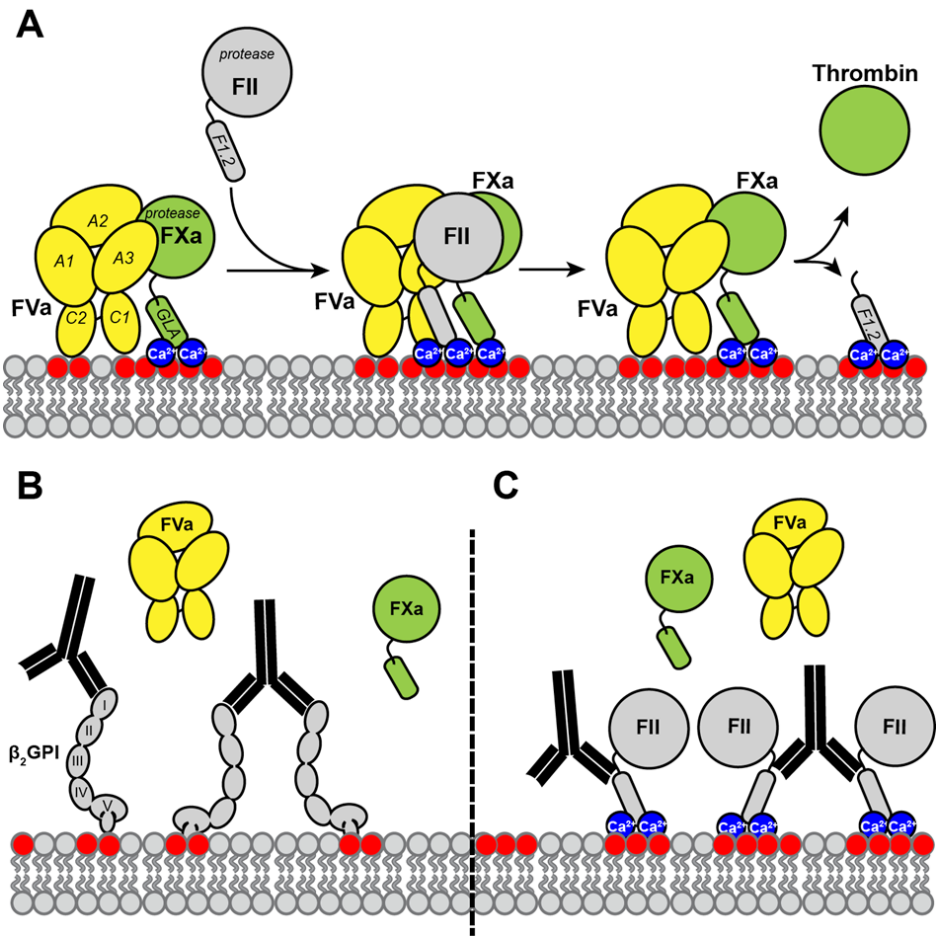

Figure 1. **The lupus anticoagulant phenomenon is caused by competition with coagulation factors for binding sites on anionic phospholipids. A**. The prothrombinase complex, consisting of FVa and FXa, assembles on PS-containing (red spheres) membranes and cleaves its substrate, prothrombin (FII), resulting in thrombin formation and its separation from the phospholipid binding domain Fragment (F)1.2. FVa interacts with PS through its C-domains, which does not require Ca^{2+} (blue spheres). Both FXa and prothrombin contain GLA-residues, which mediate the Ca^{2+}-dependent interaction with PS. **B**. Anti-β2GPI antibodies are thought to saturate PS-containing membranes, competing with coagulation factors for binding sites. However, β2GPI has a low affinity for PS in the presence of Ca^{2+}-ions. **C**. Anti-prothrombin antibodies have been shown to enhance the interaction of prothrombin with PS through an increase in avidity. This Ca^{2+}-dependent accumulation of prothrombin will lead to saturation of PS-containing membranes, thereby limiting formation of the enzymatic complexes that drive coagulation.

How competition between coagulation factors and aPL for binding sites on phospholipids would lead to thrombosis is difficult to understand. The enhanced binding of prothrombin to phospholipid surfaces through the formation of bivalent prothrombin-immune complexes has been proposed to lead to a prothrombotic state by enhancing thrombin formation under flow conditions: due to lower dissociation rates from available phospholipid surfaces, the substrate for the prothrombinase complex accumulates at the site where it is needed[64]. Although this is an attractive hypothesis, it seems unlikely, because the prothrombin concentration is not rate limiting during thrombin formation: the plasma concentration of prothrombin far exceeds the amount of prothrombinase that is likely to be present in a wound area, and blood flow ensures a constant supply of fresh prothrombin.

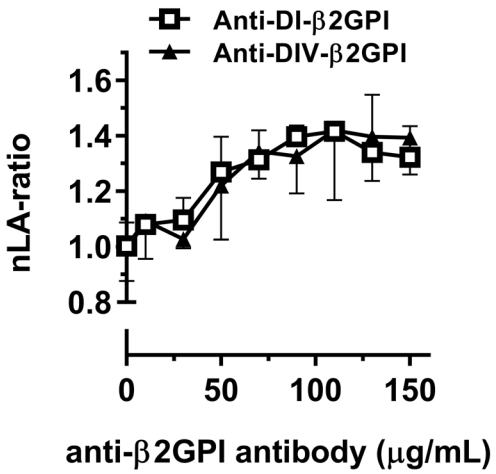

Figure 2. **Inhibitory effect of anti-β2-glycoprotein I antibodies on coagulation.** Pooled normal plasma from 200 healthy donors was spiked with monoclonal anti-β2GPI antibodies 3B7, directed against DI, or 21B2, directed against DIV. LA activity was determined with dRVVT reagents (Stago, Asnières-sur-Seine, France) and expressed as the normalized LA-ratio (nLA-ratio), which was calculated as (Screen/Screen$_{normal}$)/(Confirm/confirm$_{normal}$).

Competition with coagulation factors is not a likely scenario for LA induced by anti-β2GPI antibodies for two main reasons: first, the affinity of β2GPI for PS-containing membranes is low, especially in the presence of Ca^{2+} ions, which are essential for coagulation factor binding to phospholipid membranes[46]. Second, the effect of purified anti-β2GPI antibodies on coagulation is not as strong as would be expected for competitors of phospholipid binding: the inhibitory effect seems to saturate at antibody concentrations at which just a fraction of the β2GPI is bound (figure 2),[68] while one would expect the inhibitory effect to become stronger until all β2GPI is involved in this interaction.

These observations on the limited effects of anti-β2GPI antibodies on coagulation suggest a direct interaction between β2GPI and coagulation factors. As early as 1963, it was suggested that antibodies with LA activity prevent activation of FV by FXa[69]. Our group has confirmed these observations: we found that antibody-β2GPI complexes directly interact with FV, which prevents efficient activation of FV by FXa in the formation of the prothrombinase complex[70]. How the inhibition of FV activation would be associated with an increased risk of thrombosis remains to be determined. Factor V is an important player in thrombosis and haemostasis with both procoagulant properties as the cofactor for FXa in the prothrombinase complex, and anticoagulant properties as the binding site for tissue factor pathway inhibitor (TFPI)-α[71] and, together with protein S, as the cofactor for activated protein C during the inactivation of FVIIIa[72]. Further investigations should indicate whether the interaction between FV and β2GPI-immune complexes interferes with the anticoagulant properties of FV. Interestingly, β2GPI-dependent LA has been linked with activated protein C (APC) resistance[73] and anti-β2GPI antibodies have been shown to suppress the anticoagulant activity of TFPIα[74].

Conclusions

More than 60 years after the first description of the LA phenomenon, we are still trying to understand the mechanism behind this peculiar coagulation inhibitor. Since the first description of LA, we now know this laboratory phenomenon to counterintuitively align as a strong risk factor for thrombosis and pregnancy morbidity. Which antibody populations are responsible for clinically important LA remains to be determined. There is some evidence that LA caused by anti-β2GPI antibodies is clinically relevant, but the irrelevance of β2GPI-independent LA has not been proven. In fact, carriers of isolated LA still have a significantly higher risk of a thrombotic event than non-carriers. We should therefore be cautious to dismiss this LA variant as irrelevant. The importance of the combination of LA and antibodies against β2GPI is clear: carriers of both LA and anti-β2GPI antibodies have a higher risk for a first thrombotic event than patients with isolated LA. Whether

this is also true for the risk of recurrent cardiovascular disease, however, is unclear. It is important to bridge this knowledge gap, because the majority of patients with LA are identified after the thrombotic event has taken place. Future clinical studies should focus on the risk of recurrence in APS patients. Perhaps that unravelling of the mechanism responsible for LA will increase our ability to identify patients at risk for future disease.

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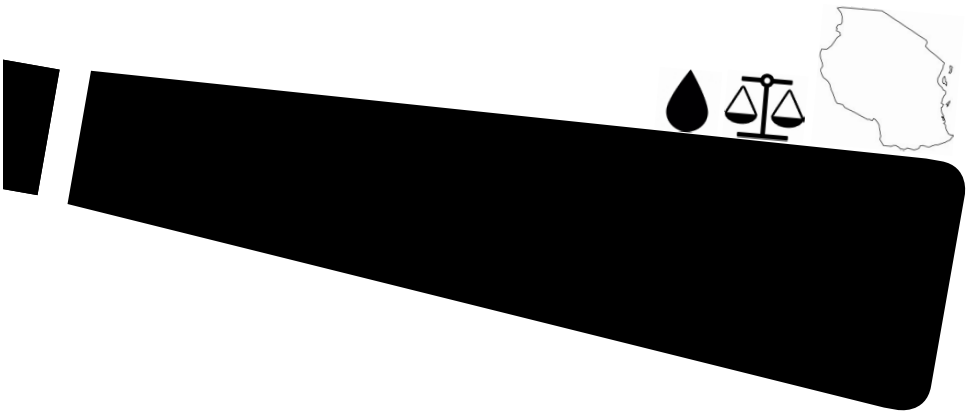
Antiphospholipid antibodies and the risk of stroke in urban and rural Tanzania: a community-based case-control study



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Abstract

Background and Purpose: The burden of stroke is high in sub-Saharan Africa (SSA) and improved knowledge of risk factors is needed. Antiphospholipid antibodies (aPL) are a common acquired stroke risk factor in young individuals. aPL may be induced by infectious diseases. SSA has a high infectious burden and we analyzed the contribution of aPL to the risk of stroke in an incident population from rural and urban Tanzania.

Methods: Stroke cases and age- and sex-matched community-acquired controls from the rural Hai district and urban Dar-es-Salaam areas of Tanzania were recruited in a wider study of stroke incidence between June 2003 and June 2006. Lupus anticoagulant (LA), anticardiolipin (aCL), anti-β2-glycoprotein I (anti-β2GPI), and anti-phosphatidylserine/prothrombin (aPS/PT) antibodies were determined in stored plasma, as well as IgG antibodies against *Treponema pallidum* (TP-IgG).

Results: Data from 158 stroke cases and 369 controls were analyzed. Thirty (19%) cases and four (1%) controls had a LA (Odds Ratio (OR) 20.8; 95% confidence interval (CI) 7.2 – 60.5). aCL IgG was the only other aPL subtype associated with increased stroke risk (OR 2.1; 95% CI 1.0 – 4.3), but this association disappeared when corrected for TP-IgG results. The prevalence of anti-β2GPI IgG antibodies in the Tanzanian healthy population was very high when Dutch cut-off values were applied (67%). The presence of anti-β2GPI IgM was associated with a reduced stroke risk (OR 0.3; 0.1 – 1.1).

Conclusions: The presence of LA is a strong, and so far unrecognized, risk factor for stroke in Tanzania, especially in young and middle-aged individuals.

Introduction

The burden of stroke is increasing in many low- and middle income countries[1]. In Tanzania, age-standardized stroke incidence rates in a rural population (Hai district) were similar to those seen in urban-dwelling African Americans, whereas incidence rates from urban Dar-es-Salaam were higher than seen in most other settings[2]. A strategy for stroke prevention is urgently needed. This requires knowledge of stroke risk factors, but data on risk factors relevant for sub-Saharan Africa (SSA) are scarce. Recent findings from the international, multicenter, INTERSTROKE study, which included four SSA countries, and a prospective study in Tanzania confirmed the importance of well-established stroke risk factors such as high blood pressure (BP), diabetes, dyslipidemia, smoking and overweight[3,4]. Nonetheless, other unique risk factors may apply to populations in SSA and contribute to the disproportionate burden of stroke. An example is HIV infection, which was recently identified as an important stroke risk factor in Tanzania by our group[4].

Antiphospholipid antibodies (aPL) are a common acquired risk factor for stroke. These antibodies comprise a heterogeneous group of antibodies that react with phospholipids or phospholipid-binding proteins of which β2-glycoprotein-I (β2GPI) is considered the main antigenic target. The presence of aPL is demonstrated either directly with enzyme-linked immunosorbent assays (anticardiolipin antibodies (aCL), or anti-β2GPI antibodies) or functionally by showing the ability of aPL to prolong *in vitro* coagulation times (lupus anticoagulant; LA). The importance of aPL, and especially LA as risk factors for stroke is increasingly recognized, particularly in young individuals. For example, a recent systematic review found an aPL prevalence of 17.4% in patients with a cerebrovascular event below the age of 50 with presence of aPL conferring a fivefold higher risk for stroke[5].

Induction of aPL may be triggered by infectious diseases[6]. SSA has a high infectious burden and we hypothesized that aPL are a common risk factor for stroke in this region. We therefore investigated whether aPL were independently associated with the risk for stroke in a rural and urban population in Tanzania.

Methods

Study design and participants

For the current study, stored citrate-anticoagulated blood samples of participants of the Tanzania Stroke Incidence Project (TSIP) were used. This study recorded stroke incidence in two well defined demographic surveillance sites in Tanzania over a 3-year period from June, 2003. These sites were the Hai district in northern Tanzania, a rural area where agriculture is the primary economic activity, and urban Dar-es-Salaam, the largest city in Tanzania. Key results, including details on study design, procedures and characteristics of study areas and participants have been

previously published[2, 4]. In short, patients with stroke were identified within the community and at health facilities using a system of community-based investigators and liaisons with local hospital and medical center staff[2]. Patients with first-ever and recurrent stroke, who fulfilled the standard World Health Organization (WHO) definition of stroke criteria were eligible for enrolment. Controls who were matched to cases for age (± 3 years) and sex were recruited from the background census population of the Hai and Dar-es-Salaam demographic surveillance sites.
Ethics approval for the TSIP study was obtained from the National Institute of Medical Research, Dar-es-Salaam, and from the Newcastle and North Tyneside Joint Ethics Committee, UK. Written informed consent was provided by each participant, or by a close relative when participants were unable to provide consent.

Measurement of established stroke risk factors
Independent significant risk factors for stroke have been identified in this population previously[4]. All cases and controls were interviewed by members of the study team using the same forms with the exception of sections relating to the stroke itself. Demographic information, social history, and past medical history were recorded and participants underwent a medical assessment and examination. Blood pressure (BP) was recorded at least seven days after stroke, to allow for elevation in BP immediately post-stroke. Three measurements were taken and the average of the second and third measurement was used. Hypertension was defined as a mean systolic or diastolic BP above 160 mm Hg and 90 mm Hg, respectively, a history of hypertension, or taking of antihypertensive drugs before stroke. Smoking habits were categorized into current smokers (smoked tobacco in the past 12 months) and former smokers (those who had smoked, but not in the past 12 months). The presence or absence of diabetes mellitus was based on self-report of a pre-stroke diagnosis by a physician. A lipid spectrum was measured at North Tyneside General Hospital with an automated biochemical analyzer. Dyslipidemia was defined as a ratio of total cholesterol to HDL cholesterol of 5.0 or more. A CT head scan was performed in stroke cases who survived long enough to undergo this[7]. Findings of ischaemia, haemorrhagic infarct, or no evidence of stroke were classified as ischaemic stroke.

Antiphospholipid antibodies
aCL IgG and IgM antibodies were measured with a commercially available kit (IBL international GmbH, Hamburg, Germany), according to the instructions of the manufacturer. Results are expressed in GPL for IgG and MPL for IgM. Antibodies against the complex of prothrombin and phosphatidylserine (IgG and IgM) were measured with the QUANTA Lite aPS/PT kit (Inova diagnostics, San Diego, CA, USA),

according to the instructions of the manufacturer. Results are expressed in AU/mL. Anti-β2GPI antibodies were measured with a home-made ELISA as previously described[8]. Cut-off was set at the 90th and 95th percentile of the value recorded for the Tanzanian control group.

The presence of LA in plasma samples was assessed with dilute Russell's Viper Venom Time (dRVVT) screen and confirm reagents (LA-screen and LA-confirm, Life Diagnostics; Clarkston, GA, USA), as described previously and in adherence to the guidelines for LA testing[8,9]. In short, plasma samples were mixed with an equal volume of pooled normal plasma to exclude coagulation factor deficiencies. Coagulation times were recorded on a MC-10 coagulometer (Merlin Medical, Lemgo, Germany). Mixed plasmas were added to cuvettes and allowed to equilibrate at 37°C for two minutes. Coagulation was initiated by addition of an equal volume of dRVVT reagent. When coagulation times obtained with LA-screen reagents were prolonged, i.e. exceeded the 99th percentile of normal as determined in samples from 120 healthy Dutch controls, coagulation tests were repeated with LA-confirm reagents. Normalized LA-ratios were subsequently calculated according to the following equation: (LA_{screen}(sample)/Mean LA_{screen}(normal)) / ($LA_{confirm}$(sample)/Mean $LA_{confirm}$(normal)). Samples were deemed LA-positive when the normalized LA-ratio exceeded the 99th percentile of normal as determined in the samples of the 120 healthy Dutch controls (normalized LA-ratio > 1.15).

High-sensitive C-reactive protein

Plasma concentrations of high-sensitive C-reactive protein (hs-CRP) were measured on a TECAN Freedom EVO robot (Tecan, Switzerland) with anti-CRP duoset antibodies from R&D systems (Abingdon, UK), as previously described[10].

Serology for Treponema pallidum

Treponema pallidum serology was assessed with a commercially available kit (*Treponema pallidum* IgG ELISA, IBL international GmbH, Hamburg, Germany) according to the instructions of the manufacturer. Samples were considered positive for anti-*Treponema pallidum* antibodies when the concentration of anti-*Treponema* IgG exceeded 11 units.

Statistical analysis

Data were analyzed with SPSS software (version 22.0; SPSS Inc, Chicago, IL, USA). Logistic regression was used to analyze LA and other aPL as stroke risk factors taking the control group as a reference. Odds ratios (OR) and 95% confidence intervals (95% CI) were adjusted for age (continuous), area (Hai or Dar-es-Salaam) and sex. Subsequently, OR for LA were also adjusted for the traditional risk factors

hypertension, smoking, diabetes mellitus and dyslipidemia. We used two-tailed tests throughout and a p-value < 0.05 signified a statistically significant difference.

Results

Data from 158 stroke cases (102 in Hai and 56 in Dar-es-Salaam) and 369 controls (223 in Hai and 146 Dar-es-Salaam) were included in this study. The median time between incident stroke and interview was 8 days (IQR 5 to 28 days) in Hai and 35 days (IQR 10 to 84 days) in Dar-es-Salaam. Table 1 shows baseline characteristics and established stroke risk factors, including their OR. As previously reported,[4] hypertension, diabetes mellitus, smoking, and a high ratio of total to HDL cholesterol were associated with stroke in univariate analysis. In addition, a positive result of anti-*Treponema pallidum* IgG antibodies (TP IgG), indicating current or past syphilis, was more common in cases (24.7%) than in controls (12.7%) and a positive TP-IgG was significantly associated with stroke risk (OR 2.8; 1.7 – 4.6). Cases from Hai also had significantly higher hs-CRP concentrations compared with controls from the same area. In contrast, hs-CRP concentrations in cases and controls from Dar-es-Salaam did not differ significantly.

Table 2 shows the results of the prevalence and OR of aPL corrected for age, gender and residence area. Given the lack of previous normative data from SSA on which to establish cut-offs for positive findings, samples were considered positive for aCL or anti-β2GPI antibodies when the concentration exceeded the 95th percentile of the values obtained in the Tanzanian controls. A positive LA was found in 30 (19%) of the cases and in four (1%) controls (OR 20.8; 7.2 – 60.5). The OR of LA was higher in stroke patients aged 65 years or below (49.0; 95% CI 6.4 –374.6) compared to those above 65 years (11.7; 95% CI 3.2 – 43.3) (table 3). LA had a trend for a higher prevalence in cases from Hai than in cases from Dar-es-Salaam (22.6% vs. 12.5%; P=0.12), but the ORs of a positive LA for stroke were similar in both areas (table 3). Further adjustment for the presence of traditional risk factors, such as diabetes, hypertension, dyslipidemia or smoking, did not affect the risk of stroke associated with LA (data not shown). A CT scan, performed within 15 days of stroke, was available in 15 LA positive stroke cases; 12 of them had an ischemic stroke and 3 a haemorrhagic stroke.

Of the other two aPL subpopulations, aCL and anti-β2GPI antibodies, only the presence of aCL IgG was associated with an increased risk for stroke (OR 2.1; 1.0 – 4.3). None of the patients had aCL IgG levels exceeding 40 GPL units. aCL antibodies are frequently found in patients with *treponemal* antibodies, and these infection-related aCL are not considered true aPL. In our study population, 12 of 33 (36%)

individuals with a positive aCL IgG also had a positive TP-IgG and the significant association of aCL IgG with stroke risk disappeared when the results were adjusted for results of TP-IgG (OR 1.7; 0.8 – 3.7). Five of 34 (14.7%) LA positive individuals had a positive TP-IgG and adjustment for TP-IgG result did not markedly change the OR for stroke of a positive LA. IgM aCL antibodies were not associated with an increased risk of stroke. Although the presence of IgG anti-β2GPI antibodies had no effect on the risk of stroke, the presence of anti-β2GPI IgM was associated with a strongly reduced risk for stroke (OR 0.3; 0.1 – 1.1).
This inverse relationship was even stronger when the 90th percentile cut-off value was used (OR 0.2; 0.0 – 0.6). Interestingly, when the Dutch diagnostic cut-off value for anti-β2GPI antibodies (determined as the 95th percentile in 40 healthy Dutch adult controls) was applied, 67% of the Tanzanian study population had an increased anti-β2GPI IgG titer, a prevalence that was similar for cases and controls from both areas.

The simultaneous presence of LA and anti-β2GPI or aCL antibodies was uncommon. Only one out of 30 patients with LA also had anti-β2GPI antibodies, and three out of 30 patients with LA had aCL antibodies. None of the controls had both LA and antibodies against β2GPI or aCL. LA has been reported to arise due to antibodies against β2GPI or prothrombin[11,12]. Given the rarity of anti-β2GPI in LA positive individuals in our cohort, we measured the prevalence of antibodies against the complex of prothrombin and the phospholipid phosphatidylserine (aPS/PT antibodies). Only three out of 30 patients with LA had aPS/PT antibodies. There was also no significant association between presence of these antibodies and stroke with 8% of cases and 5% of controls having a positive aPS/PT IgG (OR 1.9; 0.9 – 4.0) and 6% of cases and 5% of controls being positive for aPS/PT IgM (OR 1.3; 0.6 – 1.8). None of cases or controls were positive for all three antiphospholipid antibody subtypes. The presence of either anti-β2GPI antibodies, aPS/PT, or aCL antibodies next to LA did not increase the risk of stroke compared with the risk of only LA (data not shown).
LA positivity has been associated with increased concentrations of inflammatory markers[13, 14]. In the Hai population, the 23 LA positive cases had a significantly higher median hs-CRP concentration (9.3 µg/mL; IQR 3.8 - 25.2 µg/mL) than the 79 LA negative cases (5.1 µg/mL; 1.4 - 12.6 µg/mL; P=0.03). In contrast, median hs-CRP between the 7 LA-positive and 49 LA-negative cases in Dar-es-Salaam area were similar (3.0 µg/mL; IQR 2.2 - 45.6 µg/mL vs. 2.8 µg/mL; IQR 0.7 - 9.7 µg/mL; p=0.4).

Table 1. **Baseline characteristics.**

	Hai		Dar-es-Salaam		Combined		ORs (95% CI)
	cases	controls	cases	controls	cases	controls	
Number, n	102	223	56	146	158	369	
Age, years, mean (SD)	66 (15)	70 (15)	61 (13)	61 (13)	65 (15)	66 (15)	
Missing	0	0	8	17			
Male, n (%)	55 (54)	109 (49)	27 (55)	70 (53)	82 (54)	179 (51)	
Missing	0	0	7	15			
CT result, n							
Ischemic	42	NA	14	NA	56	NA	
hemorrhagic	6	NA	3	NA	9	NA	
Hs-CRP, µg/mL, median (IQR)	6.0 (2.0–14.6)	0.9 (0.3–2.3)*	2.9 (0.8–0.6)	1.6 (0.6–4.1)	4.6 (1.6–12.7)	1.1 (0.3–3.5)	
Hypertension, n (%)							1.8 (1.2 – 2.8)
Yes	52/90 (58)	101/223 (45)	30/47 (64)	58/127 (46)	82/137 (60)	159/350 (45)	
No	38/90 (42)	122/223 (55)	17/47 (36)	69/127 (54)	55/137	191/350	
Missing	12	0	9	19	21	19	
Smoking status, n (%)							Non vs current or former 3.4 (2.2 – 5.5)
Current smoker	10/98 (10)	62/206 (30)	13/34 (38)	15/111 (13)	23/132 (17)	77/317 (24)	
Former smoker	67/98 (68)	63/206 (31)	11/34 (32)	14/111 (13)	78/132 (59)	77/317 (24)	
Non-smoker	21/98 (21)	81/206 (39)	10/34 (29)	82/111 (74)	31/132 (23)	163/317 (51)	
Missing	4	17	22	35	26	52	
Diabetes, n (%)							2.5 (1.01–6.5)
Yes	5/100 (5)	5/223 (2)	5/48 (10)	5/127 (4)	10/148 (7)	10/350 (3)	
No	95/100 (95)	218/223 (98)	43/48 (90)	122/127 (96)	138/148 (93)	340/350	
Missing	2	0	8	19	10	19	
Total/HDL chol >5.0, n (%)							3.2 (2.1 – 5.0)
Yes	57/86 (66)	56/150 (37)	39/55 (71)	45/104 (43)	96/141 (68)	101/254 (40)	
No	29/86 (44)	94/150 (63)	16/55 (29)	59/104 (57)	45/141 (32)	153/254 (60)	
Missing	16	73	1	42	17	115	
TP-IgG							2.8 (1.6 – 4.6)
Positive	22/102 (22)	20/223 (9)	17/56 (30)	27/146 (18)	39/158 (25)	47/369 (13)	
Negative	80/102 (78)	203/223 (91)	39/56 (70)	119/146 (82)	119/158 (75)	322/369 (87)	

Table 1. Data are number with percentage, mean with standard deviation (SD) or median with interquartile range (IQR). Hs-CRP, high sensitive C-reactive protein; TP-IgG, *Treponema pallidum* IgG assay. *p<0.001 versus cases.

Discussion

The current findings from a large community-based, case-control study with prospective case ascertainment identifies LA as an important and independent stroke risk factor in this setting. The incident cases of stroke in this study should be broadly representative of people with stroke in Tanzania who survive long enough to give a blood sample[2,4]. Overall, 19% of the stroke cases had a positive LA compared with 1% in controls. The association of LA with stroke was highest in individuals ≤65 years. Most of the cases had an isolated LA positivity, i.e. they did not have concurrent aCL, anti-β2GPI IgG or aPS/PT antibodies and the latter three antibodies were not associated with an increased stroke risk. In contrast, presence of anti-β2GPI IgM was associated with protection against stroke. High anti-β2GPI IgG titers were common in both cases and controls in this study population. When cut-off values of a Dutch control population were applied instead of the Tanzanian controls, 67% of the entire study population would be classified as being positive for anti-β2GPI IgG.

Our current finding of a strong association of LA positivity and stroke risk is consistent with previous studies showing a high prevalence of aPL in patients with stroke and especially in young adults[8,15,16]. In a systematic review conducted by Antiphospholipid Syndrome Alliance For Clinical Trials and International Networking (APS ACTION), the estimated frequency of aPL in stroke patients of all ages was 13.5%[17]. Another recent systematic review calculated a 17.4% prevalence of aPL in patients with cerebrovascular events below the age of 50 years[5]. Most LA positive individuals in our current study had an isolated LA and this proportion was higher than reported in other studies. For example, Fabris *et al.* found that 32% of 41 LA positive individuals had concurrent aCL and/or anti-β2GPI and 56% concurrent aPS/PT[18]. LA comprises a heterogeneous group of antibodies reacting with phospholipids and the exact nature of the inhibitors responsible for the isolated LA in the participants in our study remains unknown. Regarding the pathogenic nature of an isolated LA activity, previous findings by our group as well as our current findings lend further support that LA assays are by far superior in detecting pathologic subpopulations of aPL antibodies.

Table 2. **Distribution of antiphospholipid antibodies and odds ratios for stroke.**

	Cases (n=158)	Controls (n=369)	OR (95% CI)
Lupus anticoagulant (LA-ratio$_{s/c}$ ≥1.15)	30	4	20.8 (7.2 – 60.5)
Anti-β2GPI IgG, P90	18	37	1.2 (0.6 – 2.1)
Anti-β2GPI IgG, P95	4	18	0.5 (0.2 – 1.6)
Anti-β2GPI IgM, P90	3	38	0.2 (0.0 – 0.6)
Anti-β2GPI IgM, P95	2	18	0.3 (0.1 – 1.1)
Anticardiolipin IgG, P90	21	38	1.5 (0.8 – 2.6)
Anticardiolipin IgG, P95	15	18	2.1 (1.0 – 4.3)
Anticardiolipin IgM, P90	37	37	2.9 (1.6 – 5.2)
Anticardiolipin IgM, P95	9	18	1.4 (0.6 – 3.3)

Data are number of participants or odds ratio (OR) with 95% confidence interval. ORs are corrected for age, sex and residence area. LA-ratios/c = normalized ratios between LA-screen and LA-confirm coagulation times. Cut-off anti-β2GPI and anti-cardiolipin antibodies at 90th percentile (P90) or the 95th percentile (P95) of values in the control group.

Table 3. **Odds ratio for lupus anticoagulant (LA) by age and area.**

	Cases	Controls	OR (95% CI)
Age*			
65 years, LA positive, n (%)	19/76 (25%)	1/148 (0.7%)	49.0 (6.4 – 374.6)
>65 years, LA positive, n (%)	11/74 (15%)	3/204 (1.5%)	11.7 (3.2 - 43.3)
Area			
Dar-es-Salaam, LA positive, n (%)	7/56 (13%)	1/146 (0.7%)	20.7 (2.5 – 172.6)
Hai, LA positive, n (%)	23/102 (23%)	3/223 (1.3%)	21.4 (6.2 – 73.1)

*Age missing in 8 cases and 17 controls. OR, odds ratio.

In women presenting with a first stroke under the age of 50 years, we previously showed that a positive LA was much more strongly associated with stroke (OR 43.1; 95% CI 12.2 - 152.0) compared with anti-β2GPI (OR 2.3; 95% CI 1.4 – 3.7), whereas no association was found for aCL and anti-prothrombin antibodies[8]. Most of these women had an isolated LA. A recent prospective observational study in 151 LA positive individuals also found LA positivity to be associated with increased mortality, independent of concomitant positivity for aCL or anti-β2GPI[19]. In contrast,

Pengo *et al*. recently reported that the risk of thrombo-embolic events was low in a prospective cohort of individuals with an isolated LA, suggesting that the risk associated with an isolated LA is dependent on the population studied[20].

Nearly all studies on aPL antibodies and stroke risk have been performed in high-income countries. To the best of our knowledge, there are no studies from SSA. Our current study suggests that LA may be one of the leading risk factors for stroke in SSA next to traditional stroke risk factors such as diabetes mellitus, smoking, hypertension and dyslipidemia. We also found a positive TP-IgG to be common among stroke cases (24.7%) and to be significantly associated with stroke risk. TP-IgG was determined to correct for the fact that positive aCL antibodies are frequently found in syphilis and these antibodies do not differentiate between past or current syphilis. Syphilis and especially meningovascular neurosyphilis is a well-recognized cause of stroke in young patients[21]. Our results should be interpreted with caution, because no additional diagnostic tests for (neuro)syphilis were performed, including non-treponemal tests or CSF examination. Still, our data suggest that syphilis testing should be considered part of the work-up of stroke in SSA.

Apart from syphilis, LA and other aPL subpopulations may arise in the course of other infections,[22] including those common in SSA such as HIV,[23] mycobacterial infections, syphilis,[24] malaria,[25] and viral hepatitis[26]. The observation that the prevalence of LA was higher in cases from rural Hai region may be explained by a higher infectious burden in this area, whereas traditional stroke risk factors may be more important in urban Dar-es-Salaam. A systematic screening for infections, including tuberculosis and viral hepatitis, was beyond the scope of this study. Screening for HIV was performed in this cohort previously and HIV infection turned out to be a major stroke risk factor with a prevalence of 24.5% in cases and 6.5% in controls[4]. All participants were naïve for antiretroviral therapy. Because HIV testing was performed anonymously, results could not be linked to results of aPL antibodies. Infection-associated aPL usually interact solely with phospholipids such as cardiolipin and have no anti-β2GPI activity[27]. They are usually not associated with thrombosis or other pathology. An ever increasing number of infectious diseases has also been related with the presence of anti-β2GPI antibodies. As recently reviewed,[6] many healthy individuals have low circulating titers of low-affinity anti-β2GPI antibodies. These antibodies may belong to the natural antibody repertoire and play a role in the removal of apoptotic bodies and host defense against infections. Infections are able to induce low-titer and low-affinity natural antibodies to full blown auto-antibodies[28] and even though the precise mechanisms responsible for the transition from natural-occurring to pathologic auto-antibodies is currently unknown, it is conceivable that this may occur at a higher rate in patients with chronic or

recurrent infections. In this respect, our finding that 67% of the study population had increased anti-β2GPI IgG titers when Dutch cut-off values were applied is of particular interest. Genetic factors may play a role in this high prevalence, as ethnic differences in reference values of different thrombophilia markers, including aPL, have been described earlier[29]. It is however tempting to speculate that the high burden of acute and chronic infections, including malaria, may also account for the high anti-β2GPI IgG titers in this Tanzanian population.

We cannot exclude with certainty that stroke itself may induce LA and other aPL. Stroke is associated with inflammation and earlier studies that evaluated the time course of CRP have found an increase in CRP after acute stroke[30]. This might explain why the Hai cases, in whom blood was collected earlier after stroke, had a higher hs-CRP level than the Dar-es-Salaam cases. Nonetheless, LA-positive cases also had a higher hs-CRP level compared with the LA-negative cases. Elevated levels of CRP may interfere with LA testing, but this does not occur with use of the dRVVT system, as was used in our study[31]. LA itself may also lead to inflammation through activation of the endothelium and upregulation of the expression of tissue factor and pro-inflammatory cytokines[32]. This is supported by previous studies reporting an association of isolated LA with increased levels of inflammatory markers[13,14].

Our study also reported for the first time a protective effect of anti-β2GPI IgM in stroke and this fits the same concept of naturally occurring autoantibodies having a protective effect. In line with this concept is the observation that presence of anti-β2GPI IgM protected against lupus nephritis in a large cohort of patients with systemic lupus erythematosus[33].

Particular strengths of our study are its prospective design, the fact that both functional (LA) and serologic assays (anti-β2GPI, aCL and aPS/PT) were performed and the availability of adequate control groups, which is essential for calculation of risk estimations. However, different limitations should also be acknowledged. First, blood samples for the current analyses were only available in 158 out of 200 stroke cases and 369 out of 398 controls from the originally described cohort[4]. Nonetheless, we have no reason to believe that this resulted in systematic bias. Second, the low number of LA positive controls hampered adjustment for multiple traditional stroke risk factors in one model. Adjustment of single risk factors had no effect on the risk ratio, which is not surprising as diabetes mellitus, smoking, dyslipidemia and hypertension are not expected to influence the risk for aPL. Third, we were not able to differentiate between past and active syphilis because only a TP-IgG was available. Whether participants had been treated for syphilis was not specifically asked but none self-reported that they had. Fourth, only a single sample was available and LA positivity could therefore not be confirmed after 12

weeks in a second sample as suggested in the International Society on Thrombosis and Haemostasis guidelines[9]. This also impedes classifying the proportion of participants having true antiphospholipid syndrome, as this diagnosis requires the persistent presence of antiphospholipid antibodies together with clinical criteria of vascular occlusion or pregnancy morbidity[34]. Lastly, our study does not allow us to draw definite conclusions on the contribution of aPL in stroke pathogenesis. This would require very large, prospective cohort studies which will be difficult to execute in these areas. A wealth of evidence from basic, animal and clinical studies has highlighted the prothrombotic effects of aPL and in our opinion, this makes it unlikely that aPL would merely be a 'bystander' in our stroke cohort.

Summary/Conclusions

This study identified LA as a major stroke risk factor in SSA. The pathogenic role of aPL in stroke in this area as well as contributing factors to the high aPL prevalence in SSA deserve further study.

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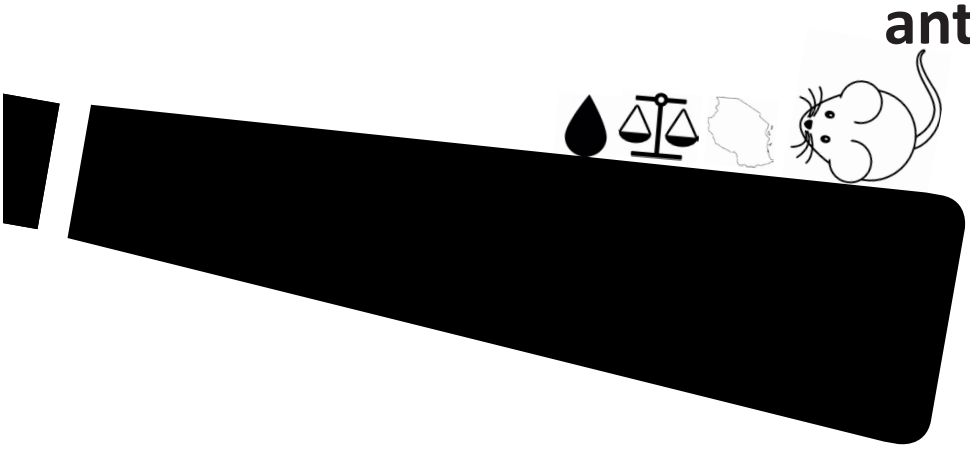
4

Streptococcus pyogenes protein H can induce a prothrombotic phenotype in mice via induction of anti-beta2-glycoprotein I antibodies



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Manuscript in preparation

The antiphospholipid syndrome (APS) is characterized by the presence of antiphospholipid antibodies in plasma of patients with thrombotic complications or pregnancy morbidity[1]. The name APS is a misnomer because the auto-antibodies are not directed against anionic phospholipids but against plasma proteins with affinity for anionic phospholipids[2]. Of the different proteins identified as target for the autoantibodies, β2-glycoprotein I (β2GPI) has been shown the most important antigen. Indeed, an epitope has been identified within domain I of β2GPI that correlates strongly with the clinical manifestations that characterize APS[3]. Animal models for APS have shown that auto-antibodies specific for this epitope in domain I are highly thrombogenic[4,5].

Many publications have linked infectious agents as cause of the induction of autoantibodies against β2GPI[6]. Different infections are accompanied by the temporary appearance of anti- β2GPI antibodies in the circulation. In a recent study we have immunised mice with different surface proteins of *Streptococcus pyogenes*. After a few boosters with surface protein H, mice start to produce auto-antibodies against domain I of murine β2GPI. This effect was specific for protein H of *Streptococcus pyogenes* and only auto-antibodies against domain I were induced, not towards other domains of β2GPI[7]. It has been shown that binding of protein H to β2GPI resulted in a conformational change in β2GPI. As a consequence of this conformational change a cryptic epitope becomes exposed within domain I. The auto-antibodies induced by immunisation with protein H were directed against this newly exposed epitope. The question remains whether these anti-domain I β2GPI antibodies induced by immunisation with protein H are thrombogenic. To answer this question we have immunized mice again with purified protein H and M1 protein and tested the thrombogenicity in these mice with a ferric chloride thrombosis model.

A mice thrombosis model was performed as described before[8]. In short: female BALB/c mice (Charles River Laboratories, the Netherlands, 4 weeks old, 10-15 grams) received standard diet and water was provided *ad libitum*. All animal experiments were approved by the Ethical Committee on Animal Experimentation of the University Medical Centre Utrecht (Utrecht, the Netherlands). 14 BALB/c mice were injected every 4 weeks with 0.2 mL of either 25 µg protein H or protein M1 of *Streptococcus pyogenes*, respectively, in the absence of any adjuvant. Protein H and M1 protein were purified as described earlier[7]. Blood was drawn in 3.2% citrate via the submandibular veins prior to the first boost, each mouse was boosted 6 times. Two weeks after the last boost the mice were weighted and anesthetized via an intraperitoneal injection with max. 0.1 mL Ketamine/Xylasine/NaCl (1:1:8; 90 mg/kg Ketamine, 4.5 mg/kg Xylasine). During the operation pain medication

was given with one subcutaneous injection containing 0.15 mg/kg Buprenorphine. The common carotid artery was exposed and vascular injury was generated via a Whatmann filter paper (1 mm^2) soaked with 5% $FeCl_3$ and applied on the carotid artery for 3 minutes. Time to occlusion was measured with a Doppler flow probe placed superior to the injury for 30 minutes. After 30 minutes a heart puncture was performed to obtain blood for experiments. Blood was centrifuged twice at 2000xg to obtain platelet poor plasma and the presence of autoantibodies was measured as described before. Analysis of the Doppler flow probe signal was performed in Labchart.

The 7 mice immunized with protein H had significant higher levels of autoantibodies against domain I of β2GPI compared to the 7 mice immunized with M1 protein (figure 1A) Immunization with protein H resulted in 5 of the 7 mice in a completely occluded artery while only 2 of the 7 mice immunized with M1 protein had a completely occluded artery (figure 1B). The level of anti-domain I antibodies weakly correlated with the time to occlusion (R^2 = 0.54).

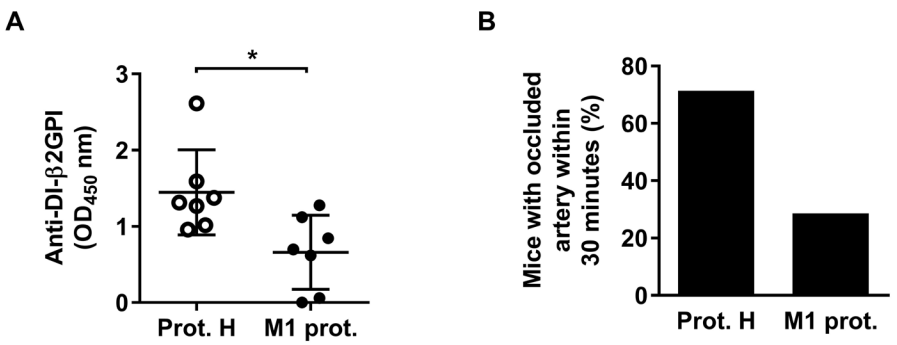

Figure 1. **Protein H of *Streptococcus pyogenes* induces anti-domain I β2GPI antibodies and more occluded arteries**. **A.** Mice were boostered 6 times with protein H or M1 protein and after the last booster the level of antibodies against domain I of β2GPI was measured **B.** Mice immunized with protein H or M1 protein were exposed to $FeCl_3$ thrombosis model and the number of occluded arteries were determined after 30 minutes. DI = domain I of β2GPI. Asterisk indicates a statistically significant difference ($p<0.05$).

This small study shows that the auto-antibodies against domain I of β2GPI that are induced after boostering with protein H of *Streptococcus pyogenes* have the potential to increase the thrombogenecity in mice. However, this association needs further investigations. We do not know whether the presence of these auto-antibodies are temporary autoantibodies or that they will persist over a longer period. The numbers in this study were too small to measure a relation between the titer of the antibodies and the thrombotic risk and we do not have any information on the presence of these induced antibodies and pregnancy complications. However, when this model is fully developed it could be an ideal model to test new therapies to treat the thrombotic risk in APS.

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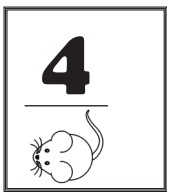
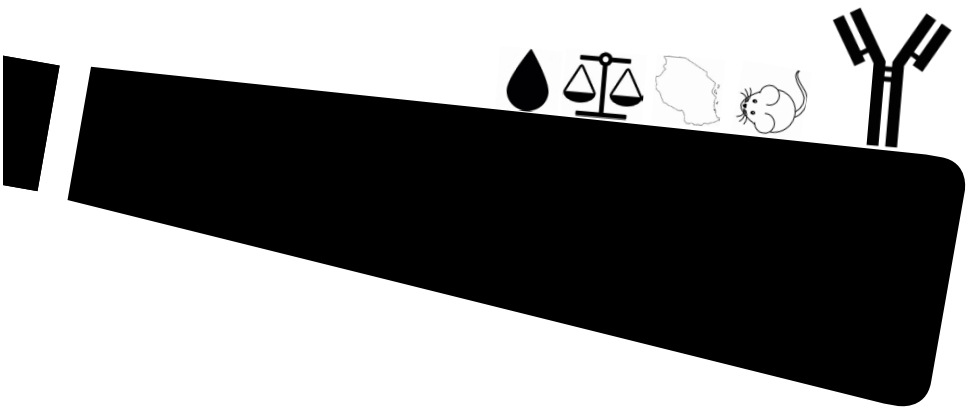


A cryptic epitope within domain V of beta2-glycoprotein I



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Manuscript in preparation

The antiphospholipid syndrome (APS) is characterized by the presence of antiphospholipid antibodies in plasma of patients with thrombotic complications or pregnancy morbidity[1]. The pathological antiphospholipid antibodies are not directed against anionic phospholipids themselves, but against plasma proteins with affinity for anionic phospholipids. It is now generally accepted that β2-glycoprotein I (β2GPI) is the principal target of the autoantibodies in APS[2]. β2GPI is a highly conserved protein in mammals. However, its physiological function is not completely clear[3]. It consists of 326 amino acid residues grouped in 5 complement control protein domains. Many studies using different animal models of APS have shown that auto-antibodies against the first domain of β2GPI are strongly prothrombotic[4,5]. β2GPI can exist in different conformations[6]. β2GPI circulates predominantly in a circular conformation in plasma. After binding to phospholipids via its V^{th} domain, β2GPI opens up and forms an elongated chain with its first auto-antibody binding domain pointing away from the surface. In the absence of an anionic surface, the antibody binding epitope within domain I is shielded from recognition. As a consequence, no immune complexes have been detected in patients with circulating antiphospholipid antibodies[7].

Analysis of the circular conformation of β2GPI showed an interaction between domains I and V[6]. As a result of the interaction with domain V, the epitope on domain I recognized by the autoantibodies that characterize the APS is cryptic. However, as domain I interacts with domain V, there should also be a cryptic epitope within domain V. We questioned whether this cryptic epitope in domain V also harbours a specific function. To investigate this, we decided to produce an antibody against domain V that only recognizes β2GPI when it is in its open conformation. Camelids, including *llamas*, produce heavy-chain only antibodies next to conventional immunoglobulins that consist of both heavy and light chain. These heavy chain only antibodies have fewer complementarity-determining regions than conventional antibodies, but are able to interact with high affinity with their targets. Moreover, they often recognize conformation-specific epitopes. For this reason, we immunized two *llamas* subcutaneously with purified β2GPI. All animal experiments were approved by the Ethical Committee on Animal Experimentation of the University Medical Centre Utrecht (Utrecht, the Netherlands). A B-cell bacteriophage library containing the heavy chain-only antibody repertoire of the immunized *llamas* was prepared as described previously[8]. Phages were produced from this library in *Escherichia coli* (TG1) and isolated via polyethylene glycol (PEG; Mr=6000) precipitation in 20% PEG with 2.5M NaCl. Phages were selected against domain V that was immobilized on a NUNC maxisorp flat bottom plate in a concentration of 5 µg/mL in PBS, in the presence of 200 µg/mL plasma-derived β2GPI in solution to prevent all phages from binding that react to β2GPI in its circular conformation. After extensive washing,

domain V-specific phages were eluted with 0.1M triethylamine (TEA; Sigma) after which pH was neutralized with 1M Tris, pH 7.5. *Escherichia coli* (TG1) were infected with eluted phages and plated on Yeast Tryptone-agar plates containing 2% glucose and 100 µg/mL ampicillin. We selected nanobody domain V (D5*), based on antigen recognition (figure 1A) and affinity for open β2GPI (figure 1B). SPR analysis indicated a kD of 10nM for open β2GPI.

D5* was cloned into an in-house expression vector pTH3.1, a PUC19 based vector, containing a N-terminal pelB leader peptide, a (His)6 affinity tag for purification purposes, and a tobacco etch virus (TEV) protease cleavage site for removal of the purification tag, as well as a C-terminal c-Myc tag for detection purposes. Nanobodies were produced in *Escherichia coli* pLyss star in a bioreactor with an auto induction protocol[9]. Nanobodies were purified with HiTrap TALON Sepharose (GE Healthcare, Eindhoven, the Netherlands), followed by removal of the terminal His-tag with TEV protease. TEV protease and uncleaved nanobodies were removed with TALON Sepharose, after which nanobodies were subjected to size exclusion chromatography on a preparative grade Superdex 200 column (GE Healthcare). Resulting nanobody preparations were >99% pure, as determined with SDS-PAGE and coomassie blue staining.

Although D5* recognized β2GPI that was immobilized on a high binding maxisorp microtiter plate (figure 1A), the nanobody did not recognize β2GPI immobilized on a low binding hydrophobic polysorp plate (data not shown). This fits with existing data, which indicate that immobilization of β2GPI on a high binding plate leads to a conformational change and exposure of the pathophysiological relevant epitopes of the protein[10]. To confirm that D5* only recognizes β2GPI in its open conformation, we immobilized the nanobody on a hydrophobic microtiter plate and compared binding of plasma-derived purified β2GPI with β2GPI that was chemically fixed in its open conformation as previously described[6] (figure 1C). These data confirm that the nanobody is specific for the open conformation of β2GPI. It has been hypothesized that binding of antiphospholipid antibodies to β2GPI stabilizes the open conformation of the protein. To test this hypothesis, we measured the amount of open β2GPI in normal plasma that was supplemented with monoclonal antibodies 3B7, directed against domain I, and 21B2, directed against domain IV of β2GPI. Both are known to form immune complexes with their antigen in solution (figure 1D). Indeed, both antibodies induced the open conformation of β2GPI, resulting in a 10-15-fold increase in binding. No open β2GPI was found when β2GPI deficient plasma was used (not shown). Similarly, addition of chemically opened β2GPI[6] resulted in a strong increase in binding.

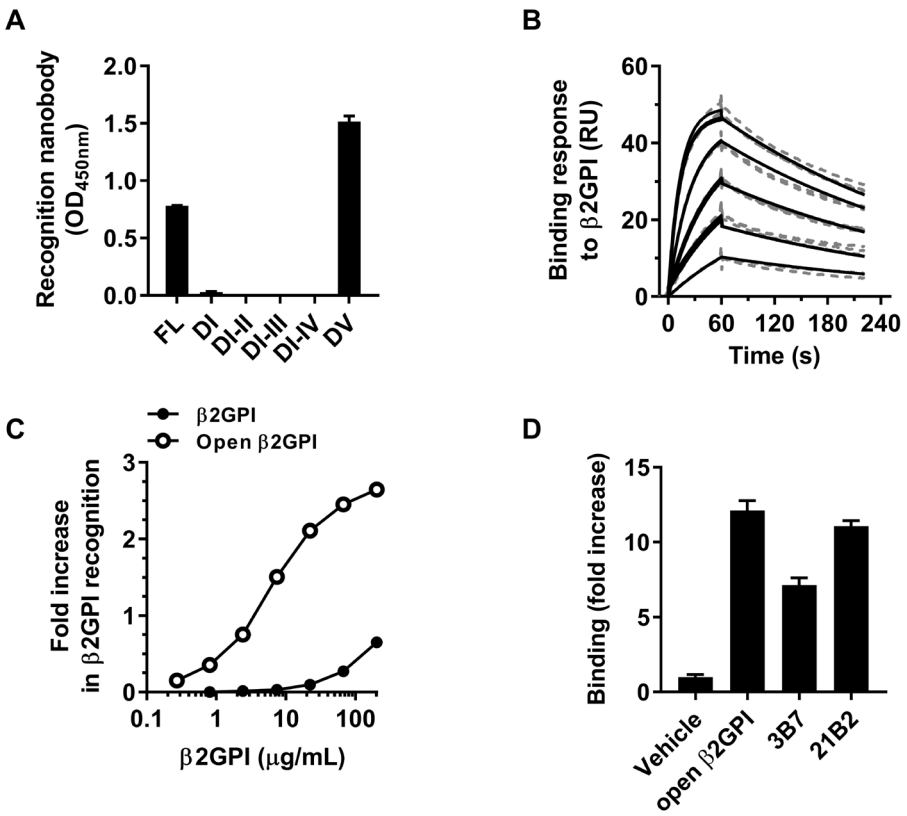


Figure 1. **Characterization of a nanobody that specifically recognizes β2-glycoprotein I in the open conformation. A**. Full length, plasma-derived β2-glycoprotein I (FL) and recombinant domain deletion mutants of β2-glycoprotein I (domain (D)I, DI-II, DI-III, DI-IV and DV) were coated on a maxisorp microtiter plate (NUNC), after which plates were blocked with 1% skimmed milk powder (Sigma Aldrich, Zwijndrecht, the Netherlands) in PBS for 1 hour. Nanobody D5* (5 μg/mL) was incubated for 1 hour at 37°C. After extensive washing, plates were incubated with mouse anti-myc, clone 9E10, followed by rabbit-anti mouse-HRP (DAKO, Glostrup, Denmark). Plates were developed with TMB and reactions were stopped by addition of 0.1M H_2SO_4. Plates were read at 450nm. **B**. SPR analyses were performed on a Biacore T100 system (GE Healthcare, Eindhoven, the Netherlands). Monoclonal antibody 3B7 (against domain I β2-glycoprotein I) of was immobilized on a CM5 series S sensorchip with amine-coupling chemistry. Prior to each run, the relevant channels were loaded with 100nM β2-glycoprotein I. Nanobody D5* was injected with a flowrate of 30 μL/min at 0, 12.5, 25, 50 and 100nM in 10mM HEPES, 150mM NaCl, 0.05% tween-20, pH 7.4. Data obtained in the reference channel, which contained only 3B7, was subtracted from the sensorgrams of the channels that contained 3B7 and β2-glycoprotein I. All injections

were performed in triplicate, and are shown in dashed grey. Sensorgrams were fitted with a 1:1 Langmuir binding model with Biacore T100 evaluation software. Fits are shown in black. **C**. Nanobody D5* was immobilized on a maxisorp microtiter plate. Plates were blocked with 1% milk powder (Protifar; Nutricia, Zoetermeer, the Netherlands) in PBS and incubated with chemically opened β2-glycoprotein I (open β2-glycoprotein I)[6] or plasma-derived β2-glycoprotein I at the indicated concentrations. Binding was detected with HRP-conjugated affinity purified goat anti-human β2-glycoprotein I antibodies (Cedarlane, Burlington, Canada). A weak signal was found when purified β2-glycoprotein I added to β2-glycoprotein I deficient plasma was tested in an ELISA set-up, however, a strong signal was found when *in-vitro* opened β2-glycoprotein I was used instead. **D**. Nanobody D5* was immobilized on a maxisorp microtiter plate. Plates were blocked with 1% milk powder (Protifar; Nutricia, Zoetermeer, the Netherlands) in PBS and incubated with human plasma (diluted 1:20), supplemented with either vehicle, 50 μg/mL 3B7, 50 μg/mL 21B2, or 10 μg/mL chemically opened β2-glycoprotein I. Data are expressed as fold-increase in binding compared with plasma. A weak signal was found in plasma which was strongly increases when *in-vitro* opened β2-glycoprotein I was added. Also addition of monoclonal antibodies 3B7 and 21B2 (directed against domain I and IV of β2-glycoprotein I, respectively) strongly increases the signal.

Next we tested whether D5* interferes with the binding of β2GPI to phospholipids or with the activation of target cells by antiphospholipid antibodies. Addition of an excess of nanobody could not inhibit the binding of β2GPI antibody complexes to immobilized phospholipids (phosphatidylserine, phospatidylethanolamine, phosphatidylcholine, in a 20:40:40 molar ratio) or cardiolipin (data not shown). Furthermore, addition of a 30-fold molar excess of D5* to monocytes did not inhibit tissue factor expression induced by 3B7-β2GPI immune complexes. A peptide covering the LPS binding site within β2GPI[6] was not recognized by the nanobody either. Since the phospholipid binding site has been reported to overlap with the receptor binding site[12] these data indicate that the neo-epitope recognized by D5* does not overlap with the major reported interaction sites on domain V.

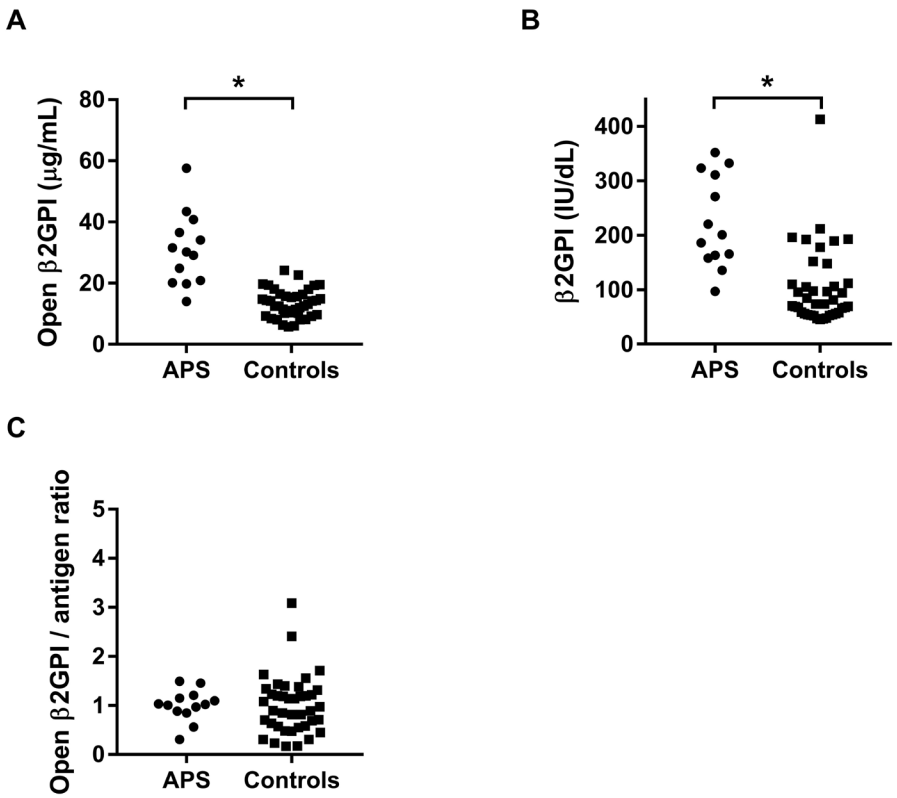
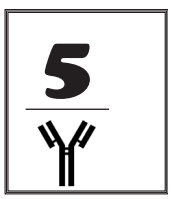


Figure 2. **The relative amount of open β2-glycoprotein I in APS patients is similar to that in controls. A**. Open β2-glycoprotein I levels were determined in plasma from 13 APS patients and 40 healthy controls with a solid phase assay, in which nanobody D5* was immobilized on a maxisorp microtiter plate. Plates were blocked with 1% milk powder (Protifar) in PBS and incubated with plasma samples (diluted 1:60 in blocking buffer). Binding was detected with affinity purified goat anti-human β2-glycoprotein I antibodies (Cedarlane), followed by incubation with HRP-conjugated rabbit anti-goat antibodies. The concentration of open β2-glycoprotein I was deduced from a calibration curve consisting of chemically opened β2-glycoprotein I and expressed in μg/mL. **B**. The concentration of total β2-glycoprotein I in plasma of the same patients and controls was determined with an ELISA, as described previously (Agar *et al.*). Data are expressed in IU/dL, with 100 IU/dL representing the mean concentration in the general population. **C**. The ratio between open and total β2-glycoprotein I. Asterisk indicates a statistically significant difference ($p<0.05$).

Since binding of an antibody to β2GPI stabilizes the open conformation of β2GPI, we investigated whether APS patients with anti-β2GPI antibodies have increased levels of open β2GPI in their circulation, we analysed β2GPI levels in 13 APS patients and 40 healthy controls. APS patients' plasma was collected at the Karolinska Institutet, Solna, Sweden. All patients had thrombotic APS, based on triple positivity. Normal levels op open β2GPI were of 13 ± 5 μg/mL, based on 40 healthy controls (figure 2A). Open β2GPI levels were 2-fold higher in APS patients (30 ± 19 μg/mL, $p<0.05$). However, total plasma β2GPI levels were higher in APS patients that in healthy controls as well ($p<0.05$)[13] (figure 2B). Indeed, the ratio between open β2GPI and total β2GPI was similar between APS patients and controls (figure 2C).

In the circulation, β2GPI predominantly circulates in a closed conformation. In this closed conformation, an epitope in domain I interacts with an epitope in domain V. The epitope in domain I harbours the binding site for the auto-antibodies that characterize the syndrome. In this study we could not identify a specific function for the cryptic epitope in domain V. However, the nanobody that is directed against the epitope in domain V could be used to estimate the amount of open or "activated" β2GPI in the circulation of healthy individuals and in patients. We found that approximately 6% of β2GPI in the circulation is in its open conformation. It has been shown that β2GPI is a very flexible protein[10] and it has been assumed that the different conformations could be in equilibrium[5]. However, this equilibrium is not shifted towards a more active conformation of β2GPI in patients with APS, an observation in agreement with the lack of circulating immune complexes in these patients.



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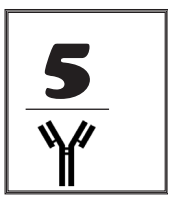
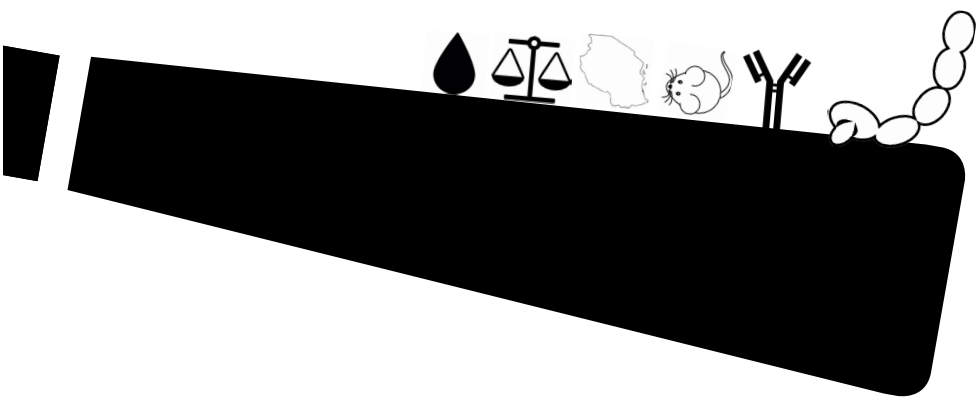


Anti-beta2-glycoprotein I antibodies cause lupus anticoagulant and activated protein C resistance via coagulation factor V



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Abstract

Background: The presence of lupus anticoagulant (LA), measured as a prolonged clotting time, correlates best with thrombotic events in the antiphospholipid syndrome. The mechanism behind the paradoxical association between thrombosis and LA is not understood. It is assumed that antibodies against β2-glycoprotein I (β2GPI) prolong clotting time by competing with coagulation factors for anionic phospholipids, but this does not explain the prothrombotic phenotype in patients with LA.

Objectives: To understand the mechanism behind the thrombotic tendency associated with lupus anticoagulant.

Methods: We assessed the effects of monoclonal anti-β2GPI IgG on coagulation in plasma and with purified coagulation factors.

Results: Experiments with factor (F)V-depleted plasma indicated that the LA effect of anti-β2GPI antibodies depends on the presence of FV. Binding studies showed that β2GPI binds to both FV and FVa. Further exploration of the effects of anti-β2GPI IgG on the prothrombinase complex showed that IgG-β2GPI complexes inhibit the activation of FV by FXa in a phospholipid-dependent manner, thereby interfering with the initiation of coagulation. In addition, the interaction between β2GPI and FV caused activated protein C (APC) resistance. Although the antibodies did not impair degradation of FVa by APC, they interfered with the cofactor role of FV in the APC-mediated degradation of FVIIIa.

Conclusion: Here, we provide an explanation for the LA paradox. The interaction between antibody-β2GPI complexes and FV causes a prolonged clotting time in LA-sensitive assays *in vitro*. This interaction also results in APC resistance, a well-known risk factor for thrombosis.

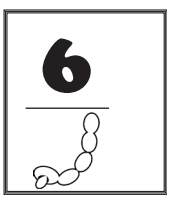
Introduction

Antiphospholipid antibodies (aPL) are acquired risk factors for thrombosis. Patients with persistent aPL and thrombosis or pregnancy-related morbidity are diagnosed with the autoimmune disorder antiphospholipid syndrome (APS)[1]. Official classification criteria for APS recognize three distinct but overlapping aPL populations: antibodies against the phospholipid binding protein β2-glycoprotein I (β2GPI), antibodies against β2GPI bound to the phospholipid cardiolipin and antibodies that prolong clotting times in a phospholipid dependent manner; so called lupus anticoagulant (LA). The persistent presence of one or more of these antibody populations in patients with thrombosis or pregnancy morbidity is sufficient for the diagnosis of APS[1,2].

Of the three serological criteria for APS, the presence of LA correlates best with the clinical features of APS: it is the aPL population that is independently associated with an increased risk of thrombosis and associated with adverse pregnancy outcome[3,4]. The clear association of LA with thrombosis represents a paradox, as prolonged clotting times are usually indicative of a bleeding tendency. The mechanism of action behind the LA phenomenon is currently incompletely understood.

LA can be detected when clotting tests are performed with limiting concentrations of phospholipids[2]. Commonly used assays for LA include the dilute Russell's Viper Venom time (dRVVT), which is based on the activation of coagulation factor (F)X by the venom of the Russell's viper, and tests based on activated partial thromboplastin time (APTT) reagents[2,5].

When LA is present in a sample, the clotting time obtained with these reagents is prolonged. Repeating the clotting test with an excess of phospholipids negates the inhibitory effects of LA, resulting in normalization of the clotting time[2,5]. Clinically relevant LA is reported to be caused by antibodies against β2GPI[6-8], although LA can be induced by antibodies against prothrombin[9]. It is generally assumed that aPL induce LA through competition with coagulation factors for binding sites on anionic phospholipids. Addition of excess phospholipids would dilute the aPL, thus lowering the likelihood that they will compete with clotting reactions[10,11]. This seems an unlikely explanation for the inhibitory effects of anti-β2GPI antibodies on coagulation for several reasons. First, anionic phospholipids and Ca^{2+} ions are essential for coagulation, but the affinity of the plasma protein β2GPI for anionic phospholipids is low in the presence of Ca^{2+} ions, despite an antibody-induced increase in avidity[10]. It is therefore unlikely that antibody-β2GPI immune complexes interfere with phospholipid dependent coagulation reactions. Second, addition of an excess of hexagonal II phase phospholipids to coagulation reactions efficiently negates the inhibitory effects of LA on coagulation[12-14], although phospholipids in this configuration do not support coagulation.



To obtain a better understanding of the pathophysiology of APS and the mechanism through which pathogenic aPL interfere with haemostasis, we have performed an in-depth analysis of the effects of anti-β2GPI antibodies on coagulation. Since aPL are readily detected with dilute Russell's viper venom time (dRVVT) reagents, which measure the common pathway of coagulation, we focused on the effects of aPL on the prothrombinase complex.

Methods

Reagents

Horse radish peroxidase (HRP)-conjugated goat anti-human β2GPI antibodies were obtained from Cedarlane (Burlington, USA). HRP-conjugated rabbit anti-mouse IgG, rabbit anti-goat IgG and goat anti-rabbit IgG were from DAKO (Glostrup, Denmark). Monoclonal anti-β2GPI antibody 3B7 was produced in-house and purified from hybridoma culture medium with protein G Sepharose (GE Healthcare, Eindhoven, the Netherlands). Pefabloc TH was from Pentapharm (Aesch, Switzerland). Human prothrombin, α-thrombin, FIXa and FX were obtained from Enzyme Research Laboratories (South Bend, USA). Human FV, FVa, FX, FXa, activated protein C (APC), protein S and monoclonal anti-FV antibodies 5112 against FV-light chain and 5146 against FV heavy-chain were obtained from Haematologic Technologies (Essex Junction, USA). Recombinant human FVIII (Advate; Baxalta, Bannockburn, USA) and hirudin (Lepirudin; CSL Behring GmbH, Marburg, Germany) were obtained as pharmaceutical formulations. Phospholipids (Coagulation reagent I) were from Avanti polar lipids (Alabaster, USA). Recombinant human relipidated tissue factor (Innovin) was from Siemens Healthcare Diagnostics (Erlangen, Germany). Chromogenic substrates for FXa (S2222) and thrombin (S2238) were from Instrumentation Laboratories (Bedford, MA, USA). DRVVT reagents (LA-screen and LA-confirm) were from Stago (Asnières sur Seine, France). Fluorogenic thrombin substrate Z-Gly-Gly-Arg-AMC was from Bachem (Bubendorf, Switzerland). Thrombin-α2-macroglobulin complexes were from Thrombinoscope (Maastricht, the Netherlands). FV depleted plasma, affinity purified sheep anti-human FV polyclonal antibodies and affinity purified sheep-anti-FVIII polyclonal antibodies were from Affinity Biologicals (Ancaster, Canada). Rabbit anti-FX polyclonal antibody was from Abcam (Cambridge, UK). Pooled normal plasma (PNP) was prepared from at least 250 healthy hospital workers. β2GPI-depleted plasma was obtained by depletion of β2GPI from PNP with affinity chromatography on 3B7-conjugated CNBr-activated Sepharose 4B (GE Healthcare). Coagulation factor levels were normal, as determined with clotting times in factor deficient plasma, and β2GPI levels were <1% as determined with ELISA. Human β2GPI was purified from fresh citrated plasma as described previously[6]. Purity was checked with SDS-PAGE and

coomassie blue staining. All β2GPI-preparations yielded a single band of 50 kDa and were >99% pure.

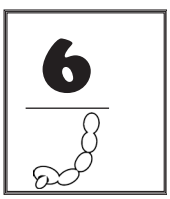
Coagulation assays

All coagulation assays were performed on an MC10-plus coagulometer (Merlin Medical, Lemgo, Germany) at 37ºC. Anti-β2GPI antibodies were incubated with plasma for 5 minutes at 37°C prior to addition of coagulation reagents. Coagulation was initiated with 250pM FXa in HBS, 0.1% BSA, 25mM $CaCl_2$ with either 4 or 500μM PL. Normalized LA-ratios were calculated according to the following equation: nLA ratio = (LA_{screen} (with antibody clotting time/without antibody clotting time))/($LA_{confirm}$ (with antibody clotting time/without antibody clotting time)).

The effect of FV on anti-β2GPI antibody-induced prolongation of the clotting time was analysed by addition of FV or FVa (0-25nM) to FV-depleted plasma with or without anti-β2GPI antibodies, after which coagulation was initiated with 250pM FXa in HBS, 0.1% BSA, 25mM $CaCl_2$ containing either 4 or 500μM PL. In some experiments, clotting was initiated in FV depleted plasma with or without anti-β2GPI antibodies with 250pM FXa and 10nM FVa in HBS, 0.1% BSA, 25mM $CaCl_2$ containing either 4 or 500μM PL.

Michaelis-Menten kinetics of the prothrombinase complex

Prothrombin (concentration ranging from 0 to 1400nM) was incubated with 0.5nM FVa, 10μM PL and 100 μg/mL 3B7 in coagulation buffer (HBS-C; 10mM HEPES, 140mM NaCl, 5mM $CaCl_2$, 0.1% BSA, pH7.5), with or without 50 μg/mL β2GPI as indicated, for 5 minutes and activated with 2.5pM FXa in HBS-C for 1 minute at 37°C. Alternatively, prothrombin was incubated with 100 μg/mL 3B7 with or without 50 μg/mL β2GPI for 5 minutes and activated with pre-formed prothrombinase complex (2.5pM FXa, 0.5nM FVa, 10μM PL in HBS-C) for 1 minute at 37°C. Reactions were stopped with EDTA, final concentration 20mM. Samples were diluted 50-fold in HBS-C and conversion of 0.2mM S-2238 was measured for 10 minutes at 37°C. Thrombin concentrations were deduced from a thrombin calibration curve with known concentrations.



Binding of β2GPI to coagulation factors V, Va, FVIII, X and Xa

All incubations were performed on an orbital shaker (600 rpm) at room temperature for 1 hour. NUNC maxisorp microtiter plates were coated with 10 μg/mL 3B7 in 50mM carbonate buffer, pH 9.6. Plates were blocked with 1% Protifar in HBS and incubated with 10 μg/mL of β2GPI in blocking buffer. After thorough rinsing, plates were incubated with PNP in several dilutions. After washing, plates were incubated with either sheep anti-FVIII or sheep anti-FV antibodies (1:2000 in blocking buffer),

followed by a washing step and incubation with HRP-conjugated rabbit anti-goat antibodies (1:2000; DAKO, Glostrup, Denmark). After a final washing step, 100 µL TMB was added to each well and colorimetric reactions were stopped by addition of 50 µL 0.2M H_2SO_4. Plates were read at 450 nm in a Spectramax M2e device (Molecular devices, Sunnyvale, CA, USA). Binding constants (kD) were estimated with similar experiments, in which PNP was substituted with FV, FVa, FX, FXa, or FVIII in sample diluent (HBS with 0.1% Protifar and 8mM $CaCl_2$) as indicated. FX and FXa were detected with rabbit anti-factor X/Xa antibodies in sample diluent, followed by incubation with HRP-conjugated goat anti-rabbit IgG (1:2000) in sample diluent.

Factor Xa-mediated factor V activation

FV (0.5nM) was activated with 0.1nM FXa in coagulation buffer (HBS-C; 10mM HEPES, 140mM NaCl, 5mM $CaCl_2$, 0.1% BSA, pH7.5) and either 4 or 500µM PL with or without 50 µg/mL β2GPI, 50-150 µg/mL 3B7, or both for 10 minutes at 37°C. Subsequently, 50 µL aliquots were transferred into 200 µL of HBS-C with 0.5nM FXa, 0.5µM prothrombin, 10µM PL, 1µM pefablock TH and incubated for 3 minutes at 37°C. To correct for confounding effects of 3B7 and β2GPI on prothrombinase reaction rates, β2GPI, 3B7 or both were supplemented to FVa-samples generated in the absence of these proteins. Prothrombinase reactions were terminated by transferring 50 µL aliquots to 150 µL stop-buffer (HBS-E; 10mM HEPES, 175mM NaCl, 20mM EDTA, 0.1% BSA, pH 7.7). Thrombin formation was measured by addition of 100 µL of this mixture to 100 µL 1mM chromogenic substrate S2238. Thrombin concentrations were deduced from a thrombin calibration curve with known concentrations. FVa concentration was calculated assuming a turnover number for prothrombinase of 6000 mole thrombin per minute per mole FXa-FVa[15]. For visualization of FV activation with western blot, 30nM FV was activated with 0.5nM FXa in 10mM HEPES, 175mM NaCl, 1% polyethyleneglycol-8000, pH 7.7, containing 10µM PL and 50 µg/mL 3B7, with or without β2GPI at 37°C. At the indicated time points, samples were taken, mixed with reducing Laemmli sample buffer and put on ice. Samples were heated at 95°C for 5 minutes, subjected to SDS-PAGE on a nuPAGE Tris-Acetate 3-8% gel (Thermo Fisher Scientific, Waltham, MA, USA), blotted over night at 4°C onto Immobilon-FL Polyvinylidene fluoride membrane (Merck Biochemicals, Darmstadt, Germany), blocked with Odyssey blocking buffer (Licor biotechnology GmbH, Bad Homburg, Germany) and incubated with either mouse anti-FV light chain or heavy chain antibodies (4 µg/mL) in Odyssey blocking buffer. After extensive washing, blots were incubated with IRDye680-conjugated donkey antimouse antibodies (Licor) in Odyssey blocking buffer and rinsed thoroughly. Bands were visualized on an Odyssey infrared imager (Licor).

Factor Va inactivation by APC

FVa (0.5nM) was incubated with 4μM PL and 0.5nM APC in HBS-C with or without 50 μg/mL β2GPI, 50-150 μg/mL anti-β2GPI antibodies, or both, for 10 minutes at 37°C. Subsequently, 50 μL aliquots were transferred into 200 μL of HBS-C with 0.5nM FXa, 0.5μM prothrombin, 10μM PL, 1μM Pefabloc TH and incubated for 3 minutes at 37°C. β2GPI and anti-β2GPI antibodies were supplemented to FVa-samples generated in the absence of these proteins to exclude confounding effects on prothrombinase reaction rates. Prothrombinase reactions were terminated by transferring 50 μL aliquots to 150 μL HBS-E (pH 7.7). Thrombin formation was measured by addition of 100 μL of this mixture to 100 μL 1mM chromogenic substrate S2238. Thrombin concentrations were deduced from a thrombin calibration curve with known concentrations. FVa concentration was calculated assuming a turnover number for prothrombinase of 6000 mole thrombin per minute per mole FXa-FVa[15].

Factor VIIIa inactivation by APC Factor VIIIa degradation assay was performed as described[16] with a few modifications. FVIII (0.6 U/mL) was activated with thrombin (0.002 U/mL) in the presence of 0.05 U/mL FIXa and 56μM PL in 50mM Tris, 10mM $CaCl_2$, 0.02% BSA, pH 7.3 at 37 °C for 2 minutes. FVIII activation was stopped with hirudin (20 U/mL). FVIIIa was subsequently incubated with 4nM APC and 4nM protein S with or without 2nM FV, 50 μg/mL β2GPI, 50 μg/mL 3B7, or combinations thereof, at 25°C. At the indicated time points, FX (10 μg/mL) was added to the reaction and incubated for an additional minute, after which reactions were terminated with EDTA (16mM). FXa formation was measured with chromogenic substrate S2222. FXa concentrations were deduced from a calibration curve of FXa with known concentrations. Results are expressed as relative FVIIIa activity compared with baseline.

Calibrated automated thrombography and activated protein C sensitivity ratios

Calibrated automated thrombography was performed as described[17] with a few modifications. In short, 20 μL mix A (5pM recombinant relipidated tissue factor (TF) in HBS with 0.1% BSA, 4μM PL with or without 5nM APC and 50 μg/mL anti-β2GPI antibodies, final concentration in the assay) was added to 80 μL of plasma in a black NUNC maxisorp 96-well microtiter plate and incubated at 37°C for 10 minutes. 20 μL of thrombin calibrator (α2-macroglobulin-thrombin complexes; activity 700nM) instead of TF was added to control wells. Reactions were initiated by addition of 20 μL pre-warmed FluCa mix (16.6mM $CaCl_2$ with 0.4mM fluorogenic substrate Z-Gly-Gly-Arg-AMC, final concentration in the assay) to each well.

Plates were read in a SpectraMAX M2e with λ_{ex} 390nm and λ_{em} 460nm at 37ºC for one hour with a 15 second interval. Fluorescence data were converted to thrombin generation curves as previously described[18]. Normalized APC sensitivity ratios were calculated as described[19].

Statistical analyses

Statistics were analyzed with the unpaired Student t test. A p-value of less than 0.05 was considered significant. Kinetic constants K_m and V_{max} were calculated with non-linear regression. Binding data were analyzed with non-linear regression assuming a 1:1 interaction. All data analyses were performed with GraphPad Prism v7.02 software (La Jolla, USA).

Results

Anti-β2GPI antibodies interfere with the formation of the prothrombinase complex.

Anti-β2GPI antibodies readily prolong dRVVT-based clotting times, suggesting interference with the common pathway of coagulation. To investigate the effect of anti-β2GPI antibodies on the common pathway, FXa-initiated clotting times were measured in FV-depleted plasma spiked with monoclonal anti-β2GPI antibody 3B7 that was reconstituted with either FV or FVa.

At a limiting PL concentration, addition of 3B7 to FV-depleted plasma reconstituted with FV caused a 2-fold increase in FXa-initiated clotting time (figure 1A). In contrast, 3B7 had no effect on FXa initiated clotting times in the presence of an excess of PL (500µM), which resulted in an increased LA-ratio and signifies LA-activity of 3B7 (figure 1D). Addition of 3B7 to FV-depleted plasma reconstituted with FVa caused a 1.5-fold prolongation of the clotting time, which did not correct when an excess of PL was used and was independent of FVa concentration (figure 1B). As a result, 3B7 did not result in an increased LA-ratio in FVa reconstituted plasma (figure 1D). 3B7 had no effect on the clotting time when coagulation was initiated with a combination of PL, $CaCl_2$, FXa and FVa (prothrombinase complex) (figure 1C). These data suggest that anti-β2GPI antibody-induced LA depends on the presence of FV. In the absence of FV, Russell's viper venom still clots plasma in a factor Xa-dependent manner. The lack of effect of 3B7 on dRVVT clotting times in FV-depleted plasma is in line with a FV-dependent inhibition of the coagulation reaction (figure 1E).

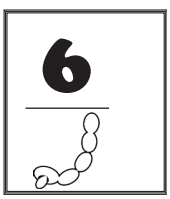
Antibody-β2GPI complexes bind to FV(a) with high affinity

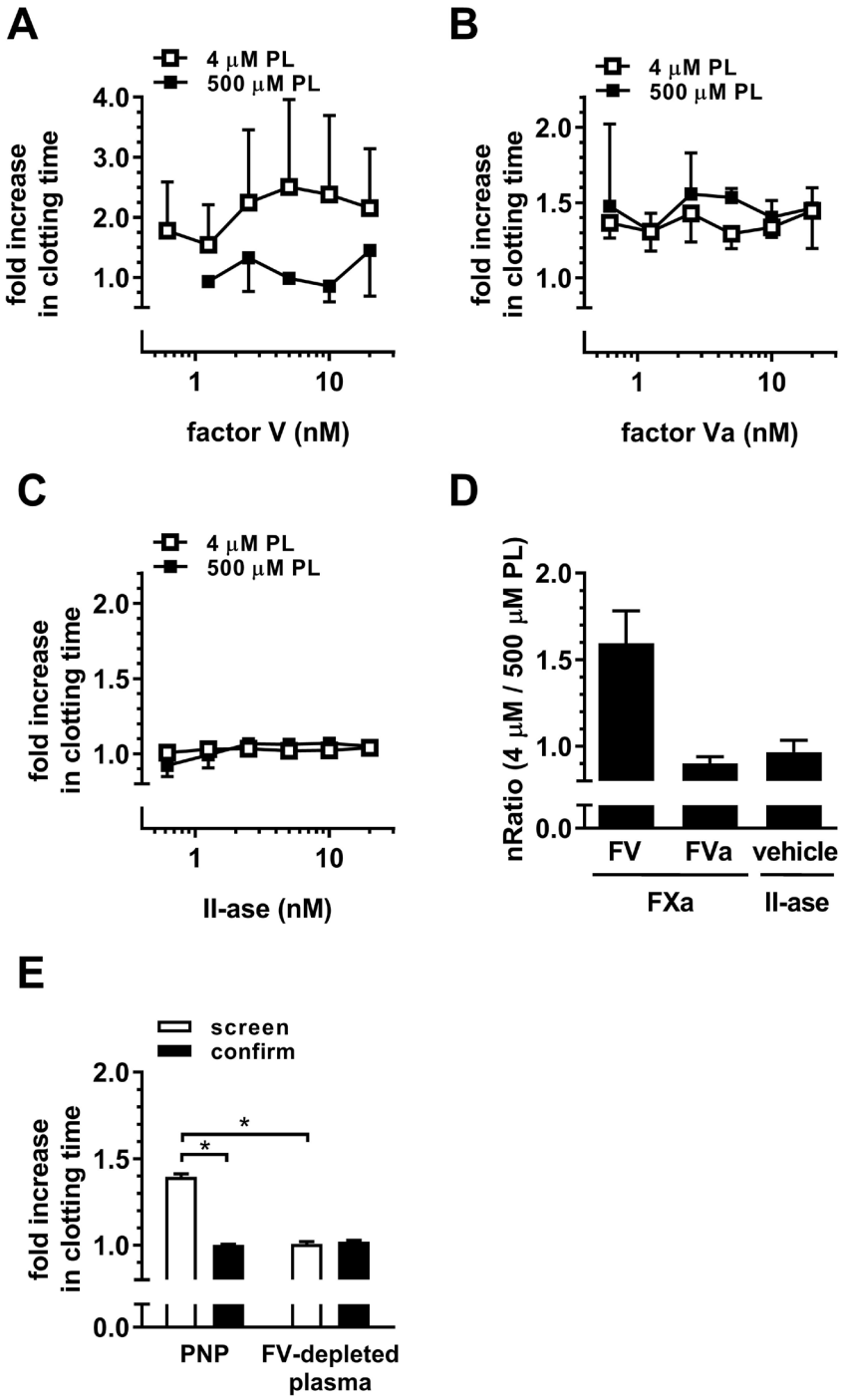
The observation that 3B7 inhibited FXa-initiated clotting reactions in the presence of both FV and FVa, but not when the prothrombinase complex was already formed, suggests a direct interaction between β2GPI-antibody complexes and the

individual components of the prothrombinase complex, FV(a) or FXa. Therefore, the interaction between FV, FVa, FX and FXa with β2GPI was investigated with solid phase binding assays. Binding experiments in plasma showed that FV interacts with immobilized β2GPI-3B7 complexes, whereas its homologue FVIII does not (figure 2A). More detailed analysis of the interaction between immobilized antibody-β2GPI complexes and FV and FVa showed that both proteins interacted with β2GPI-3B7 complexes with high affinity: The apparent kD was 15 ± 9nM for FV and 46 ±11nM for FVa. Interestingly, 3B7-β2GPI complexes also bound with high affinity to purified recombinant FVIII (apparent kD 1.4 ± 0.5nM), although this is not expected to lead to meaningful interactions at plasma FVIII-levels (figure 2B). No binding was observed between FX or FXa and β2GPI (data not shown).

Anti-β2GPI antibodies attenuate FXa-mediated FV activation

The inhibitory effects of 3B7 on FXa-initiated clotting reactions were more pronounced in the presence of FV than in the presence of FVa. Combined with the observation that β2GPI interacts with FV, one can hypothesize that antibody-β2GPI complexes interfere with the formation of the prothrombinase complex by preventing FV activation. FV can be activated by both FXa and thrombin. Although thrombin is a more efficient activator of FV, the initial activation of FV depends on FXa[20]. To analyse the effect of 3B7-β2GPI complexes on FXa mediated FV activation, FV was activated with FXa in the presence (figure 3A) or absence of 3B7 and β2GPI (figure 3B). At low phospholipid concentration (4μM), FV activation was inhibited in a dose-dependent manner in the presence of both β2GPI and 3B7 (figure 3C), but was not influenced by either β2GPI or 3B7 alone. In contrast, 3B7-β2GPI complexes had no effect on FXa-mediated FV activation in the presence of excess phospholipid (500μM) (figure 3D). These data suggest that anti-β2GPI antibodies interfere with the initiation of coagulation, rather than with propagation. We therefore analyzed the effect of 3B7 on calibrated automated thrombography (table 1). Consistent with these results, 3B7 increased the lag time and slightly increased the peak height, but had no effect on endogenous thrombin potential.




A
4 μM PL
500 μM PL
fold increase in clotting time
4.0
3.0
2.0
1.0
1
10
factor V (nM)
B
4 μM PL
500 μM PL
fold increase in clotting time
2.0
1.5
1.0
1
10
factor Va (nM)
C
4 μM PL
500 μM PL
fold increase in clotting time
2.0
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1.0
1
10
II-ase (nM)
D
nRatio (4 μM / 500 μM PL)
2.0
1.5
1.0
0.0
FV
FVa
vehicle
FXa
II-ase
E
screen
confirm
fold increase in clotting time
2.0
1.5
1.0
0.0
*
*
PNP
FV-depleted plasma

Figure 1. **Anti-β2GPI antibodies interfere with coagulation FV/FVa.** Clotting was initiated with 0.25nM FXa and $CaCl_2$ (8.3mM final concentration) or the combination of FVa, FXa, PL and $CaCl_2$ (8.3mM final concentration) (prothrombinase complex: II-ase) at the indicated concentrations in FV-depleted plasma spiked with monoclonal anti-β2GPI antibody 3B7 that was reconstituted with either FV (**A**), FVa (**B**) or buffer (**C**) in the presence of 4 or 500μM PL. Data are expressed as fold increase in clotting time ± SD. **D.** Normalized LA-ratios (nLA-ratio) were calculated from clotting times obtained in FV-depleted plasma spiked with 3B7 that was reconstituted with 2.5nM FV, 2.5nM FVa or vehicle and either 4μM PL or 500μM PL. Coagulation was initiated with 0.25nM FXa, PL and 8.3mM $CaCl_2$ (final concentration) or 0.25nM prothrombinase complex as indicated. Clotting times were normalized on the clotting time obtained in the absence of 3B7. nLA-ratios were calculated as the ratio between normalized clotting times obtained with 4μM PL and normalized clotting times obtained with 500μM PL. **E.** Clotting times were measured in pooled normal plasma (PNP) or FV-depleted plasma with or without 3B7 with dRVVT screen (low PL content) and confirm (high PL content) reagents. Data are expressed as fold increase ± SD in clotting time in the presence of 3B7 as compared with control. Data represent at least three independent experiments. Asterisk indicates a statistically significant difference ($p<0.05$).

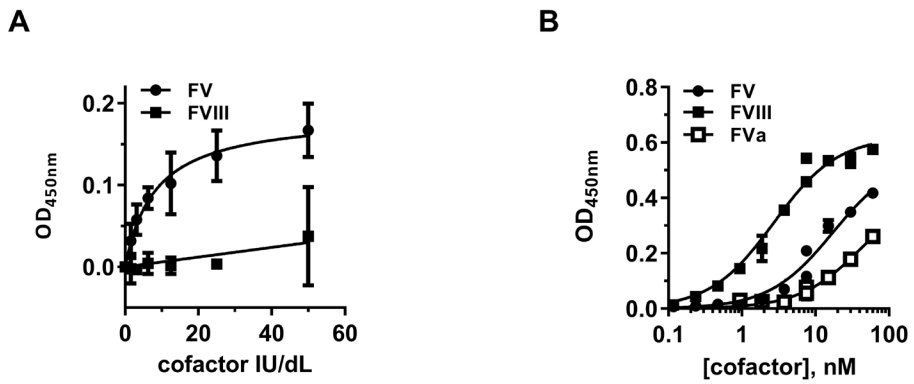
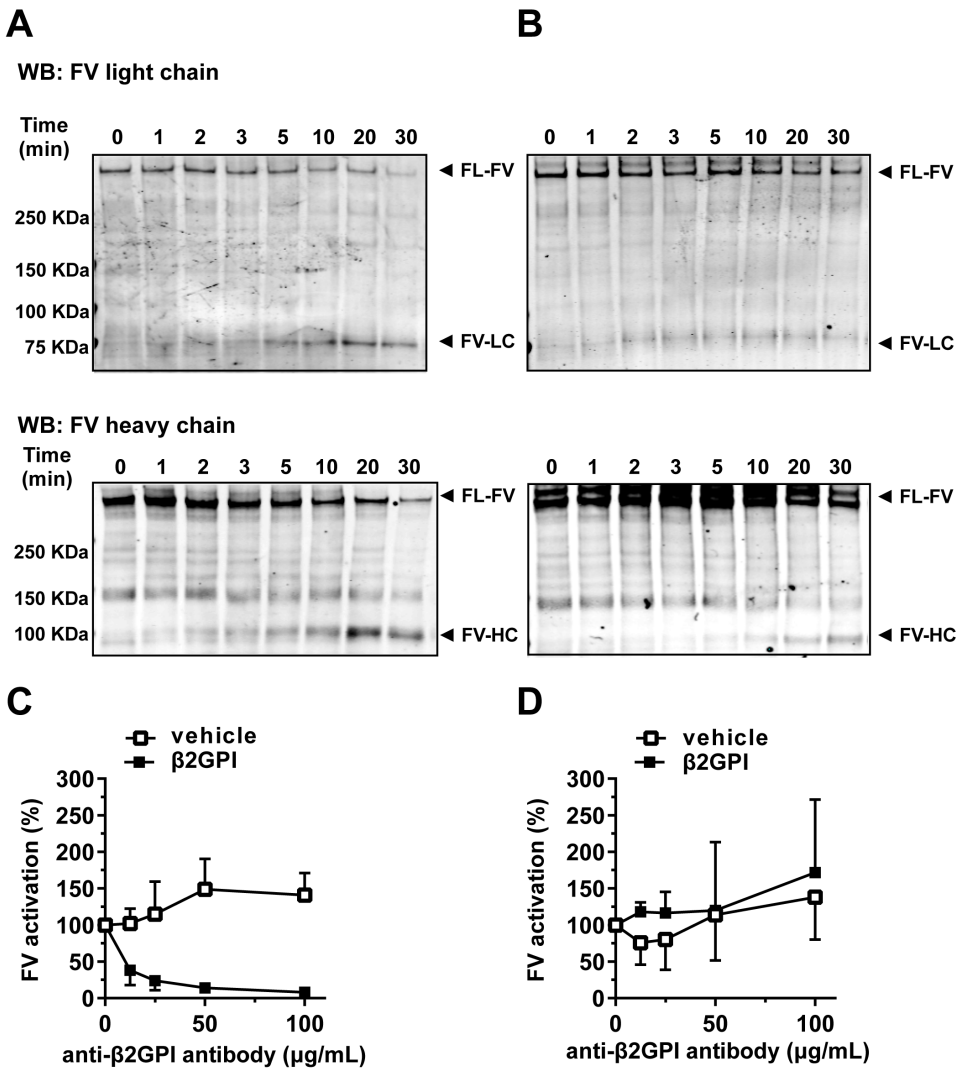


Figure 2. **β2GPI binds to coagulation factor V and Va with high affinity.** 3B7 was immobilized on a microtiter plate and saturated with β2GPI. **A.** Plates were incubated with several dilutions of normal plasma, indicated by the concentration of the cofactor (IU/mL), and binding of FV or FVIII was assessed. **B.** Plates were incubated with purified FV, FVa, or FVIII at the indicated concentrations and binding was assessed with polyclonal antibodies. Data are representative of at least three independent experiments.


A
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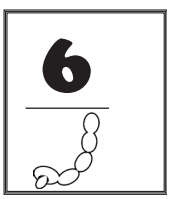
WB: FV light chain
Time (min)
0 1 2 3 5 10 20 30
250 KDa
150 KDa
100 KDa
75 KDa
FL-FV
FV-LC
WB: FV heavy chain
250 KDa
150 KDa
100 KDa
FV-HC
C
D
vehicle
β2GPI
FV activation (%)
anti-β2GPI antibody (μg/mL)

Figure 3. **Anti-β2GPI antibodies attenuate FXa-mediated FV activation.** 30nM FV was activated with 0.5nM FXa in the presence of PL, $CaCl_2$ and 3B7, without (**A**) or with β2GPI (**B**) at 37°C. Samples were taken at the indicated time points, mixed with reducing sample buffer, heated at 95°C for 5 minutes and subjected to SDS-PAGE on a 3-7% Tris-Acetate nuPAGE gel. After Western blotting, membranes were incubated with either anti-FV light chain antibody 5112 or anti-FV heavy chain antibody 5146, followed by IRDye680-conjugated donkey anti-mouse antibodies. Bands were visualized on an Odyssey infrared imager. **C.** 0.5nM FV was activated with 0.1nM FXa in 10mM HEPES, 140mM NaCl, 5mM $CaCl_2$, 0.1% BSA, pH7.5 and 4μM PL at the indicated concentration of 3B7, with or without 50 μg/mL β2GPI for 10 minutes at 37°C. **D.** Similar experiments were performed with 500μM PL. Data are expressed as relative FV activity compared with the conditions without 3B7. Data represent at least three independent experiments and are expressed as mean ± SD.

Table 1. **Anti-β2GPI antibody 3B7 influences lag time of thrombin generation.**

/	Lag time (min)	Peak height (nM IIa)	ETP (nM IIa.min)
Vehicle	1.1 ± 0.2	329 ± 35	1347 ± 70
3B7	1.8 ± 0.1*	371 ± 16*	1360 ± 68

The influence of anti-β2GPI antibodies on thrombin generation in PNP was measured with calibrated automated thrombography (CAT). Coagulation was initiated with 5pM tissue factor, 4μM PL, with or without 50 μg/mL 3B7. Data are expressed as mean ± SD and represent at least three independent experiments. Asterisk indicates a statistically significant difference ($p<0.05$).



Antibody-β2GPI complexes interfere with assembly of the prothrombinase complex

The observation that 3B7 prolonged clotting times in FV-depleted plasma reconstituted with FVa suggests that interference of β2GPI-antibody complexes with the prothrombinase complex is not limited to the attenuation of FV activation. To investigate whether antibody-β2GPI complexes interfere with assembly of the prothrombinase complex, the kinetic constants of the prothrombinase reaction were determined in the presence and absence of β2GPI-3B7 complexes. Pre-incubation of FVa with both β2GPI and 3B7 prior to addition of FXa lowered the V_{max} of the prothrombinase complex from 3553 ± 625 to 1998 ± 764 mol IIa x mol Xa^{-1} x min^{-1} ($p < 0.05$) (figure 4A), but had no effect on the K_m, which suggests interference with the cofactor function of FVa, not the PL surface and is consistent with the plasma-based experiments shown in figure 1C. However, when the prothrombinase complex was allowed to form prior to exposure to antibody-β2GPI complexes, the V_{max} of the

prothrombinase complex in the absence of 3B7 and β2GPI (8310 ± 733 mol IIa x mol Xa^{-1} x min^{-1}) was similar to the V_{max} obtained in the presence of antibody-β2GPI complexes (7460 ± 128 mol IIa x mol Xa^{-1} x min^{-1}) (figure 4B). These data suggest that the interaction between FVa and β2GPI-antibody complexes interferes with assembly of the prothrombinase complex.

Anti-β2GPI antibodies induce APC resistance through the interaction with FV

APC resistance is frequently observed in patients with anti-β2GPI antibodies and thought to contribute to thrombotic risk[21], but the mechanism behind APC resistance in APS remains incompletely understood. APC regulates coagulation through degradation of FVa and FVIIIa. Based on the observation that β2GPI-dependent LA is caused by a direct interaction of β2GPI antibody complexes with FV and FVa, we investigated whether anti-β2GPI antibodies could induce APC resistance. Addition of anti-β2GPI antibodies to PNP increased the normalized APC-sensitivity ratio to 2.8 (figure 5A). These effects were β2GPI-dependent, as depletion of β2GPI from PNP completely prevented anti-β2GPI antibody-induced APC resistance. Reconstitution of β2GPI in β2GPI-depleted plasma fully restored the capacity of 3B7 to induce APC resistance (normalized APC-sensitivity ratio of 3). Combined, these data confirm that anti-β2GPI antibodies induce APC resistance. Next, the effects of β2GPI and anti-β2GPI antibodies on FVa-inactivation by APC were analyzed in a purified system. APC was added to FVa and residual FVa activity was assessed over time. Under these conditions, APC readily inactivated FVa with 37% ± 13% residual FVa activity after 10 minutes (figure 5B). Addition of β2GPI, 3B7 or both did not influence FVa inactivation, suggesting anti-β2GPI-induced APC-resistance is not due to attenuated FVa inactivation. Since APC also degrades FVIIIa, for which FV is a cofactor, we next investigated the effects of anti-β2GPI antibodies on the inactivation of FVIIIa. APC, protein S and/or FV were added to FVIIIa and residual FVIIIa activity was assessed over time.

FV augmented the inactivation of FVIIIa by APC (figure 5C) and addition of β2GPI and 3B7 completely abolished the enhancing effect of FV on the inactivation of FVIIIa (figure 5D).

Discussion

Here, we provide an explanation for the LA paradox, which involves the cofactor FV. This protein has a dual role in coagulation, as it has both anticoagulant properties as a cofactor for APC and procoagulant properties as a cofactor for FXa during thrombin formation[22,23]. We have found that antibody-β2GPI complexes bind to FV. This attenuates FXa-dependent FV activation and delays activation of the prothrombinase complex, leading to prolonged clotting times *in vitro*. At the

same time, the interaction between antibody-β2GPI complexes and FV causes APC resistance, a known risk factor for venous thrombosis.

Lupus anticoagulants are phospholipid-dependent coagulation inhibitors. It is generally assumed that aPL induce LA through competition with coagulation factors for binding sites on anionic phospholipids. This is an unlikely explanation, because β2GPI has a low affinity for PL in the presence of Ca^{2+} ions[10], which contradicts with a supposed displacement of FXa from the PL. Moreover, hexagonal II phase PL negate the inhibitory effects of LA[12-14], while PL in this configuration do not support coagulation. Instead, we have found that antibody-β2GPI complexes interfere with activation of FV in a phospholipid-dependent manner.

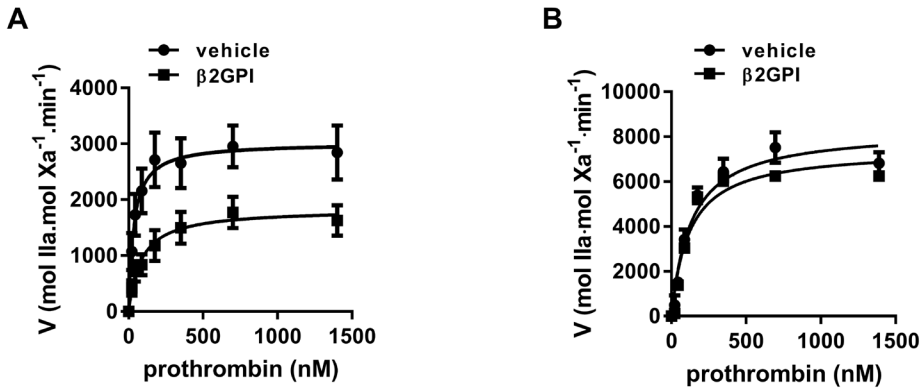


Figure 4. **Anti-β2GPI antibodies attenuate assembly of the prothrombinase complex. A**. Prothrombin was incubated with 0.5nM FVa, 10μM PL and 100 μg/mL 3B7 in 10mM HEPES, 140mM NaCl, 5mM $CaCl_2$, 0.1% BSA, pH7.5, with or without 50 μg/mL β2GPI as indicated, for 5 minutes and activated with 2.5pM FXa for 1 minute at 37°C. Reactions were stopped with EDTA and thrombin was assayed as described in the methods section. Reaction velocity V (in mol thrombin x mol FXa^{-1} x min^{-1}) was plotted as a function of prothrombin concentration. **B.** Prothrombin was incubated with 100 μg/mL 3B7, with or without 50 μg/mL β2GPI as indicated, for 5 minutes and activated with pre-formed prothrombinase complex (2.5pM FXa, 0.5nM FVa, 10μM PL) for 1 minute at 37°C. Reactions were stopped with EDTA, final concentration 20mM and thrombin was assayed as described in the methods section. Reaction velocity V (in mol thrombin x mol FXa^{-1} x min^{-1}) was plotted as a function of prothrombin concentration.

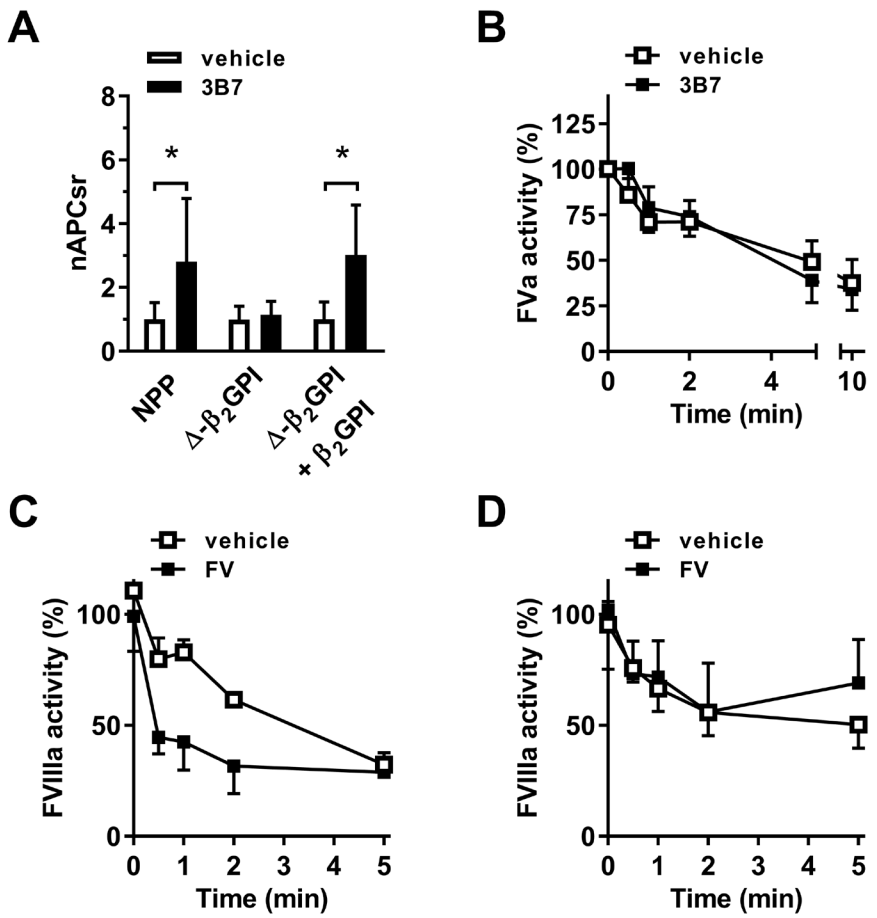
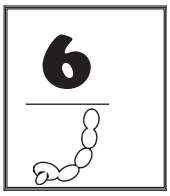


Figure 5. **Anti-β2GPI antibodies induce APC resistance via coagulation factor V. A.** Normalized APC sensitivity ratios (nAPCsr) were measured with calibrated automated thrombography in PNP, β2GPI-depleted plasma (Δ-β2GPI) and Δ-β2GPI plasma reconstituted with β2GPI in the presence of 3B7. **B.** FVa (0.5nM) was incubated with 4μM PL and 0.5nM APC in 10mM HEPES, 140mM NaCl, 5mM $CaCl_2$, 0.1% BSA, pH7.5 and 50 μg/mL 3B7 with or without 50 μg/mL β2GPI at 37°C. At the indicated time points, FVa activity was assayed as described in the methods section. Data are expressed as mean ± SD residual FVa-activity compared with the condition without APC. **C.** FVIII was activated as described in the methods section and was subsequently incubated with 4nM APC, 4nM protein S, with or without 2nM FV at 25°C. Aliquots were taken at the indicated time points and residual FVIIIa activity was assayed as described in the methods section. **D.** FVIIIa was incubated with 4nM APC and 4nM PS in the presence of 50 μg/mL β2GPI, 50 μg/mL 3B7 with or without FV. Data are expressed as mean

± SD residual FVIIIa-activity compared with the condition without APC. Data represent at least three independent experiments. Asterisk indicates a statistically significant difference ($p<0.05$).

During the initiation of coagulation, the pro-cofactor FV is activated through limited proteolysis by FXa, leading to assembly of the prothrombinase complex and subsequent thrombin generation[20]. The activation of FV by FXa is much less efficient than the activation of FV by thrombin, but minute amounts of FVa are sufficient for substantial thrombin formation. Our data indicate that anti-β2GPI antibodies inhibit the activation of FV by FXa, but do not influence coagulation when the prothrombinase complex has already been formed, which suggests that anti-β2GPI antibodies interfere with the initiation of coagulation, not the propagation phase. This is in line with the effect of antiphospholipid antibodies on calibrated automated thrombography, where addition of anti-β2GPI antibodies to normal plasma leads to a prolonged lag time, which reflects the initiation phase of coagulation, with a minor influence on peak height, which reflects the propagation phase.

Although our data indicate that LA is caused by impaired FXa-mediated FV activation, clotting times in FV-depleted plasma reconstituted with FVa that was supplemented with anti-β2GPI antibodies were slightly prolonged as well, albeit much less than the prolongation observed in plasma reconstituted with FV. Our data indicate that the interaction between β2GPI and FVa impairs efficient prothrombinase assembly in addition to the inhibitory effects on FV activation. This interaction does not appear to be phospholipid-dependent, which could explain why a lupus anticoagulant is rarely fully corrected in the presence of an excess of phospholipid during diagnostic testing. Our data indicate that anti-β2GPI antibodies induce significant APC resistance, with normalized APCsr similar to those obtained in plasma from carriers of the FV Leiden (R506Q) mutation[19]. The association of the presence of antiphospholipid antibodies with APC resistance has been frequently observed in patients with thrombotic APS[24-28]. Several mechanisms have been proposed to explain acquired APC resistance in APS, including a direct interaction between APC and β2GPI[29]. Although we did not investigate the direct interaction between APC and β2GPI, our data indicate that cleavage of FVa by APC is not influenced by the presence of antibody-β2GPI complexes, raising doubt on the relevance of such an interaction. Instead, we found that anti-β2GPI antibodies impaired the cofactor function of FV during APC-mediated FVIIIa inactivation, which fits with a direct interaction between FV and β2GPI. The clinical importance of the role of FV as an APC-cofactor is illustrated by the prothrombotic state associated



with the R506Q mutation in FV, which can no longer function as an APC cofactor during FVIIIa degradation[30,31].

Despite the clear inhibitory effects of anti-β2GPI antibodies on coagulation *in vitro*, patients with APS do not have a bleeding tendency. We hypothesize that this is due to platelet derived FV/FVa, which is released at the site of injury and plays an important role during physiological hemostasis[32]. This platelet pool of FV has already been subjected to limited proteolysis, leading to the release of FVa from activated platelets[33], which is much less susceptible to the inhibitory effects of anti-β2GPI antibodies. The fact that LA can be efficiently corrected with platelet lysate *in vitro*, the so-called platelet neutralization procedure, supports this hypothesis. Combined with APC resistance, this explains why anti-β2GPI antibodies cause excessive thrombin formation rather than insufficient clotting reactions *in vivo*.

APC resistance is a strong risk factor for venous thrombosis, but the association with arterial thrombotic disease is less clear[34-37]. While some studies indicate an association between APC resistance and early onset stroke, others indicate no relationship. Although the majority of patients with APS presents with venous thromboembolic disease, a large proportion of patients presents with cerebral ischemia or other arterial thrombotic events[38]. It is highly likely that the increased risk of arterial thrombosis is mediated predominantly through activation of leukocytes, platelets and endothelial cells. Nevertheless, it is likely that APC resistance contributes to the risk of an arterial event. We previously reported a substantially increased risk of stroke in young women with LA who take oral contraceptives compared with women with LA who do not take oral contraceptives[39]. As both β2GPI-dependent LA and oral contraceptives cause APC resistance through different mechanisms, we cannot exclude a role for APC resistance in APS-associated stroke.
Although β2GPI-dependent LA is considered the clinically most relevant aPL subtype, a substantial proportion of patients with LA does not have antibodies against β2GPI[40]. Moreover, there are several studies that show LA is a risk factor for thrombosis independent from the presence of anti-β2GPI or anticardiolipin antibodies[3,39,41]. The mechanism responsible for the prolongation of coagulation and the prothrombotic phenotype in these patients remains to be determined.

In conclusion, we provide an explanation for the lupus anticoagulant paradox in patients with a β2GPI-dependent LA. These finding might help to identify those patients with aPL who are at risk for a thrombotic event.

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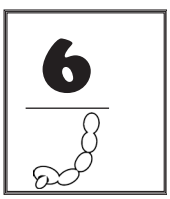

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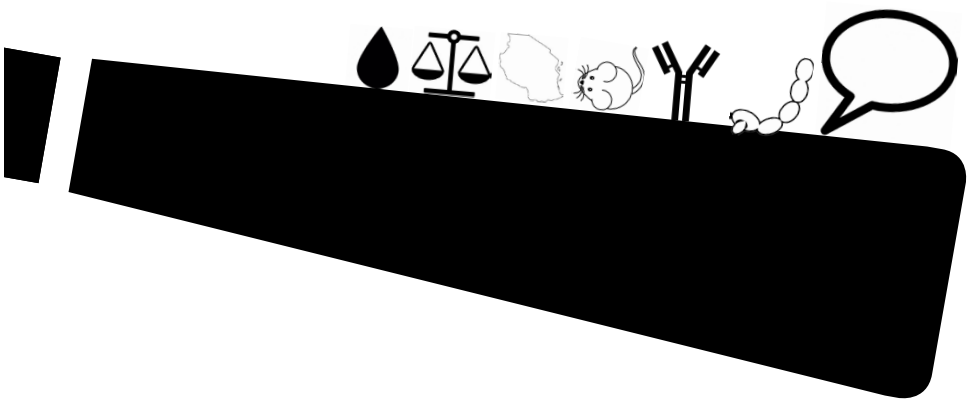
Discussion and future perspectives



65 years ago two patients with systemic lupus erythematosus were described with a prolonged clotting time that could not be corrected with the addition of pooled normal plasma[1]. Interestingly, despite the prolonged clotting times, the patients did not bleed. Two decades later it was established that the presence of such an inhibitor was relatively common and it got its present name: lupus anticoagulant[2,3]. Importantly, it was also found that this inhibitor was a strong risk factor for thrombotic complications. Ten years after the discovery of an alternative way to measure the presence of this inhibitor with an ELISA (anti-cardiolipin antibodies), Graham Hughes defined the combination of this inhibitor with thrombotic complications or pregnancy morbidity as the antiphospholipid syndrome (APS)[4]. The next important step was the discovery that the real antigen for the antibodies was not a phospholipid but a protein named β2-glycoprotein I (β2GPI)[5,6]. Three assays are now available to measure the presence of these prothrombotic auto-antibodies: a clotting assay: lupus anticoagulant and two ELISA assays: anti-β2GPI antibodies and anti-cardiolipin antibodies. Both in retrospective and prospective studies the best association was found between lupus anticoagulant and thrombotic manifestations, hardly any significant correlation was found for anti-cardiolipin or anti-β2GPI antibodies and thrombotic manifestations. Twenty years after the discovery of β2GPI as an important player in APS, the physiological function of this protein has not been established. Different possibilities have been proposed of which a role in complement activation[7-9] or as scavenger for lipopolysaccharide (LPS)[10] or microparticles[11] are the most promising ones. β2GPI is a strongly conserved protein in evolution and it is present at high concentrations in plasma[12,13], indicating that an important function for β2GPI is very likely.
The research described in this thesis aimed to understand one of the enigmas of APS, a prolonged clotting time (lupus anticoagulant) associated with a thrombotic risk (**chapter 2**).

Lupus anticoagulant
Many epidemiological studies have been performed on the association between thrombosis and antiphospholipid antibodies as measured with the different available assays: lupus anticoagulant, anti-cardiolipin or anti-β2GPI antibodies. The different assays measure distinct and only partly overlapping autoantibodies. In earlier studies we have shown that lupus anticoagulant correlates much better with thrombosis than the other assays. One of the remaining questions is why the presence of LA is high in the healthy population. A theory is that the occurrence of autoantibodies is the consequence of infectious diseases[14,15]. The high prevalence of both stroke and infectious diseases in Sub-Saharan Africa stimulated us to start a study in Tanzania on the relation between stroke, lupus anticoagulant and infectious

diseases. In **chapter 3** we have again confirmed that lupus anticoagulant is an important the risk factor for stroke, also in Tanzania. However, the study was too small and the incidence of infections too common to correlate a specific infectious disease with the presence of lupus anticoagulant.
An interesting finding in our study was a protective effect of anti-β2GPI IgM on stroke risk. In the past, several studies have reported comparable findings on this protective effect of IgM antibodies. A study on lupus nephritis by Parodis *et al.* showed no protective effect[16] whereas Mehrani *et al.* did identify a protective effect of IgM antibodies in lupus nephritis[17]. Sène *et al.* identified a protective effect in infectious related diseases as HIV[18,19]. Also one study found for anti-cardiolipin antibodies a protective effect on coronary events[20]. Many studies show a positive correlation between IgM and thrombosis or pregnancy complications. Further studies should solve this enigma. Our results suggest that the induction of anti-phospholipid antibodies after an infection might be one of the mechanisms to protect ourselves against (complications of) infections. However, when these antibodies become permanent, they turn into a risk factor for thrombotic complications. Further and larger studies should answer the role of antiphospholipid antibodies in our fight against bacteria and viruses and why transient antibodies become permanently present antibodies that are a risk for the individual him-or herself. In this respect animal models for APS should be very helpful.

In vivo thrombosis models
All data above are obtained from *in vitro* experiments or cohort studies. It is important to verify all the *in vitro* effects in a mouse model of APS. A previous study was performed in which anti-β2GPI antibodies were induced in mice by surface proteins of *Streptococcus pyogenes*[21], a strain known to induce lupus anticoagulant[22,23]. In the study of van Os *et al*., mice were injected with surface protein H and M1 protein resulting in anti-β2GPI antibodies in protein H injected mice. This indicated the ability of protein H to cause anti-β2GPI antibodies and lupus anticoagulant. In this paper they did not investigate the effect on thrombosis. To answer this important issue, we have performed a comparable study and studied the thrombogenicity in these mice with a ferric chloride thrombosis model in **chapter 4**. Unfortunately, these experiments failed to give a definite answer. We could show that mice injected with protein H and not with M1 protein develop antibodies against domain I of β2GPI. In addition, more mice that were injected with protein H showed an occluded artery 30 minutes after induction of a thrombotic event, however, the numbers were too small to find significant differences. A second set of mice showed confusing results, i.e. mice injected with cardiolipin did not develop auto-antibodies against β2GPI. In literature, injection with cardiolipin resulted in the appearance of

anti-β2GPI antibodies[24]. In this paper they injected cardiolipin simultaneously with human β2GPI. It could be that mice β2GPI is not recognizing the injected cardiolipin and no anti-β2GPI antibodies can arise. To be sure whether injection of an infectious agents will result in pathological relevant anti-β2GPI antibodies, these experiments should be repeated.

Insights into the function of ß2-glycoprotein I

β2GPI is an interesting protein because it can exist in different structural conformations. Originally it was thought that, based on the crystal structure, it exists in a J-shape conformation[25]. SAXS experiments suggested that in solution it occurs as an S-shaped molecule[26] while electron microscopy studies showed a circular conformation[26]. Apparently, β2GPI is a flexible molecule. We reasoned that when β2GPI is in a circular conformation, there should be cryptic epitopes in the domains that hold β2GPI in this conformation, within the first and last domain of β2GPI. Indeed, extensive studies have shown that the pathological autoantibodies against β2GPI recognize a cryptic epitope within domain I of β2GPI. To further investigate the structure and function of β2GPI, we developed an antibody against the proposed cryptic epitope in domain V. We identified a nanobody that discriminates between the closed and open conformation of β2GPI, as described in **chapter 5**. This antibody was generated in *llamas*. Camelids, including *llamas*, produce heavy chain only antibodies in addition to conventional immunoglobulins that consist of both heavy and light chains. These heavy chain only antibodies have fewer complementarity-determining regions than conventional antibodies, but are still able to interact with high affinity with their targets. Moreover, they often recognize conformation-specific epitopes. Nanobodies are small antibody fragments (15 kD) containing the antigen binding site derived from heavy chain antibodies[27]. When the selected nanobody was bound to an ELISA plate it did not recognize plasma β2GPI, but after addition of a murine monoclonal antibody to plasma that has been shown to stabilize β2GPI in its J-shape conformation, a strong signal was detected. We could not describe a specific function to the cryptic epitope in domain V of β2GPI, as none of the functional assays in which β2GPI participates was inhibited when the nanobody was added in excess. We also used this nanobody to set-up an ELISA to measure the amount of open β2GPI in plasma of healthy individuals and in patients diagnosed with APS[27-29]. We could show that about 6% of β2GPI in the circulation is in its open conformation and that there is no difference between patients and healthy controls. This fits with the observation that there are no circulating immune complexes found in patients. Within immune complexes, the autoantibodies are expected to stabilize β2GPI in its open conformation.

The general consensus is that lupus anticoagulant causing antibodies prolong coagulation times because the complex between antibodies and β2GPI competes with clotting factors for the catalytic phospholipids in a clotting assay. However, clotting factors bind to phospholipids in their hexagonal I phase while β2GPI binds preferentially to phospholipids in their hexagonal II phase. Therefore we decided to reinvestigate lupus anticoagulant and how these antibodies interfere with clotting. In **chapter 6** we showed that when factor V deficient plasma was reconstituted with factor V, addition of β2GPI and anti-β2GPI antibodies are able to generate a lupus anticoagulant whereas reconstitution with factor Va did not. These results indicate a direct effect of anti-β2GPI antibodies on factor V. We showed that β2GPI is able to bind to both factor V and Va. Further exploration of the effects of anti-β2GPI IgG on the prothrombinase complex showed that IgG-β2GPI complexes inhibit the activation of FV by FXa in a phospholipid-dependent manner, thereby interfering with the initiation of coagulation. In addition, the interaction between β2GPI and FV caused activated protein C (APC) resistance which is often observed in APS patients[31,32]. Although the antibodies did not impair degradation of FVa by APC, they interfered with the cofactor role of FV in the APC-mediated degradation of FVIIIa. Our results showing an effect of lupus anticoagulant on the initiation phase were in line with results obtained with calibrated automated thrombography by Devreese *et al*[33], as the addition of anti-β2GPI antibodies led to a prolonged lag time which reflects the initiation phase. As factor V activation is also possible via thrombin and β2GPI is able to bind this protein[34], this effect is also relevant to investigate as so many mechanism intermingle. These findings may help in our understanding of the contradiction between lupus anticoagulant as a clotting prolonger and thrombosis. It should be emphasized that this is only true for a subset of APS patients as we have only studied the β2GPI-dependent lupus anticoagulant and a substantial part of the APS patients does not have anti-β2GPI antibodies[35]. Keeling *et al.* already suggested that APS patients might benefit from treatment of APC resistence[36]. However, our results suggested that the effects of antibodies against β2GPI on APC resistance are not mediated by an effect on the prothrombinase complex but on the tenase complex, which opens alternative treatment options.

Future perspectives

In this thesis, we have studied auto-antibodies against β2GPI. The prolongation of clotting assays by these antibodies, known as lupus anticoagulant, is one of the most important characteristics to diagnose patients with the antiphospholipid syndrome. We have found an alternative explanation why these antibodies prolonged *in vitro* clotting assays and we also showed that these antibodies induce protein C resistance. We have shown *in vitro* that the APC resistance is not an effect on the

prothrombinase complex but on the tenase complex. The next step is to show this in an animal model. To do so, the induction of anti-β2GPI antibodies in a haemophilia A mouse should give the answers: does impaired factor VIII inactivation play a role in APS and is it responsible for the increased risk for (venous) thrombosis. We have started to study the origin of anti-β2GPI antibodies. Infectious agents are among the main triggers for the induction of these autoantibodies. The molecular mimicry hypothesis is the general accepted hypothesis to explain the relation between autoantibodies and infections. In earlier studies we have shown that induction of a conformational change and exposure of a cryptic epitope could also explain the induction of anti-β2GPI antibodies. This was repeated in a small *in vivo* study and showed that this could coincide with an increased thrombogenicity. However, the number of animals was too small to draw a conclusion. These experiments should be repeated, because these experiments could show that the presence of anti-β2GPI antibodies is a risk factor for thrombosis, independently of their time in the circulation. The criterion that defines APS requires the presence of these antibodies in two samples taken at least twelve weeks apart. If our experiments are true, this criterion could be left out because they showed that anti-β2GPI antibodies are always a risk factor for thrombosis when they are present. It would be interesting when it could be shown that when these antibodies disappeared, APS has disappeared. The APS model which was developed in our lab opens many possibilities for studying this but also the effect of different treatments.

There are many different antibody profiles present in APS patients and we have only studied the role of anti-β2GPI antibodies. Prudence is in order to make any conclusion for the whole population of APS patients. Future studies should be focussed on unravelling the effect of the different antibody profiles. For example, the presence of anti-prothrombin antibodies is also commonly found in APS patients and we do not know their effects on APC resistance. Moreover, their effects on thrombogenicity in animal models have been hardly studied. It is not known whether the human autoantibodies used, do recognize murine prothrombin. In the studies performed in this thesis we have tried to better understand the anti-β2GPI antibodies that cause the prolongation of clotting assays (lupus anticoagulant), why they prolonged these assays and why a prolongation of a clotting assay could explain a thrombotic tendency. We have made progress, but not all answers are yet available.

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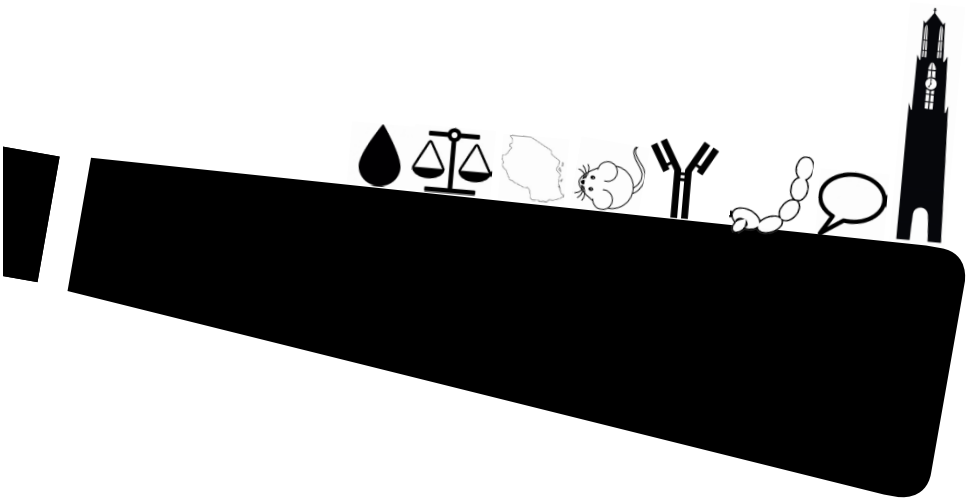
Nederlandse samenvatting



Dikgedrukte woorden zijn opgenomen in de verklarende woordenlijst op bladzijde 110.

Bloedstolling

Iedereen heeft zich wel eens verwond en gemerkt dat je lichaam in staat is de wond weer te dichten door bloed te laten stollen. Bloedstolling wordt in het menselijk lichaam goed gereguleerd door een samenwerking van verschillende **eiwitten** en cellen. Als eerste hechten **bloedplaatjes** aan de beschadiging en wordt er een bloedplaatjesprop gevormd die de wond tijdelijk afdicht. Deze bloedplaatjesprop wordt pas stabiel zodra er fibrine draden doorheen gevormd worden. Het stolsel met fibrine draden wordt na herstel van het weefsel weer opgeruimd door het **enzym** plasmine die het stolsel afbreekt door de fibrinedraden kapot te knippen. Dit mechanisme is ook goed gereguleerd. Het kan voorkomen dat er in mindere of meerdere mate de regulatie van vorming of afbraak van een stolsel uit balans raakt. Als gevolg hiervan kan er **trombose** of een **bloedingsneiging** ontstaan. Er zijn verschillende ziekten die kunnen ontstaan als het ergens in de regulatie van de bloedstolling niet goed gaat en het systeem uit balans raakt. Een voorbeeld hiervan is het **antifosfolipiden syndroom** (APS).

Het antifosfolipiden syndroom

Het antifosfolipiden syndroom is een **auto-immuun ziekte** waarbij er een verhoogde kans is op trombose en **zwangerschapsmorbiditeit**. Bij dit syndroom komt er vooral in de diep gelegen aderen in de benen (**veneuze** trombose) en in de hersenslagader-circulatie (**arteriële** trombose) trombose voor. In de officiële criteria staat dat er antifosfolipiden **autoantistoffen** aanwezig moeten zijn, die 12 weken na eerste ontdekking nog steeds aanwezig moeten zijn om de diagnose APS te krijgen. Deze autoantistoffen kunnen gericht zijn tegen **cardiolipine**, **β2-glycoproteïne I** (β2GPI) of kunnen een **lupus anticoagulant veroorzaken**. Lupus anticoagulant is een **fosfolipiden** afhankelijke **stollingsinhibitor**, die leidt tot een verlengde stoltijd. Bij antifosfolipiden syndroom patiënten komen veel verschillende combinaties voor tussen de autoantistoffen, zo kunnen er bijvoorbeeld patiënten zijn met alleen anti-β2GPI autoantistoffen of anti-β2GPI autoantistoffen en een lupus anticoagulant.

De rol van het eiwit β2GPI in APS is ontdekt in de jaren 90 door Galli, Matsuura en McNeil, en is gedefinieerd als een **cofactor** plasma eiwit betrokken in APS. Het is in een hoge concentratie aanwezig in plasma en is goed geconserveerd in de evolutie. Toch is de fysiologische functie onbekend. β2GPI is opgebouwd uit vijf **domeinen** en het vijfde domein bevat een bindingsplaats voor negatief geladen fosfolipiden, bestaande uit een cluster van positief geladen lysines. Na binding verandert het

eiwit van vorm, de circulaire structuur wordt een J-vorm en er wordt een **cryptisch epitoop** geëxponeerd welke gelegen is in het eerste domein. Tegen het cryptische epitoop kunnen antilichamen ontstaan die uiteindelijk karakteristiek zijn voor het syndroom. De antistoffen zijn verantwoordelijk voor de klinische verschijnselen van het syndroom.

Dit proefschrift

Dit proefschrift behandelt lupus anticoagulant en zijn relatie tot trombose in het antifosfolipiden syndroom. In dit proefschrift is onderzoek gedaan naar de effecten van de autoantistoffen op de verlengde stoltijd (lupus anticoagulant) en de correlatie met trombose of beroerte om uiteindelijk APS beter te diagnosticeren en waarom er een risico ontstaat voor trombose beter te begrijpen.

De algemeen geaccepteerde verklaring voor het ontstaan van een lupus anticoagulant is dat de aanwezige autoantistoffen samen met β2GPI binden aan **anionische fosfolipiden** en daardoor voorkomen dat stolfactoren kunnen binden aan deze voor een optimale stolling noodzakelijke **katalysatoren**. Het is erg tegenstrijdig dat er tijdens diagnose een verlengde stoltijd wordt gevonden wat duidt op bloedingen terwijl deze patiënten last hebben van trombose en zwangerschapsmorbiditeit. In **hoofdstuk 2** is op deze tegenstrijdigheid ingegaan en wordt er beschreven wat er tot nu toe over lupus anticoagulant bekend is. Lupus anticoagulant is een verlengde stoltijd die fosfolipiden afhankelijk is en wordt veroorzaakt door een **antistof**. Van de drie criteria voor het antifosfolipiden syndroom (lupus anticoagulant, anti-β2GPI autoantistoffen en anti-cardiolipine antistoffen) correleert lupus anticoagulant het beste met trombose. Omdat lupus anticoagulant het best correlerende criterium is, is het van belang om goed uit te zoeken wat het mechanisme achter lupus anticoagulant is wat voor meer begrip over het mechanisme kan zorgen welke uiteindelijk leidt tot een betere behandeling.

Een van de veroorzakers van het antifosfolipiden syndroom zijn infectieziekten. In de sub-Sahara in Afrika komen veel infectieziekten voor en is het voorkomen van herseninfarcten hoger dan in de Westerse wereld. We veronderstelden dat de infectieziekten zorgden voor de aanwezigheid van anti-β2GPI antistoffen, anticardiolipin antistoffen en lupus anticoagulant, en dat dit de toegenomen incidentie van herseninfarcten zou kunnen verklaren. De standaard risico factoren zoals hoge bloeddruk, **diabetes** en roken werden in het onderzoek meegenomen. In **hoofdstuk 3** hebben we onderzocht of er een verband is tussen antifosfolipiden antistoffen en beroertes in Tanzania. In deze **cohort** studie hebben we een hoge

correlatie gevonden tussen lupus anticoagulant en het risico op een beroerte. Dit was vooral sterk aanwezig in jonge en middelmatige leeftijdscategorieën. In deze studie hebben we gevonden dat lupus anticoagulant een nog niet herkend risico factor is voor beroertes in de sub-Sahara in Afrika buiten de standaard risico factoren om. Door de omvang van onze studie kunnen we een goede indicatie geven over de betrokkenheid van lupus anticoagulant in beroertes, echter om conclusies te trekken over de rol van infectieziekten bij het ontstaan van antifosfolipiden antistoffen dient er een grotere vervolg studie te worden uitgevoerd.

Eerder was al genoemd dat infectieziekten de veroorzaker kunnen zijn van het antifosfolipiden syndroom en de bijbehorende autoantistoffen, dit geldt ook voor een *Streptococcus pyogenes* infectie. Eerder onderzoek heeft laten zien dat deze infectie kan leiden tot anti-β2GPI autoantistoffen die specifiek gericht zijn tegen het cryptisch epitoop in het eerste domein. In ons onderzoek hebben wij de invloed van deze autoantistoffen op trombose in muizen onderzocht (**hoofdstuk 4**). Om deze autoantistoffen op te wekken tegen β2GPI hebben we de muizen geïnjecteerd met twee eiwitten van ***Streptococcus pyogenes*** (M1-protein (controle) en protein H (pathogeen)). Beide eiwitten kunnen β2GPI binden, echter alleen protein H zorgt voor de noodzakelijke **conformatieverandering** van β2GPI. Het bleek dat de muizen ingespoten met protein H inderdaad autoantistoffen hebben ontwikkeld, gericht tegen het eerste domein van β2GPI, en dat het aantal muizen met een afgesloten bloedvat hoger was. Uit deze data konden we concluderen dat de muizen met autoantistoffen gericht tegen het eerste domein van β2GPI meer trombose hadden gekregen dan de muizen zonder deze autoantistoffen. Dit houdt in dat autoantistoffen gericht tegen het eerste domein van β2GPI in staat zijn het risico op trombose te verhogen. Deze resultaten komen overeen met correlatie studies uitgevoerd bij APS patiënten.

Het eiwit β2GPI komt voor in verschillende vormen, een belangrijke conformatieverandering vindt plaats als het eiwit bindt aan negatief geladen oppervlakten zoals anionische fosfolipiden. De circulaire vorm (O-vorm) gaat over in een uitgestrekte vorm (J-vorm). Om een onderscheid te kunnen maken tussen beide vormen hebben we in **hoofdstuk 5** een **nanobody** geselecteerd tegen het vijfde domain die dit mogelijk maakt. Met deze nanobody is aangetoond dat in patiënten met APS weinig J-vorm aanwezig is van β2GPI in plasma. Ongeveer 6% van het circulerend β2GPI is in de J-vorm en we vonden geen verschil tussen gezonde vrijwilligers en patiënten met APS. De aanwezigheid van een cryptisch epitoop in domein V van β2GPI is een aanvullend bewijs voor het bestaan van een O-vorm van β2GPI. We konden geen specifieke functie vinden voor het cryptisch

epitoop in domain V. Verder onderzoek door middel van **epitoop mapping** is nodig om te achterhalen waar onze nanobody bind en wat dus het cryptische epitoop is waaraan onze nanobody bindt.

Lupus anticoagulant is de beste assay voor het voorspellen van trombose. De gangbare theorie om lupus anticoagulant te verklaren is competitie mat stolfactoren voor anionische fosfolipiden. Echter, deze theorie is nooit overtuigend bewezen en er zijn indicaties die tegen deze theorie spreken. Zo binden stolfactoren aan fosfolipiden in **hexagonale I fase** terwijl β2GPI juist bindt aan fosfolipiden in de **hexagonale II fase**. Wij hebben in **hoofdstuk 6** uitgezocht waarom patiënten met het antifosfolipiden syndroom een verlengde stoltijd (lupus anticoagulant) hebben. Het samenspel van eiwitten in de bloedstolling wordt verstoord door **complexen** van β2GPI met autoantistoffen tegen dit eiwit. We vonden dat de activatie van **factor V** door **factor Xa** wordt verhinderd waardoor er minder **trombine** gevormd kan worden. Dit heeft tot gevolg dat antifosfolipiden syndroom patiënten een verlengde stoltijd (lupus anticoagulant) hebben. Dit verklaard nog niet het verhoogde risico op trombose en zwangerschapsmorbiditeit. We hebben verder onderzoek gedaan om dit te begrijpen en vonden dat complexen van β2GPI met autoantistoffen tegen dit eiwit, de functie van factor V als cofactor in de afbraak van **factor VIII** door **actief proteïne C** kunnen verhinderen. De antistoffen zorgen dus voor een factor VIII afhankelijke proteïne C resistentie en dit zou een verklaring kunnen zijn voor veneuze trombose. De verklaring die wij hebben gevonden is niet van toepassing op alle patiënten maar alleen voor patiënten met β2GPI afhankelijke antistoffen, en of het de belangrijkste oorzaak is moet verder worden uitgezocht.

Conclusies
Meer kennis over het mechanisme en de invloed van β2GPI op de verlengde stoltijd (lupus anticoagulant) en de invloed van autoantistoffen hierop kan de behandeling van patiënten met APS verbeteren. Dit proefschrift beschrijft de verkregen kennis over de correlatie van lupus anticoagulant met beroerte en geeft meer inzicht in het mechanisme achter lupus anticoagulant. Op deze manier levert het proefschrift aanknopingspunten voor verder onderzoek naar een nog veelal onbegrepen ziekte. Dit kan op den duur leiden tot betere behandeling en medicatie voor patiënten met het antifosfolipiden syndroom.

Verklarende woordenlijst

Actief proteïne C	Antistollingsfactor; is in staat factor V en VIII te knippen en de vorming van een bloedpropje vertraagt. Met te weinig actief proteïne C kan er trombose ontstaan.
Anionische fosfolipiden	Een negatief geladen structurele lipide betrokken als bouwonderdeel bij cellen in het membraan.
Antifosfolipiden syndroom	Auto-immuunziekte gekenmerkt door trombose.
Arteriële trombose	Trombose in een slagader.
(Auto)antistoffen	Immunoglobuline; eiwitten geproduceerd als reactie op lichaamsvreemde stoffen. In het geval van autoantistoffen ziet je lichaam zijn eigen cellen en stoffen als lichaamsvreemd.
Auto-immuun ziekte	Je lichaam maakt antistoffen tegen eigen cellen en stoffen omdat die als lichaamsvreemd worden gezien.
β2-glycoproteïne I	Plasma eiwit dat kan binden aan negatief geladen oppervlakten en een rol speelt in het antifosfolipiden syndroom.
Bloedingsneiging	Een beschadiging van een bloedvat waardoor het bloed naar buiten treedt en dit niet voldoende gestopt kan worden door bloedstolling.
Bloedplaatjes	De kleinste cellen in het bloed zonder celkern, welke zorgen voor het dichten van de wond.
Cardiolipine	Is een fosfolipide aanwezig in de mitochondriën van cellen.
Cofactor	Nodig voor biologische activiteit van een ander eiwit welke gewoonlijk enzymen zijn betrokken bij biochemische reacties.
Cohort	Een groep mensen die een gemeenschappelijk karakteristiek hebben.

Complexen	Een verbinding tussen meerdere eiwitten.
Conformatieverandering	Een eiwit dat verandert van vorm door een externe oorzaak.
Cryptisch epitoop	Epitoop dat niet beschikbaar is wanneer een eiwit in zijn 'normale' toestand circuleert en pas wordt geëxponeerd als het eiwit van vorm verandert door een externe oorzaak.
Diabetes	Suikerziekte; Ziekte gekenmerkt door verhoogde bloedglucose waarden.
Domeinen	Een terugkerend patroon van opeenvolgende aminozuren in een eiwit.
Eiwitten	Biologische moleculen bestaande uit ketens van aminozuren verbonden via peptidebindingen (chemische verbinding).
Enzym	Een eiwit dat fungeert als katalysator bij een chemische reactie in of buiten de cel. Het enzym maakt de reactie mogelijk en kan zorgen voor een versnelling van de reactie.
Epitoop	Deel van een eiwit dat herkent wordt door o.a. antistoffen.
Epitoop mapping	Identificeren van de bindingsplekken (epitopen) van antistoffen op hun target.
Factor V	Eiwit betrokken in de stolling, welke niet enzymatisch actief is maar helpt als een cofactor.
Factor VIII	Essentiële cofactor betrokken in de stolling. Een defect/afwezigheid van dit eiwit veroorzaakt bloedingsziekten zoals hemofilie.
Factor Xa	Eiwit met een essentiële rol in de stolling, welke enzymatisch actief is.

Fosfolipiden	Een structurele lipide betrokken als bouwonderdeel bij cellen in het membraan. Bestaan uit een hydrofiele kop (positief geladen) en hydrofobe staart (negatief geladen).
Hexagonale I fase	Moleculaire organisatie van fosfolipiden met hydrofobe staart naar binnen.
Hexagonale II fase	Moleculaire organisatie van fosfolipiden met hydrofiele staart naar binnen.
Katalysatoren	Een stof die de snelheid van een chemische reactie beïnvloedt zonder zelf verbruikt te worden.
Lupus anticoagulant	Een verlengde stoltijd die kan worden geneutraliseerd door het toevoegen van extra fosfolipiden.
Nanobody	Fragment van een antistof die het antigeen herkend. De antistof wordt opgewekt in lama's en de selectie en productie vindt plaats door middel van moleculaire biologie.
Plasmine	Een glycoproteïne dat een grote rol speelt in de fibrinolyse.
Stollingsinhibitor	Een eiwit dat kan binden aan een enzym in de bloedstollingscascade waardoor het zijn activiteit verliest.
Streptococcus pyogenes	Een bacterie die ziekten kunnen veroorzaken in het menselijk lichaam, van een huidinfectie tot aan een systemische infectie.
Trombine	Onderdeel van de bloedstollingscascade en zorgt voor de activatie van bloedplaatjes en fibrine draden.

Trombose	Een aandoening waarbij er in de bloedvaten een bloedstolsel, de zogenaamde **trombus**, gevormd wordt, waardoor de bloedtoevoer van het achterliggend weefsel wordt geblokkeerd. Dit kan leiden tot het afsterven van het achterliggende weefsel.
Trombus	Bloedprop in een intact bloedvat.
Veneuze trombose	Trombose in een ader of in de longslagader.
Zwangerschapsmorbiditeit	Aandoeningen tijdens de zwangerschap.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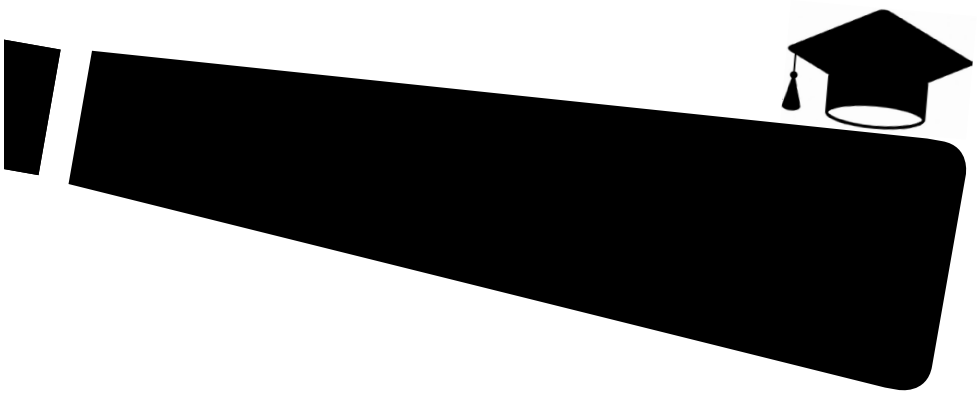
Appendices



Curriculum Vitae

Jessica Molhoek was born on the 30th of May 1989 in Amstelveen, The Netherlands. In 2007 she finished her VWO education focused on Health and Science at Helen Parkhurst Almere, and started her Bachelor's program Biomedical Sciences at Vrije Universiteit Amsterdam. She obtained her degree in 2010 and started a Master's program in Biomolecular Sciences at Vrije Universiteit Amsterdam. During her Master's, she completed a 7-month research project at the Department of Cell Biology II at the Netherlands Cancer Institute. This project was aimed to develop a method to study antigen cross presentation using Green Fluorescent Protein. After this, she started her 6-month research project at the Department of Experimental Immunology at the Academic Medical Center Amsterdam. This project was aimed to study the influence of DC-SIGN signaling in HIV-1 and the influence of HIV-1 on Th1/Th2 skewing. She obtained her Master's degree in 2012 and started her PhD project in 2013 at the Department of Clinical Chemistry and Haematology at University Medical Center Utrecht. In her project she worked on lupus anticoagulant and its relation to thrombosis in the antiphospholipid syndrome. The results of this work are presented in this thesis. She continued her career as postdoctoral researcher at the Department of Translational Immunology, where she investigates new markers to identify minimal residual disease (MRD) in acute myeloid leukemia (AML) patients.

List of publications

Publications from this thesis

de Mast Q*, **Molhoek JE***, van der Ven AJ, et al. *Antiphospholipid Antibodies and the Risk of Stroke in Urban and Rural Tanzania: A Community-Based Case-Control Study.* Stroke 2016;47:2589-2595.
*Authors contributed equally

Molhoek JE, de Groot PG and Urbanus RT. *The lupus anticoagulant paradox.* Accepted in Seminars in Thrombosis and Hemostasis. June 2017.

Molhoek JE, de Groot PG, Verhoef S, et al. *Beta2-glycoprotein I antibodies cause lupus anticoagulant and activated protein C resistance via coagulation factor V.* Submitted to Journal of Thrombosis and Haemostasis. July 2017.

Other publications

Lauder SN, Allen-Redpath K, Slatter DA, Aldrovandi M, O'Conner A, Farewell D, Percy CL, **Molhoek JE** et al. Networks of pro-coagulant enzymatically-oxidized phospholipids (eoxPL) from circulating blood cells are elevated and immunogenic in antiphospholipid syndrome. *Accepted in Science Signaling*. July 2017.

Selected abstracts

de Mast Q*, **Molhoek JE***, van der Ven AJ, et al. *Anti-phospholipid antibodies and the risk of ischemic stroke in Tanzania: results from a community-based case-control study.* Oral presentation; Dutch Society for Thrombosis and Haemostasis symposium, Koudekerke, the Netherlands, April 2015.
*Authors contributed equally

Molhoek JE, de Groot PG, Verhoef S, et al. *Lupus anticoagulant activity can be explained by interference of anti-B2GPI antibodies with the prothrombinase complex.* Oral presentation; International Society for Thrombosis and Haemostasis XXV Congress, Toronto, Canada, June 2015.

de Mast Q*, **Molhoek JE***, van der Ven AJ, et al. *Anti-phospholipid antibodies and the risk of ischemic stroke in Tanzania: results from a community-based case-control study.* Poster presentation; International Society for Thrombosis and Haemostasis XXV Congress, Toronto, Canada, June 2015.
*Authors contributed equally

Molhoek JE, de Groot PG, Verhoef S, et al. *Anti-beta2 glycoprotein I antibodies cause lupus anticoagulant and activated protein C resistance through an interaction with coagulation factor V.* Poster presentation; European Congress on Thrombosis and Haemostasis, Den Haag, the Netherlands, September 2016.

Awards

Molhoek JE, de Groot PG, Verhoef S, et al. *Lupus anticoagulant activity can be explained by interference of anti-B2GPI antibodies with the prothrombinase complex.* International Society for Thrombosis and Haemostasis XXV Congress, Toronto, Canada, June 2015.
Young Investigator Award

de Mast Q*, **Molhoek JE***, van der Ven AJ, et al. *Anti-phospholipid antibodies and the risk of ischemic stroke in Tanzania: results from a community-based case-control study.* Dutch Society for Thrombosis and Haemostasis symposium, Koudekerke, the Netherlands, April 2015.
*Authors contributed equally
Award of Scientific Excellence for Clinical Research

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Oplossing:_____

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ZONNE

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