Arterial thrombosis in the antiphospholipid syndrome

Rolf T. Urbanus

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Arterial thrombosis in the antiphospholipid syndrome

Arteriële trombose in het anti-fosfolipiden syndroom
(met een samenvatting in het Nederlands)

Proefschrift

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Just ’cause you feel it
doesn’t mean it’s there

Radiohead

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1

Current insight into diagnostics and pathophysiology of the antiphospholipid syndrome

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Summary

The diagnosis of the antiphospholipid syndrome, a non-inflammatory autoimmune disease characterized by thrombosis or pregnancy morbidity in the presence of antiphospholipid antibodies, depends greatly upon laboratory diagnostics. The diagnostic value of all available assays to detect antiphospholipid antibodies and the anticardiolipin assay in particular, is a matter of ongoing debate. Although the presence of lupus anticoagulant correlates best with thrombosis, accurate determination is not always possible due to anticoagulant treatment. Data on the predictive value of alternatives such as the anti-β_2-glycoprotein I and the anti-prothrombin antibody assay are insufficient and prospective cohort studies are needed. Determining antiphospholipid antibody profiles seems to increase diagnostic specificity.

Substantial progress has been made in unravelling the pathophysiological mechanisms underlying the antiphospholipid syndrome. Several cellular receptors for antibody-β_2-glycoprotein I complexes have been identified and their roles in cellular activation are being investigated. In vivo data should provide more insight into the importance of the interaction with individual receptors.

Introduction

The antiphospholipid syndrome is defined as the association between the persistent presence of circulating antiphospholipid antibodies in plasma and the presence of a history of thrombosis and /or pregnancy morbidity including foetal loss.[1] The first description of antiphospholipid antibodies dates back to 1952, when Moore et al.[2] described patients suffering from Systemic Lupus Erythematosus (SLE) with a persistently false positive VDRL flocculation test for syphilis, a test based on the detection of antibodies against a phospholipid extracted from beef heart called cardiolipin. In the same year, Conley et al.[3;4] described two SLE patients with a peculiar circulating inhibitor of coagulation. These anticoagulants could inhibit in vitro coagulation assays, did not influence the activity of coagulation factors and were not associated with a bleeding diathesis. Feinstein and Rapaport introduced the term lupus anticoagulant (LAC) to describe this phenomenon in 1972.[5] Although the relation between thrombosis and the presence of these anticoagulants in SLE patients was already noticed in 1963,[6] it took until 1980 before the association between LAC and thrombosis was widely recognized.[7] As LAC was found to be associated with a persistently false positive syphilis test, this led to the development of an anticardiolipin immunoassay and the establishment of the association between thrombosis and anti-cardiolipin antibodies.[8] From then on, patients presenting with thrombosis and / or pregnancy loss in combination with persistently positive anti-cardiolipin antibodies and / or circulating LAC were considered to have the antiphospholipid syndrome.[9] Originally described in patients with SLE, it was soon recognized to occur in patients without underlying autoimmune disease as well.

Although many researchers took an interest in the syndrome, classification criteria for patient inclusion in clinical studies were lacking. It was not until 1999 that an international consensus meeting formulated the definitive classification criteria for patients with the antiphospholipid syndrome, contributing greatly to the value of experimental data obtained from population studies.[9] Since then, these criteria have been updated in 2004 at another international consensus meeting (table 1).[1] One of the goals of the classification criteria for the antiphospholipid syndrome was the exclusion of several clinical symptoms that had been associated with the syndrome over the years without being sufficiently based on clinical or experimental evidence. Amongst these were thrombocytopenia, haemolytic anaemia, livedo reticularis, transient cerebral ischemia, cardiac valve disease, chorea and migraine.[9] One of the missed opportunities of this consensus meeting, however, was that a thorough analysis of the scientific evidence for maintaining and extending the serological criteria was not performed. Although almost 25 years have passed since the original definition of the antiphospholipid syndrome, poor standardization of available diagnostic tools still is a major problem. The lack of a golden standard continues to complicate diagnostics. Despite difficulties in identifying the patients at risk for thrombosis, however, great progress is being made in the unravelling of the pathophysiological mechanisms underlying the antiphospholipid syndrome.

This review deals with the current insights in the available diagnostic assays and the pathophysiology of the syndrome.

Detection of antiphospholipid antibodies

The antiphospholipid syndrome is a unique syndrome as the diagnosis is dependent on the co-occurrence of only one clinical event: vascular thrombosis or pregnancy morbidity, and only one laboratory marker: the persistent presence of antiphospholipid antibodies. Whether a patient has the syndrome or not is predominantly determined by the laboratory results, as the incidence and prevalence of the clinical features of APS are high in the general population. Not only is the standardisation of the available assays to detect the presence of antiphospholipid antibodies poor, the cut-off for a positive test, i.e. laboratory results higher than the 99th percentile of a normal population, automatically means that 1% of the population is positive for antiphospholipid antibodies. The risk of overdiagnosis of the antiphospholipid syndrome is great.

According to the Sapporo criteria[9] for the diagnosis of the antiphospholipid syndrome, antiphospholipid antibodies should be detected in one of two ways: either an enzyme-linked immunosorbent assay (ELISA) in which antibodies against cardiolipin-bound β_2-Glycoprotein I (β_2-GPI) are detected, or a sensitive assay in which phospholipid dependent coagulation inhibitors can be picked up. The recent Sydney update on the Sapporo criteria has added a direct anti-β_2-GPI ELISA to this list.[1] To minimize the risk of detection of transiently present antiphospholipid antibodies, the recommendations are to perform assays twice, with different samples, taken at least twelve weeks apart.

Despite efforts to standardize assays for the diagnosis of the antiphospholipid syndrome, the inter-laboratory variation in test results remains great especially for the presence of weak antibodies,[10-12] making the diagnosis of the syndrome dependent on the laboratory that performs the assay. Due to the consequences for treatment of patients diagnosed with the antiphospholipid syndrome, this is an unwanted situation. Efforts should be made to improve standardization and increase the specificity of diagnostic assays for the antiphospholipid syndrome.

Lupus anticoagulant

Tests for LAC are based on the principle that antiphospholipid antibodies compete with vitamin K-dependent clotting factors for binding sites on anionic phospholipids, thereby increasing coagulation time. Since the amount and composition of the phospholipids are qualifying determinants in this assay, it is important to minimize the amount of residual platelets in the test plasmas. As no available test for LAC is 100% sensitive, the recommendations are to perform at least two different assays.[13]

Several tests are available to measure LAC, all with different sensitivity. The test most often used is based on the activated partial thromboplastin time (APTT) with lower

phospholipid content, the dilute APTT, making it more sensitive for phospholipid-dependent coagulation inhibitors. The sensitivity of an APTT for LAC, however, also depends on the activator used. Tests using ellagic acid as an activator are less sensitive for lupus anticoagulant than tests employing other activators, such as kaolin or silica.[14-16] A second popular assay to detect lupus anticoagulant is the dilute Russell's viper venom time (dRVVT), an assay that depends on the activation of coagulation factor X by a snake venom.

The presence of lupus anticoagulant should always be confirmed by performing the same assay in the presence of excess phospholipids, during which the prolongation of the clotting time should be corrected. The absolute percentage of correction, i.e. the ratio between the clotting time without and with excess phospholipids, indicative for the presence of a lupus anticoagulant, however, is still a matter of debate.[17]

LAC has been shown to correlate much better with the occurrence of thrombo-embolic events and pregnancy morbidity than the anticardiolipin or the anti-β_2-GPI assay.[18;19] The relation between LAC and venous thrombosis has been studied more extensively than the relation between LAC and arterial thrombosis.[18] Antibody subsets responsible for the lupus anticoagulant effect overlap with, but are not the same as, the antibodies detected in the anticardiolipin assay,[20;21] which is partly due to anti-prothrombin antibodies that, while causing a LAC effect, are not detected in the anticardiolipin assay. To discriminate between the two major antibody subsets in LAC positive patients, the groups of Simmelink et al.[22] and Pengo et al.[23] devised two assays based on the phospholipid binding characteristics of β_2-GPI. Using the assay described by Simmelink et al.,[22] it was shown that anti-β_2-GPI dependent lupus anticoagulants correlate better with a history of thrombo-embolic events than β_2-GPI independent lupus anticoagulants,[24] which supports the idea that β_2-GPI is the most relevant antigen in the syndrome.

Although correlating best with the occurrence of thrombotic events, the lupus anticoagulant assay has one major drawback. Based on the process of coagulation, the assay is sensitive for the presence of anticoagulant drugs, such as vitamin K antagonists and heparin. Even though the recommendations are to dilute patient plasma with normal pooled plasma to normalize clotting factor levels, a high INR (> 3.0) makes accurate determination of the presence of lupus anticoagulant impossible. As a result, confirmation of the diagnosis of APS in patients presenting with a thrombotic event with this assay becomes difficult once the patient is treated with anticoagulant drugs. The anti-β_2-GPI ELISA might therefore be favoured during oral anticoagulant treatment above the lupus anticoagulant.

Anticardiolipin antibodies

The diagnostic value of the anticardiolipin assay is currently under debate. Due to the setup of the assay, the anticardiolipin ELISA has several methodological problems. The coating of an ELISA tray with a reproducible phospholipid layer is not easy. Furthermore, a sample is considered positive when anticardiolipin levels are above the 99th

percentile of the normal population, which means that by definition 1 % of the entire population will test positive. In itself this would not represent a problem, as this is true for most reference values for diagnostic purposes. When the clinical criteria on which the diagnosis depend have as high a prevalence and incidence as is the case for thrombosis or pregnancy morbidity, a cut-off level of the 99th percentile results in decreased assay specificity. To discriminate between patients at high risk for thrombosis and those with a normal risk, it is argued that patients at risk tend to have higher anticardiolipin antibody levels than healthy individuals.[25]

The Sapporo classification criteria for the antiphospholipid syndrome clearly stated that anticardiolipin antibodies should be β_2-GPI dependent,[9] a criterion that was, regrettably, omitted in the Sydney update.[1] The β_2-GPI dependency of anticardiolipin antibodies was added to the Sapporo criteria to increase assay specificity, as the risk of detecting infection related anticardiolipin antibodies would decrease. Using the Sydney criteria, however, infection related anticardiolipin antibodies can not be excluded.[1] One could argue that omission of β_2-GPI dependency from the criteria is understandable, as it is almost impossible to prove β_2-GPI dependency in the anticardiolipin assay. Al-

Table 1. Inclusion criteria for definitive antiphospholipid syndrome (Miyakis et al.1).

Clinical Criteria	
1	Vascular thrombosis
	One or more clinical episodes of arterial, venous, or small vessel thrombosis§, in any tissue or organ. Thrombosis must be confirmed by objective validated criteria (i.e. unequivocal findings of appropriate imaging studies or histopathology). For histopathologic confirmation, thrombosis should be present without significant evidence of inflammation in the vessel wall.
2	Pregnancy morbidity
	A) One or more unexplained deaths of a morphologically normal foetus at or beyond the 10th week of gestation, with normal foetal morphology documented by ultrasound or by direct examination of the foetus, or
	B) One or more premature births of a morphologically normal neonate before the 34th week of gestation because of: (i) eclampsia or severe pre-eclampsia defined according to standard definitions, or (ii) recognized features of placental insufficiency, or
	C) Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation, with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded.
Laboratory Criteria	
1	Lupus anticoagulant (LA) present in plasma, on two or more occasions at least 12 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Haemostasis (Scientific Subcommittee on LAs/phospholipid-dependent antibodies).[120;121]
2	Anticardiolipin (aCL) antibody of IgG and/or IgM isotype in serum or plasma, present in medium or high titer (i.e. >40 GPL or MPL, or >the 99th percentile), on two or more occasions, at least 12 weeks apart, measured by a standardized ELISA.[25;122;123]
3	Anti-β_2-glycoprotein I antibody of IgG and/or IgM isotype in serum or plasma (in titer >the 99th percentile), present on two or more occasions, at least 12 weeks apart, measured by a standardized ELISA, according to recommended procedures.[124]
Definite antiphospholipid antibody syndrome is considered to be present if at least 1 of the clinical criteria and 1 of the laboratory criteria are met.	

though commercially available assays provide ELISA trays saturated with β_2-GPI, none of these assays provide the necessary control, which is the same assay in a β_2-GPI-free environment to rule out reactivity towards phospholipids themselves.

Apart from shortcomings in assay performance and interpretation, there are also epidemiological arguments against the use of the anticardiolipin ELISA for the detection of patients at risk for thrombosis. A meta-analysis of 25 studies on the correlation between antiphospholipid antibodies and thrombosis showed no correlation between anticardiolipin antibodies and venous thrombo-embolic events.[18] One could argue that the studies examined in this review often used home-made, non-standardized assays, making the results of this meta-analysis questionable. Furthermore, most studies included in that meta-analysis determined anticardiolipin antibodies only once, after the thrombo-embolic event. Two large prospective population-based studies, however, also failed to show an increased risk for venous thrombosis in the presence of anticardiolipin antibodies.[26;27] Moreover, several groups reported a lack of correlation between anticardiolipin antibodies and thrombosis since the publication of the meta-analysis.[28-30] Despite the apparent lack of correlation between anticardiolipin antibodies and thrombosis, a recent meta-analysis on the association between anticardiolipin antibodies and pregnancy morbidity reports a significant correlation with both early and late recurrent foetal loss.[19] The studies included in this analysis, however, suffer from the same lack of standardization as those studied in the meta-analysis by Galli et al.[18]

In its present application, the anticardiolipin assay does not seem to be the assay of choice for detection of the antiphospholipid syndrome.

Anti-β_2-Glycoprotein I antibodies

Although recently added to the diagnostic criteria for the antiphospholipid syndrome, the value of the direct anti-β_2-GPI antibody ELISA in diagnosing the antiphospholipid syndrome remains to be established. In theory, the direct anti-β_2-GPI antibody ELISA should be preferred over the anticardiolipin assay, as the assay has less standardization issues with only β_2-GPI coated on a plate; not a sequence of cardiolipin followed by β_2-GPI, and no need for an internal control, i.e. elimination of antibodies that interact with cardiolipin. Although there is room for improvement in standardization of anti-β_2-GPI assays, a comparison of different anti-β_2-GPI antibody assay kits currently on the market show the results of these different assays are in better agreement when measuring the same samples than anticardiolipin antibody assays.[31;32] Despite improved assay performance compared to the anticardiolipin assay, the anti-β_2-GPI ELISA still has some weaknesses. The issue of the choice of the cut-off level between positive and negative values remains for the anti-β_2-GPI ELISA, as the occurrence of low avidity anti-β_2-GPI antibodies is quite common in the normal population. Furthermore, some commercial assays use bovine β_2-GPI preparations, which is not the protein of choice when searching for anti-human β_2-GPI antibodies. The ELISA tray used in the assay is another important issue, as the orientation of β_2-GPI coated on a surface appears to be related

to the antibodies detected.[33] In an effort to gain specificity by decreasing detection of low affinity anti-β_2-GPI antibodies, De Laat et al.[34] performed the anti-β_2-GPI ELISA using a high saline concentration in a well described patient cohort to identify the risk for thrombo-embolism. High saline concentrations decrease the binding of low affinity, charge-dependent anti-β_2-GPI antibodies, leaving only the high affinity anti-β_2-GPI antibodies for detection. Indeed, the risk for thrombotic complications was increased in patients with salt-resistant antibodies.

A large meta-analysis by Galli et al.[35] showed that the clinical value of the anti-β_2-GPI antibody ELISA as an independent risk factor for thrombosis is still uncertain. Several studies do show a relation between thrombosis and the presence of anti-β_2-GPI antibodies, but most of these studies are performed in patient groups with a history of autoimmune disease. A recent paper on the relation between anti-β_2-GPI antibodies and venous thrombosis in a general population showed an increased risk of a first episode of venous thrombosis for anti-β_2-GPI positive patients.[36] The role of anti-β_2-GPI antibodies in arterial thrombosis remains to be established. Prospective studies in a general population are lacking altogether. Although there is not much data available, no correlation was found between anti-β_2-GPI antibodies and pregnancy morbidity.[19]

Other "antiphospholipid" antibodies

A significant percentage of anti-prothrombin antibodies show lupus anticoagulant activity.[37] Whereas lupus anticoagulant is a known thrombotic risk factor, the currently available data on anti-prothrombin antibodies as risk factors for thrombosis are inconclusive. Some studies fail to establish anti-prothrombin antibodies as independent risk factors for thrombo-embolic events,[35;38] other studies do report an increased risk for thrombosis when anti-prothrombin antibodies are present.[29;39] Most studies only report an increased risk for thrombosis if antiprothrombin antibodies occur in conjunction with an established prothrombotic risk factor, such as LAC, or a systemic autoimmune disease.[30;36;40] The precise role of antiprothrombin antibodies as a marker for thrombosis needs to be determined.

Antibodies directed against Annexin A5 have also been shown to be present in plasma of some patients suspected of having the antiphospholipid syndrome. Although not much data are available, anti-Annexin A5 antibodies seem to be related to the occurrence of foetal loss.[30;41] Further studies are needed to clarify the role of anti-annexin A5 antibodies in the antiphosholipid syndrome.

Determine antibody profiles?

The currently available assays for detection of antiphospholipid antibodies identify patient subgroups at risk for thrombosis. Evidence is building that patients testing positive for more than one single antiphospholipid antibody assay have an increased risk of thrombosis[28;29;36;42;43] or pregnancy morbidity.[44] It is therefore argued that antibody profiles should be determined in patients suspected of having the antiphospholipid syn-

drome, rather than determining only lupus anticoagulant, anti-β_2-GPI antibodies or anticardiolipin antibodies. Although a combined positive test result seems to identify patients at high risk for thrombosis, it remains a matter of debate whether determining antibody profiles is indicated in every patient presenting with thrombosis or pregnancy morbidity, including those without a history of autoimmunity.

Future directions in the serology of the syndrome

Lupus anticoagulant is the best single test for antiphospholipid antibodies to detect patients at risk for thrombo-embolic complications and pregnancy morbidity. The diagnostic value of the anticardiolipin ELISA is currently under debate. It is doubtful whether every patient with thrombosis or pregnancy morbidity should be screened for anticardiolipin antibodies. Proof that a positive test result in only an anti-β_2-GPI antibody or only an antiprothrombin antibody assay has predictive value should also be provided. At the moment, the best available option to detect patients at risk for thrombotic events seems to be a combination of positive test results, in particular the combination of a positive LAC assay and a positive anti-β_2-GPI antibody ELISA. New assays, such as an ELISA that specifically detects antibodies directed against domain I of β_2-GPI[45] or assays specific for a β_2-GPI-dependent lupus anticoagulant,[23;24] seem to be very promising and results from multicenter studies on the correlation between these antibodies and the clinical manifestations are awaited. We do not know, however, whether these more specific assays will pick up all pathological antibodies, because we do not know whether only one subpopulation of antiphospholipid antibodies is responsible for all the clinical manifestations or whether there are different pathological subpopulations of antibodies with different clinical interferences. Studies on the pathophysiology of the syndrome should answer this question.

Pathophysiology

Major antigens in the antiphospholipid syndrome

Antiphospholipid antibodies are not, as the term implies, directed against phospholipids, but rather against a wide variety of protein cofactors. Amongst these are β_2-GPI, prothrombin,[37;46] protein C,[47] protein S,[47-49] annexin V[50] and coagulation factor XII.[51]
The 45 kDa plasma protein β_2-GPI is considered the most important antigenic target in the antiphospholipid syndrome.[52;53] Circulating at a concentration of 150-300 µg mL^{-1} in human plasma, β_2-GPI has no known physiological function. Although the protein has been ascribed both pro-[54;55] and anticoagulant[56-58] properties in vitro, humans[59] and mice[55] deficient in this protein do not have a phenotype. β_2-GPI, which circulates as a monomer, consists of five highly homologous complement control protein repeats, or sushi domains.[60-62] The fifth sushi domain contains a phospholipid insertion loop, which enables it to interact with anionic phospholipids such as phosphatidylserine or cardiolipin. As surfaces expressing anionic phospholipids are usually hidden from the

circulation, β_2-GPI can circulate freely.

Although anti-prothrombin antibodies can cause LAC, there is currently no convincing evidence that they are involved in the pathogenesis of the antiphospholipid syndrome.

Pathological antiphospholipid antibodies

Patients with the antiphospholipid syndrome have a highly heterogeneous pool of antibodies, most of which have only low to moderate affinity for β_2-GPI.[63] There is increasing evidence, however, that only the high affinity antibodies are pathologically relevant.[34] Interestingly, no one has been able to purify antibody-β_2-GPI complexes from patient plasma. The explanation is that native β_2-GPI first needs to bind a surface such as the anionic phospholipids exposed on the membrane of activated cells before antibodies can recognize it (Figure 1). Upon phospholipid binding, β_2-GPI changes conformation and exposes a cryptic epitope to which high affinity antibodies can bind.[64;65] Several epitopes have been described,[63;66-68] although the positively charged region surrounding Arginine 43 in the first domain of β_2-GPI seems to be the best candidate.[45;69-72] β_2-GPI is subsequently dimerized by the autoantibodies and is probably fixed in an activated conformation, as the affinity of the complex for anionic phospholipids is over a hundredfold greater than the affinity of monomer β_2-GPI.[73] Furthermore, the antibody-β_2-GPI complex is able to interact with several cellular receptors to which the circulating monomer does not bind.[74;75]

Cellular activation in the antiphospholipid syndrome

The antibody-β_2-GPI complex has been reported to bind several cell types, amongst others endothelial cells, monocytes and platelets, all of which play an important role in haemostasis. The list of potential binding sites for the β_2-GPI-antibody complex is ever increasing and includes Annexin A2,[76] Apolipoprotein E receptor 2' (ApoER2'),[74;77] Glycoprotein Ibα[78;79] (GPIbα), Low density lipoprotein Receptor related Protein (LRP),[75] Megalin,[75] Toll-like receptor 2 (TLR2),[80] Toll-like receptor 4 (TLR4),[81] the Very low density lipoprotein (VLDL) receptor[75] and P-selectin glycoprotein ligand-1 (PSGL-1).[82] Most of these receptors are expressed on various cell types in different combinations.

Endothelial cell activation in the antiphospholipid syndrome has been reported to be mediated by TLR4,[81] TLR2[83] and Annexin A2,[76;84] resulting in a prothrombotic and proinflammatory endothelial phenotype.[81;85-91] TLR4, a pattern recognition receptor involved in the innate immune response against LPS, has never been shown to directly interact with β_2-GPI. However, after incubation with antiphospholipid antibodies, cultured endothelial cells show the activation of a signalling pathway known to be downstream of TLR4.[81] In a set of elegant experiment using dominant negative mutants of the TLR signalling pathway, Raschi et al.[81] show involvement of the signalling molecules MyD88 and TRAF6 in endothelial cells incubated with antiphospholipid antibodies. Interestingly, data from in vitro studies on the effect of anti-β_2-GPI antibodies on the

activation of fibroblasts show a role for TLR2,[80] which also signals via MyD88,[92] and not for TLR4. Although there is substantial evidence that TLR signalling pathways are involved in endothelial activation by antiphospholipid antibodies, further studies are needed to clarify the role of the Toll-like receptor family.

Annexin A2 is a membrane bound protein without a transmembrane domain. Annexin A2 is a potent fibrinolytic receptor and binds tissue type plasminogen activator (tPA) and its ligand plasminogen.[93] It has also been shown to bind monomer β_2-GPI, without the need for the presence of anti-β_2-GPI antibodies.[76] Endothelial activation by the antibody-β_2-GPI complex has been described to occur via dimerization of Annexin A2, as F(ab')2 fragments of anti-β_2-GPI antibodies caused an equal amount of endothelial activation as the whole IgG molecule and single Fab fragments had no effect.[84] Due to the impossibility of Annexin A2 to transmit signals across the cell membrane, however, it is difficult to envision how the binding of antibody-β_2-GPI complexes could cause cellular activation. The most likely explanation is that other receptors are involved in the activation of the cell, whereas annexin A2 serves as a docking site for β_2-GPI on endothelial cells.

Monocytes have been shown to become activated upon stimulation with antiphospholipid antibodies, although experimental data on the receptor involved is lacking. Zhou et al.[94] report the involvement of Annexin A2, but the same arguments hold for monocyte activation as for endothelial cell activation: Annexin A2 cannot translate a signal from outside to inside the cell. Other receptors must be involved. Monocytes are regarded as the source of the majority of circulating TF-bearing microparticles.[95-97] Indeed, aside from the production of proinflammatory cytokines,[98] TF upregulation is a major feature of monocyte activation in the antiphospholipid syndrome.[99–103] The involvement of the NFκB pathway in monocyte activation shows that monocytic TF exposure is due to de novo synthesis rather than to release of an intracellular storage pool.[104]

Platelet activation in the antiphospholipid syndrome has been extensively studied. Addition of antiphospholipid antibodies or a recombinant dimeric β_2-GPI construct mimicking the antibody-β_2-GPI complex[73] to blood resulted in increased platelet adhesion to a collagen surface in an in vitro thrombosis model.[77] Later studies showed that this was mediated by ApoER2', the only member of the LDL-receptor family present on platelets, as addition of Receptor Associated Protein, an inhibitor of ligand binding to members of the LDL-receptor family, reduced platelet adhesion to background level.[74;77] Interestingly, two groups simultaneously identified a second receptor involved in platelet activation by antiphospholipid antibodies; the platelet adhesive receptor GPIbα.[78;79] Although both groups showed the importance of GPIbα in mediating platelet activation in the antiphospholipid syndrome, there are some marked differences in the reported binding sites for β_2-GPI on GPIbα. Shi et al.[79] report that β_2-GPI interacts via sushi domains II to V with the leucine rich repeats of GPIbα, thereby blocking the Von Willebrand Factor (vWF) binding site. This finding directly opposes previous work by

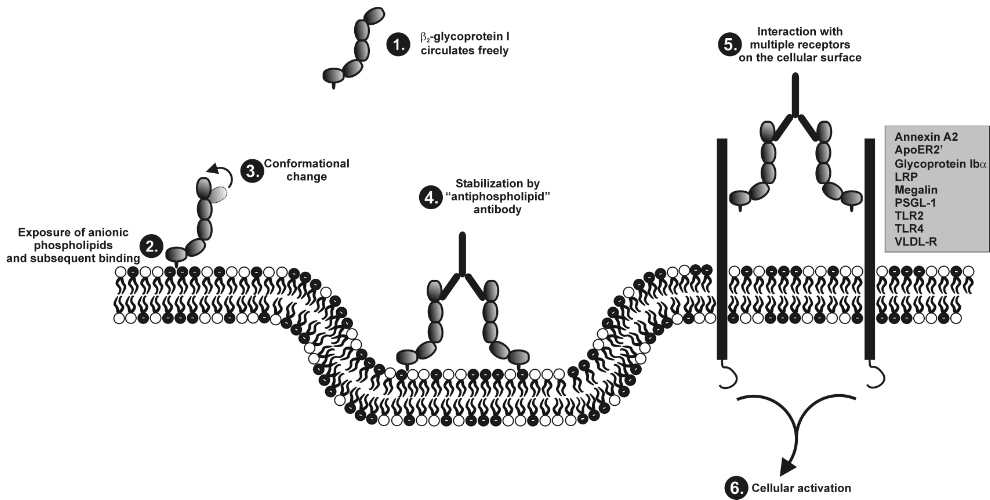

Figure 1. Chain of events in cellular activation by anti-β_2-glycoprotein I antibodies. 1. β_2-glycoprotein I circulates freely in its native conformation. **2.** Upon surface exposure of anionic phospholipids, β_2-glycoprotein I binds and **3.** undergoes a conformational change, exposing a cryptic epitope. **4.** Anti-β_2-glycoprotein I antibodies are now able to bind β_2-glycoprotein I at a region surrounding Arginine 43 on domain I, resulting in dimerization and stabilization of its conformation. **5.** The antibody-β_2-glycoprotein I complex is then able to interact with several cellular receptors. **6.** Interaction of the complex with these receptors results in cellular activation.

Lutters et al.,[77] where platelet adhesion to a collagen surface under conditions of flow at arterial shear stress, a process dependent upon the interaction between GPIbα and vWF, was shown to increase upon addition of antibody-β_2-GPI complexes to blood. Moreover, Pennings et al.[78] show that dimeric β_2-GPI interacts with GPIbα in close proximity to the thrombin binding site via its fifth domain. Despite differences in localizing the binding site on GPIbα, both groups assign a major role to the fifth domain of β_2-GPI in binding to GPIbα.

Activation of cells mediated by a simultaneous interaction with multiple receptors

If different cells can be activated via the interaction of antibody-β_2-GPI complexes with several different receptors, this might explain the wide distribution of thrombosis throughout the vasculature. All known other thrombotic risk factors show vascular bed specific thrombosis.[105] Both the members of Toll-like receptor family and the LDL-receptor family show an uneven distribution throughout the vasculature, but they can be found in almost every type of vessel. It is conceivable that the observed thrombotic risk is due to interactions of β_2-GPI-antibody complexes with different receptors, depending on the local availability of certain receptors. It is, however, incomprehensible why β_2-GPI-antibody complexes, and not β_2-GPI alone, have such a broad specificity for different receptor families. Apparently, the antibodies induce a pattern recognition site in β_2-GPI.

While the interaction of β_2-GPI with members of the Toll-like receptor family still needs to be shown, the binding characteristics of dimeric β_2-GPI with the LDL-receptor family have been determined.[75] LRP, which is regarded as an endocytic receptor, might be responsible for the clearance of surface bound antibody-β_2-GPI complexes, while VLDL-R and ApoER2 might be involved in activation mechanisms. Perhaps the interaction of the antibody-β_2-GPI complex with a member of the LDL-R family and the Toll-like receptor family is important in mediating endothelial and leukocyte activation. Interestingly, platelet GPIbα shows structural similarities to the Toll-like receptor family as both contain leucine rich repeats in their ectodomain.[106]

Inflammation; a link to thrombosis?

The inclusion criteria for the diagnosis of the antiphospholipid syndrome clearly state that evidence of histological inflammation near an occluded vessel is a reason for exclusion.[1] Nevertheless, there is increasing experimental evidence that antiphospholipid antibodies influences inflammation in the microenvironment.[81;86;88;89;107] Data from in vitro experiments show up-regulation of adhesive receptors such as VCAM-1[68;86;89] and E-selectin[68;86;89;90;107] upon incubation of human umbilical vein endothelial cells with antiphospholipid antibodies. Increased production of cytokines such as interleukine-6[85;91;107] (IL-6) by endothelial cells has also been reported. Several groups have shown increased adhesion of leukocytes to activated endothelium in in vivo[89] and in vitro[90] settings. As leukocytes, and especially monocytes and neutrophils, are important sources of Tissue Factor (TF), the inflammatory system could play a role in pathology of the antiphospholipid syndrome without overt histological inflammation of the vasculature. Interestingly, IL-6 is known to induce monocytic TF production.[108] Another key player in innate immunity, the complement system, has recently been linked to coagulation. C5a, an important mediator of the inflammatory response, has been shown to induce the expression of TF on endothelial cells[109] and neutrophils.[110] The complement system has also been associated with thrombosis and foetal loss in the antiphospholipid syndrome. Fischetti et al.[111] show involvement of the complement system in fibrin deposition in a growing thrombus in an in vivo thrombosis model in rats after injection of antiphospholipid antibodies, presumably through the induction of TF expression. Likewise, C5a is thought to trigger TF production by neutrophils, leading to inflammation of placental tissue in mice injected with antiphospholipid antibodies, which results in foetal loss.[112]

Involvement of Fc-receptors

Surface bound antibodies are prone to cause cellular activation via Fc-receptors.[113] As antiphospholipid antibodies are predominantly of the IgG2 isotype,[114] it has been hypothesized that the FcγRIIa might be involved in the pathology of the antiphospholipid syndrome. No such relationship has been found, however.[115] In vitro data do not support the involvement of Fc-receptors in the antiphospholipid syndrome either. Al-

though endothelial cell activation has mostly been studied by the addition of patient derived antiphospholipid antibodies or monoclonal anti-β_2-GPI antibodies, there is no evidence for involvement of Fcγ receptors; blockage of the most common endothelial Fc-receptor, FcγRIIa, does not influence the effect of antiphospholipid antibodies[90] and F(ab')$_2$ fragments of antiphospholipid antibodies have the same effects on cellular activation as the whole antibody.[116;117]

Second hit model

Patients with the antiphospholipid syndrome tend to suffer from occasional, highly localized thrombotic episodes, rather than presenting with widespread disseminated thrombosis, despite the persistent presence of antiphospholipid antibodies. This might be explained by the absence of antibody-β_2-GPI complexes in the circulation, as complex formation only takes place on anionic surfaces. As these negatively charged surfaces are usually not exposed to the circulation, priming of the vascular surface by cytokines, vascular injury or the induction of local apoptosis might be the initiating steps in inducing cellular activation by antiphospholipid antibodies. Experimental data suggest that antiphospholipid antibodies are not capable of cellular activation by themselves. The need for a priming factor has been shown both in vitro and in vivo; Fischetti et al.[111] show a growing thrombus after infusion of antiphospholipid antibodies in rats only after a prior stimulation with intraperitoneal LPS and Vega-Ostertag et al.[118] show platelet aggregation caused by antiphospholipid antibodies only in the presence of a suboptimal dose of thrombin. The possible involvement of TLR2 in endothelial cell activation is in line with this view, as this receptor is not constitutively expressed by endothelium in its quiescent state; only after activation is TLR2 expression upregulated.[119]

Unanswered questions regarding the pathophysiology of the syndrome

β_2-Glycoprotein I is the major antigen in the antiphospholipid syndrome. Although the circulating plasma protein is not able to interact with cellular receptors, once it is dimerized by auto-antibodies it gains several binding capabilities. The precise mechanisms underlying this change in affinity need to be investigated, not only because it may contain the clue why people develop anti-β_2-GPI antibodies, it can also provide vital information for the development of anti-thrombotic treatment for the antiphospholipid syndrome without the side-effects of the vitamin K antagonist currently in use. Knowledge of the precise amino acids involved in receptor binding might provide a valuable tool for the development of drugs.

Dimerized β_2-GPI has been shown to interact with several cellular receptors. As most of the data was generated in in vitro settings, animal models should provide more information on the importance of the interaction between dimerized β_2-GPI and each of its receptors.

Practice points:

- Lupus anticoagulant is the assay of choice to detect patients at risk for thrombosis.
- Determine antiphospholipid antibody profiles using the lupus anticoagulant and the anti-β_2-GPI antibody ELISA to detect patients at high risk.
- Always determine INR and check for the presence of heparin when performing Lupus Anticoagulant assays in anticoagulated patients; use the anti-β_2-GPI ELISA when INR > 3, as LAC cannot be reliably tested under these circumstances.

Research agenda

- Evaluation of the diagnostic value of the anticardiolipin assay.
- Validation of the anti-β_2-Glycoprotein I ELISA.
- Determine predictive value of all available antiphospholipid antibody assays for the occurrence of thrombosis and pregnancy complications in the general population.
- Development and validation of new reproducible assays with better sensitivity and specificity.
- Clarify which combination of receptors is responsible for activation of different cell types
- Develop inhibitors of cellular binding of β_2-GPI-antibody complexes.

References

1. Miyakis S, Lockshin MD, Atsumi T et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J.Thromb.Haemost. 2006;4:295-306
2. Moore JE, Mohr CF. Biologically false positive serologic tests for syphilis; type, incidence, and cause. J.Am.Med.Assoc. 1952;150:467-473.
3. Conley CL, Hartmann RC. A hemorrhagic disorder caused by circulating anticoagulant in patients with disseminated lupus erythematosus. J.Clin.Invest. 1952;31:621-622.
4. Conley CL. Disorders of the blood in disseminated lupus erythematosus. Am.J.Med. 1952;13:1-2.
5. Feinstein DI, Rapaport SI. Acquired inhibitors of blood coagulation. Prog.Hemost.Thromb. 1972;1:75-95.
6. Bowie EJ, Thompson JH, Jr., Pascuzzi CA, Owen CAJ. Thrombosis in systemic lupus erythematosus despite circulating anticoagulants. J.Lab Clin.Med. 1963;62:416-430.
7. Mueh JR, Herbst KD, Rapaport SI. Thrombosis in patients with the lupus anticoagulant. Ann.Intern. Med. 1980;92:156-159.
8. Harris EN, Gharavi AE, Boey ML et al. Anticardiolipin antibodies: detection by radioimmunoassay and association with thrombosis in systemic lupus erythematosus. Lancet 1983;2:1211-1214.
9. Wilson WA, Gharavi AE, Koike T et al. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop. Arthritis Rheum. 1999;42:1309-1311.
10. Kutteh WH, Franklin RD. Assessing the variation in antiphospholipid antibody (APA) assays: comparison of results from 10 centers. Am.J.Obstet.Gynecol. 2004;191:440-448.
11. Audrain MA, Colonna F, Morio F, Hamidou MA, Muller JY. Comparison of different kits in the detection of autoantibodies to cardiolipin and beta2glycoprotein 1. Rheumatology.(Oxford) 2004;43:181-185.
12. Reber G, Arvieux J, Comby E et al. Multicenter evaluation of nine commercial kits for the quantitation of anticardiolipin antibodies. The Working Group on Methodologies in Haemostasis from the GEHT (Groupe d'Etudes sur l'Hemostase et la Thrombose). Thromb.Haemost. 1995;73:444-452.
13. Brandt JT, Barna LK, Triplett DA. Laboratory identification of lupus anticoagulants: results of the Second International Workshop for Identification of Lupus Anticoagulants. On behalf of the Subcommittee on Lupus Anticoagulants/Antiphospholipid Antibodies of the ISTH. Thromb. Haemost. 1995;74:1597-1603.
14. Brandt JT, Triplett DA, Rock WA, Bovill EG, Arkin CF. Effect of lupus anticoagulants on the activated partial thromboplastin time. Results of the College of American Pathologists survey program. Arch.Pathol.Lab Med. 1991;115:109-114.
15. Brandt JT, Triplett DA, Musgrave K, Orr C. The sensitivity of different coagulation reagents to the presence of lupus anticoagulants. Arch.Pathol.Lab Med. 1987;111:120-124.
16. Denis-Magdelaine A, Flahault A, Verdy E. Sensitivity of sixteen APTT reagents for the presence of lupus anticoagulants. Haemostasis 1995;25:98-105.
17. Jacobsen EM, Barna-Cler L, Taylor JM, Triplett DA, Wisloff F. The Lupus Ratio test--an interlaboratory study on the detection of Lupus anticoagulants by an APTT-based, integrated, and semi-quantitative test. Fifth International Survey of Lupus Anticoagulants--ISLA 5. Thromb.Haemost. 2000;83:704-708.
18. Galli M, Luciani D, Bertolini G, Barbui T. Lupus anticoagulants are stronger risk factors for thrombosis than anticardiolipin antibodies in the antiphospholipid syndrome: a systematic review of the literature. Blood 2003;101:1827-1832.
19. Opatrny L, David M, Kahn SR, Shrier I, Rey E. Association between antiphospholipid antibodies and recurrent fetal loss in women without autoimmune disease: a metaanalysis. J.Rheumatol. 2006;33:2214-2221.
20. McNeil HP, Chesterman CN, Krilis SA. Anticardiolipin antibodies and lupus anticoagulants comprise separate antibody subgroups with different phospholipid binding characteristics. Br.J.Haematol. 1989;73:506-513.
21. Chamley LW, Pattison NS, McKay EJ. Separation of lupus anticoagulant from anticardiolipin antibodies by ion-exchange and gel filtration chromatography. Haemostasis 1991;21:25-29.
22. Simmelink MJ, Derksen RH, Arnout J, de Groot PG. A simple method to discriminate between beta2-glycoprotein I- and prothrombin-dependent lupus anticoagulants. J.Thromb.Haemost. 2003;1:740-747.
23. Pengo V, Biasiolo A, Pegoraro C, Iliceto S. A two-step coagulation test to identify antibeta-glycoprotein I lupus anticoagulants. J.Thromb.Haemost. 2004;2:702-707.
24. de Laat HB, Derksen RH, Urbanus RT, Roest M, de Groot PG. beta2-glycoprotein I-dependent lupus anticoagulant highly correlates with thrombosis in the antiphospholipid syndrome. Blood 2004;104:3598-3602.
25. Harris EN, Pierangeli SS. Revisiting the anticardiolipin test and its standardization. Lupus 2002;11:269-275.
26. Naess IA, Christiansen SC, Cannegieter SC, Rosendaal FR, Hammerstroem J. A prospective study of anticardiolipin antibodies as a risk factor for venous thrombosis in a general population (the HUNT study). J.Thromb.Haemost. 2006;4:44-49.
27. Runchey SS, Folsom AR, Tsai MY, Cushman M, McGovern PD. Anticardiolipin antibodies as a risk factor for venous thromboembolism in a population-based prospective study. Br.J.Haematol. 2002;119:1005-1010.
28. Pengo V, Biasiolo A, Pegoraro C et al. Antibody profiles for the diagnosis of antiphospholipid syndrome. Thromb.Haemost. 2005;93:1147-1152.
29. Bizzaro N, Ghirardello A, Zampieri S et al. Anti-prothrombin antibodies predict thrombosis in patients with systemic lupus erythematosus: a 15-year longitudinal study. J.Thromb.Haemost. 2007;5:1158-1164.
30. Galli M, Borrelli G, Jacobsen EM et al. Clinical significance of different antiphospholipid antibodies in the WAPS (warfarin in the antiphospholipid syndrome) study. Blood 2007;110:1178-1183.
31. Reber G, Tincani A, Sanmarco M, de Moerloose P, Boffa MC. Variability of anti-beta2 glycoprotein I antibodies measurement by com-

mercial assays. Thromb.Haemost. 2005;94:665-672.

32. Pengo V, Biasiolo A, Bison E, Chantarangkul V, Tripodi A. Antiphospholipid antibody ELISAs: survey on the performance of clinical laboratories assessed by using lyophilized affinity-purified IgG with anticardiolipin and anti-beta2-Glycoprotein I activity. Thromb.Res. 2007;120:127-133.
33. Iverson GM, Matsuura E, Victoria EJ, Cockerill KA, Linnik MD. The orientation of beta2GPI on the plate is important for the binding of anti-beta2GPI autoantibodies by ELISA. J.Autoimmun. 2002;18:289-297.
34. de Laat B, Derksen RH, de Groot PG. High-avidity anti-beta glycoprotein I antibodies highly correlate with thrombosis in contrast to low-avidity anti-beta glycoprotein I antibodies. J.Thromb. Haemost. 2006;4:1619-1621.
35. Galli M, Luciani D, Bertolini G, Barbui T. Anti-beta 2-glycoprotein I, antiprothrombin antibodies, and the risk of thrombosis in the antiphospholipid syndrome. Blood 2003;102:2717-2723.
36. de Groot PG, Lutters BC, Derksen RH et al. Lupus anticoagulants and the risk of a first episode of deep venous thrombosis. J.Thromb.Haemost. 2005;3:1993-1997.
37. Bevers EM, Galli M, Barbui T, Comfurius P, Zwaal RF. Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin. Thromb. Haemost. 1991;66:629-632.
38. Previtali S, Barbui T, Galli M. Anti-beta2-glycoprotein I and anti-prothrombin antibodies in antiphospholipid-negative patients with thrombosis: a case control study. Thromb.Haemost. 2002;88:729-732.
39. Forastiero R, Martinuzzo M, Pombo G et al. A prospective study of antibodies to beta2-glycoprotein I and prothrombin, and risk of thrombosis. J.Thromb.Haemost. 2005;3:1231-1238.
40. Hudson M, Herr AL, Rauch J et al. The presence of multiple prothrombotic risk factors is associated with a higher risk of thrombosis in individuals with anticardiolipin antibodies. J.Rheumatol. 2003;30:2385-2391.
41. Nojima J, Kuratsune H, Suehisa E et al. Association between the prevalence of antibodies to beta(2)-glycoprotein I, prothrombin, protein C, protein S, and annexin V in patients with systemic lupus erythematosus and thrombotic and thrombocytopenic complications. Clin.Chem. 2001;47:1008-1015.
42. Zoghlami-Rintelen C, Vormittag R, Sailer T et al. The presence of IgG antibodies against beta2-glycoprotein I predicts the risk of thrombosis in patients with the lupus anticoagulant. J.Thromb. Haemost. 2005;3:1160-1165.
43. Sailer T, Zoghlami C, Kurz C et al. Anti-beta2-glycoprotein I antibodies are associated with pregnancy loss in women with the lupus anticoagulant. Thromb. Haemost. 2006;95:796-801.
44. Ruffatti A, Tonello M, Del Ross T et al. Antibody profile and clinical course in primary antiphospholipid syndrome with pregnancy morbidity. Thromb.Haemost. 2006;96:337-341.
45. de Laat B, Derksen RH, Urbanus RT, de Groot PG. IgG antibodies that recognize epitope Gly40-Arg43 in domain I of beta 2-glycoprotein I cause LAC, and their presence correlates strongly with thrombosis. Blood 2005;105:1540-1545.
46. Fleck RA, Rapaport SI, Rao LV. Anti-prothrombin antibodies and the lupus anticoagulant. Blood 1988;72:512-519.
47. Oosting JD, Derksen RH, Bobbink IW et al. Antiphospholipid antibodies directed against a combination of phospholipids with prothrombin, protein C, or protein S: an explanation for their pathogenic mechanism? Blood 1993;81:2618-2625.
48. Ruiz-Arguelles GJ, Ruiz-Arguelles A, Perez-Romano B, arcon-Segovia D. Protein S deficiency associated to anti-protein S antibodies in a patient with mixed connective-tissue disease and its reversal by danazol. Acta Haematol. 1993;89:206-208.
49. Sorice M, Griggi T, Arcieri P et al. Protein S and HIV infection. The role of anticardiolipin and anti-protein S antibodies. Thromb.Res. 1994;73:165-175.
50. Nakamura N, Kuragaki C, Shidara Y, Yamaji K, Wada Y. Antibody to annexin V has anti-phospholipid and lupus anticoagulant properties. Am.J.Hematol. 1995;49:347-348.
51. Bertolaccini ML, Mepani K, Sanna G, Hughes GR, Khamashta MA. Factor XII autoantibodies as a novel marker for thrombosis and adverse obstetric history in patients with systemic lupus erythematosus. Ann.Rheum.Dis. 2007;66:533-536.
52. Galli M, Comfurius P, Maassen C et al. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. Lancet 1990;335:1544-1547.
53. McNeil HP, Simpson RJ, Chesterman CN, Krilis SA. Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2-glycoprotein I (apolipoprotein H). Proc.Natl.Acad.Sci.U.S.A 1990;87:4120-4124.
54. Mori T, Takeya H, Nishioka J, Gabazza EC, Suzuki K. beta 2-Glycoprotein I modulates the anticoagulant activity of activated protein C on the phospholipid surface. Thromb.Haemost. 1996;75:49-55.
55. Sheng Y, Reddel SW, Herzog H et al. Impaired thrombin generation in beta 2-glycoprotein I null mice. J.Biol.Chem. 2001;276:13817-13821.
56. Shi T, Iverson GM, Qi JC et al. Beta 2-Glycoprotein I binds factor XI and inhibits its activation by thrombin and factor XIIa: loss of inhibition by clipped beta 2-glycoprotein I. Proc.Natl.Acad. Sci.U.S.A 2004;101:3939-3944.
57. Hulstein JJ, Lenting PJ, de Laat B et al. Beta2-Glycoprotein I inhibits von Willebrand factor-dependent platelet adhesion and aggregation. Blood 2007;110:1483-1491.
58. Lopez-Lira F, Rosales-Leon L, Martinez VM, Ruiz Ordaz BH. The role of beta2-glycoprotein I (beta2GPI) in the activation of plasminogen. Biochim.Biophys. Acta 2006;1764:815-823.
59. Bancsi LF, van der Linden I, Bertina RM. Beta 2-glycoprotein I deficiency and the risk of thrombosis. Thromb.Haemost. 1992;67:649-653.
60. Bouma B, de Groot PG, van den Elsen JM et al. Adhesion mechanism of human beta(2)-glycoprotein I to phospholipids based on its crystal structure. EMBO J. 1999;18:5166-5174.
61. Schwarzenbacher R, Zeth K, Diederichs K et al. Crystal structure of human beta2-glycoprotein I: implications for phospholipid binding and the antiphospholipid syndrome. EMBO J. 1999;18:6228-6239.
62. Hammel M, Kriechbaum M, Gries A et al. Solution structure of human and bovine beta(2)-glycoprotein I revealed by small-angle X-ray scattering. J.Mol.Biol. 2002;321:85-97.

63. Arvieux J, Regnault V, Hachulla E et al. Heterogeneity and immunochemical properties of anti-beta2-glycoprotein I autoantibodies. Thromb.Haemost. 1998;80:393-398.
64. Gamsjaeger R, Johs A, Gries A et al. Membrane binding of beta2-glycoprotein I can be described by a two-state reaction model: an atomic force microscopy and surface plasmon resonance study. Biochem.J. 2005;389:665-673.
65. de Laat B, Derksen RH, van Lummel M, Pennings MT, de Groot PG. Pathogenic anti-beta2-glycoprotein I antibodies recognize domain I of beta2-glycoprotein I only after a conformational change. Blood 2006;107:1916-1924.
66. Koike T, Ichikawa K, Kasahara H et al. Epitopes on beta2-GPI recognized by anticardiolipin antibodies. Lupus 1998;7 Suppl 2:S14-S17.
67. Yang CD, Chen SL, Shen N, Qi M, Xu F. Detection of anti-recombinant beta 2-glycoprotein 1 and anti-recombinant beta 2-glycoprotein 1 fifth-domain antibodies in sera from patients with systemic lupus erythematosus. Rheumatol.Int. 1998;18:5-10.
68. Blank M, Shoenfeld Y, Cabilly S et al. Prevention of experimental antiphospholipid syndrome and endothelial cell activation by synthetic peptides. Proc.Natl.Acad.Sci.U.S.A 1999;96:5164-5168.
69. Reddel SW, Wang YX, Sheng YH, Krilis SA. Epitope studies with anti-beta 2-glycoprotein I antibodies from autoantibody and immunized sources. J.Autoimmun. 2000;15:91-96.
70. Iverson GM, Victoria EJ, Marquis DM. Anti-beta2 glycoprotein I (beta2GPI) autoantibodies recognize an epitope on the first domain of beta2GPI. Proc.Natl.Acad.Sci.U.S.A 1998;95:15542-15546.
71. McNeeley PA, Dlott JS, Furie RA et al. Beta2-glycoprotein I-dependent anticardiolipin antibodies preferentially bind the amino terminal domain of beta2-glycoprotein I. Thromb.Haemost. 2001;86:590-595.
72. Ioannou Y, Pericleous C, Giles I et al. Binding of antiphospholipid antibodies to discontinuous epitopes on domain I of human beta(2)-glycoprotein I: mutation studies including residues R39 to R43. Arthritis Rheum. 2007;56:280-290.
73. Lutters BC, Meijers JC, Derksen RH, Arnout J, de Groot PG. Dimers of beta 2-glycoprotein I mimic the in vitro effects of beta 2-glycoprotein I-anti-beta 2-glycoprotein I antibody complexes. J.Biol.Chem. 2001;276:3060-3067.
74. van Lummel M, Pennings MT, Derksen RH et al. The binding site in {beta}2-glycoprotein I for ApoER2' on platelets is located in domain V. J.Biol.Chem. 2005;280:36729-36736.
75. Pennings MT, van Lummel M, Derksen RH et al. Interaction of beta2-glycoprotein I with members of the low density lipoprotein receptor family. J.Thromb.Haemost. 2006;4:1680-1690.
76. Ma K, Simantov R, Zhang JC et al. High affinity binding of beta 2-glycoprotein I to human endothelial cells is mediated by annexin II. J.Biol.Chem. 2000;275:15541-15548.
77. Lutters BC, Derksen RH, Tekelenburg WL et al. Dimers of beta 2-glycoprotein I increase platelet deposition to collagen via interaction with phospholipids and the apolipoprotein E receptor 2'. J.Biol.Chem. 2003;278:33831-33838.
78. Pennings MT, Derksen RH, van Lummel M et al. Platelet adhesion to dimeric beta-glycoprotein I under conditions of flow is mediated by at least two receptors: glycoprotein Ibalpha and apolipoprotein E receptor 2'. J.Thromb.Haemost. 2007;5:369-377.
79. Shi T, Giannakopoulos B, Yan X et al. Anti-beta2-glycoprotein I antibodies in complex with beta2-glycoprotein I can activate platelets in a dysregulated manner via glycoprotein Ib-IX-V. Arthritis Rheum. 2006;54:2558-2567.
80. Satta N, Dunoyer-Geindre S, Reber G et al. The role of TLR2 in the inflammatory activation of mouse fibroblasts by human antiphospholipid antibodies. Blood 2007;109:1507-1514.
81. Raschi E, Testoni C, Bosisio D et al. Role of the MyD88 transduction signaling pathway in endothelial activation by antiphospholipid antibodies. Blood 2003;101:3495-3500.
82. Pendu RP, Weeterings C, Urbanus RT et al. PSGL-1 and LDL-receptors mediate adhesion of leucocytes to Beta2-Glycoprotein I [abstract]. J.Thromb.Haemost. 2007;5 supplement 2:P-M-463.
83. Satta N, Dunoyer-Geindre S, Fish RJ et al. Antiphospholipid antibodies and TLR2 colocalized in HUVEC endosomes [abstract]. J.Thromb.Haemost. 2007;5 supplement 2:O-S-010.
84. Zhang J, McCrae KR. Annexin A2 mediates endothelial cell activation by antiphospholipid/anti-beta2 glycoprotein I antibodies. Blood 2005;105:1964-1969.
85. Del Papa N, Guidali L, Sala A et al. Endothelial cells as target for antiphospholipid antibodies. Human polyclonal and monoclonal anti-beta 2-glycoprotein I antibodies react in vitro with endothelial cells through adherent beta 2-glycoprotein I and induce endothelial activation. Arthritis Rheum. 1997;40:551-561.
86. Dunoyer-Geindre S, de Moerloose P, Galve-de Rochemonteix B, Reber G, Kruithof EK. NFkappaB is an essential intermediate in the activation of endothelial cells by anti-beta(2)-glycoprotein 1 antibodies. Thromb.Haemost. 2002;88:851-857.
87. Ferrara DE, Swerlick R, Casper K et al. Fluvastatin inhibits up-regulation of tissue factor expression by antiphospholipid antibodies on endothelial cells. J.Thromb.Haemost. 2004;2:1558-1563.
88. Gharavi AE, Pierangeli SS, Espinola RG et al. Antiphospholipid antibodies induced in mice by immunization with a cytomegalovirus-derived peptide cause thrombosis and activation of endothelial cells in vivo. Arthritis Rheum. 2002;46:545-552.
89. Pierangeli SS, Colden-Stanfield M, Liu X et al. Antiphospholipid antibodies from antiphospholipid syndrome patients activate endothelial cells in vitro and in vivo. Circulation 1999;99:1997-2002.
90. Simantov R, LaSala JM, Lo SK et al. Activation of cultured vascular endothelial cells by antiphospholipid antibodies. J.Clin.Invest 1995;96:2211-2219.
91. Vega-Ostertag M, Casper K, Swerlick R et al. Involvement of p38 MAPK in the up-regulation of tissue factor on endothelial cells by antiphospholipid antibodies. Arthritis Rheum. 2005;52:1545-1554.
92. Shigeoka AA, Holscher TD, King AJ et al. TLR2 is constitutively expressed within the kidney and participates in ischemic renal injury through both MyD88-dependent and -independent pathways. J.Immunol. 2007;178:6252-6258.
93. Cesarman-Maus G, Rios-Luna NP, Deora AB et al. Autoantibodies against the fibrinolytic receptor, annexin 2, in antiphospholipid syndrome. Blood 2006;107:4375-4382.
94. Zhou H, Ling S, Yu Y, Wang T,

Hu H. Involvement of annexin A2 in anti-beta(2)GPI/beta(2) GPI-induced tissue factor expression on monocytes. Cell Res. 2007;17:737-739.

95. Giesen PL, Rauch U, Bohrmann B et al. Blood-borne tissue factor: another view of thrombosis. Proc.Natl.Acad.Sci.U.S.A 1999;96:2311-2315.

96. Morel O, Toti F, Hugel B et al. Procoagulant microparticles: disrupting the vascular homeostasis equation? Arterioscler.Thromb. Vasc.Biol. 2006;26:2594-2604.

97. Falati S, Liu Q, Gross P et al. Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin. J.Exp.Med. 2003;197:1585-1598.

98. Bohgaki M, Atsumi T, Yamashita Y et al. The p38 mitogen-activated protein kinase (MAPK) pathway mediates induction of the tissue factor gene in monocytes stimulated with human monoclonal anti-beta2Glycoprotein I antibodies. Int.Immunol. 2004;16:1633-1641.

99. Conti F, Sorice M, Circella A et al. Beta-2-glycoprotein I expression on monocytes is increased in anti-phospholipid antibody syndrome and correlates with tissue factor expression. Clin.Exp. Immunol. 2003;132:509-516.

100. Cuadrado MJ, Lopez-Pedrera C, Khamashta MA et al. Thrombosis in primary antiphospholipid syndrome: a pivotal role for monocyte tissue factor expression. Arthritis Rheum. 1997;40:834-841.

101. Kornberg A, Blank M, Kaufman S, Shoenfeld Y. Induction of tissue factor-like activity in monocytes by anti-cardiolipin antibodies. J.Immunol. 1994;153:1328-1332.

102. Reverter JC, Tassies D, Font J et al. Effects of human monoclonal anticardiolipin antibodies on platelet function and on tissue factor expression on monocytes. Arthritis Rheum. 1998;41:1420-1427.

103. Zhou H, Wolberg AS, Roubey RA. Characterization of monocyte tissue factor activity induced by IgG antiphospholipid antibodies and inhibition by dilazep. Blood 2004;104:2353-2358.

104. Lopez-Pedrera C, Buendia P, Cuadrado MJ et al. Antiphospholipid antibodies from patients with the antiphospholipid syndrome induce monocyte tissue factor expression through the simultaneous activation of NF-kappaB/Rel proteins via the p38 mitogen-activated protein kinase pathway, and of the MEK-1/ERK pathway. Arthritis Rheum. 2006;54:301-311.

105. Aird WC. Vascular bed-specific thrombosis. J.Thromb.Haemost. 2007;5 Suppl 1:283-291.

106. Matsushima N, Tanaka T, Enkhbaya P et al. Comparative sequence analysis of leucine-rich repeats (LRRs) within vertebrate toll-like receptors. BMC.Genomics 2007;8:124.

107. Meroni PL, Raschi E, Testoni C et al. Statins prevent endothelial cell activation induced by antiphospholipid (anti-beta2-glycoprotein I) antibodies: effect on the proadhesive and proinflammatory phenotype. Arthritis Rheum. 2001;44:2870-2878.

108. Neumann FJ, Ott I, Marx N et al. Effect of human recombinant interleukin-6 and interleukin-8 on monocyte procoagulant activity. Arterioscler.Thromb.Vasc.Biol. 1997;17:3399-3405.

109. Ikeda K, Nagasawa K, Horiuchi T et al. C5a induces tissue factor activity on endothelial cells. Thromb.Haemost. 1997;77:394-398.

110. Ritis K, Doumas M, Mastellos D et al. A novel C5a receptor-tissue factor cross-talk in neutrophils links innate immunity to coagulation pathways. J.Immunol. 2006;177:4794-4802.

111. Fischetti F, Durigutto P, Pellis V et al. Thrombus formation induced by antibodies to beta2-glycoprotein I is complement dependent and requires a priming factor. Blood 2005;106:2340-2346.

112. Redecha P, Tilley R, Tencati M et al. Tissue factor: a link between C5a and neutrophil activation in antiphospholipid antibody-induced fetal injury. Blood 2007;prepublished online May 29:-DOI 10.1182/blood-2007-01-070631.

113. Ravetch JV, Bolland S. IgG Fc receptors. Annu.Rev.Immunol. 2001;19:275-290.

114. Sammaritano LR. Significance of aPL IgG subclasses. Lupus 1996;5:436-439.

115. Atsumi T, Caliz R, Amengual O, Khamashta MA, Hughes GR. Fcgamma receptor IIA H/R131 polymorphism in patients with antiphospholipid antibodies. Thromb.Haemost. 1998;79:924-927.

116. Jankowski M, Vreys I, Wittevrongel C et al. Thrombogenicity of beta 2-glycoprotein I-dependent antiphospholipid antibodies in a photochemically induced thrombosis model in the hamster. Blood 2003;101:157-162.

117. Kornberg A, Renaudineau Y, Blank M, Youinou P, Shoenfeld Y. Anti-beta 2-glycoprotein I antibodies and anti-endothelial cell antibodies induce tissue factor in endothelial cells. Isr.Med.Assoc.J. 2000;2 Suppl:27-31.

118. Vega-Ostertag M, Harris EN, Pierangeli SS. Intracellular events in platelet activation induced by antiphospholipid antibodies in the presence of low doses of thrombin. Arthritis Rheum. 2004;50:2911-2919.

119. Faure E, Thomas L, Xu H et al. Bacterial lipopolysaccharide and IFN-gamma induce Toll-like receptor 2 and Toll-like receptor 4 expression in human endothelial cells: role of NF-kappa B activation. J.Immunol. 2001;166:2018-2024.

120. Wisloff F, Jacobsen EM, Liestol S. Laboratory diagnosis of the antiphospholipid syndrome. Thromb.Res. 2002;108:263-271.

121. Brandt JT, Triplett DA, Alving B, Scharrer I. Criteria for the diagnosis of lupus anticoagulants: an update. On behalf of the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the ISTH. Thromb. Haemost. 1995;74:1185-1190.

122. Tincani A, Allegri F, Sanmarco M et al. Anticardiolipin antibody assay: a methodological analysis for a better consensus in routine determinations--a cooperative project of the European Antiphospholipid Forum. Thromb. Haemost. 2001;86:575-583.

123. Wong RC, Gillis D, Adelstein S et al. Consensus guidelines on anticardiolipin antibody testing and reporting. Pathology 2004;36:63-68.

124. Reber G, Tincani A, Sanmarco M, de Moerloose P, Boffa MC. Proposals for the measurement of anti-beta2-glycoprotein I antibodies. Standardization group of the European Forum on Antiphospholipid Antibodies. J.Thromb. Haemost. 2004;2:1860-1862.

2

Aim of this thesis

The antiphospholipid syndrome is characterized by vascular thrombosis or pregnancy morbidity associated with persistently present antiphospholipid antibodies.[1] Antiphospholipid antibodies are a heterogeneous population of antibodies and are categorized based either on their effects, i.e. phospholipid-dependent inhibition of coagulation assays by so-called lupus anticoagulants, or on antigen specificity, e.g. anticardiolipin, anti-β_2-glycoprotein I or antiprothrombin antibodies. Lupus anticoagulants are associated with the highest risk of thrombosis and fetal loss.[2] The plasma protein β_2-glycoprotein I is considered the most relevant antigen in the antiphospholipid syndrome. There are indications that anti-β_2-glycoprotein I antibodies that prolong coagulation in a phospholipid-dependent manner (viz. have lupus anticoagulant activity) are the pathogenic antibody population.[3]

Both arterial and venous thrombosis occur in patients with the antiphospholipid syndrome. Nevertheless, as the effect of antiphospholipid antibodies on endothelial cell activation is studied most often , the focus of antiphospholipid antibody-related research lies predominantly on the etiology of venous thromboembolic disease and fetal loss. Much less data are available on the role of antiphospholipid antibodies in the mechanisms of arterial thrombosis.

This thesis aims to provide more insight into the role of antiphospholipid antibodies in arterial thrombosis. The first half of this thesis has a more patient-oriented approach and describes the relation between antiphospholipid antibodies and clinical manifestations in patients: Chapter 3 describes the paradoxical effects of antiphospholipid antibodies on a diagnostic test of primary haemostasis in patients with lupus anticoagulant and chapters 4 and 5 deal with the influence of antiphospholipid antibodies on the risk of ischemic stroke (chapter 4) or myocardial infarction (chapters 4 and 5) in the general population. The second part of this thesis focuses upon a potential molecular mechanism for a prothrombotic effect of antiphospholipid antibodies: Chapter 6 describes the signaling cascade downstream from the platelet receptors Apolipoprotein E Receptor 2' (ApoER2') and glycoprotein Ibα upon stimulation of human platelets with a recombinant dimer of β_2-glycoprotein I that mimicks the function of anti-β_2-glycoprotein I antibody/β_2-glycoprotein I immune complexes.[4] The role of ApoER2' in anti-β_2-glycoprotein I antibody-mediated thrombosis in the antiphospholipid syndrome is further explored in chapter 7, in which the influence of dimeric β_2-glycoprotein I on in vivo thrombus formation is investigated in a murine model. In chapter 8, the implications of the findings reported in the preceding chapters are discussed in a broader context.

References

1. Miyakis S, Lockshin MD, Atsumi T et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 2006; 4(2):295-306.
2. Galli M, Luciani D, Bertolini G, Barbui T. Lupus anticoagulants are stronger risk factors for thrombosis than anticardiolipin antibodies in the antiphospholipid syndrome: a systematic review of the literature. Blood 2003; 101(5):1827-1832.
3. de Laat HB, Derksen RH, Urbanus RT, Roest M, de Groot PG. beta2-glycoprotein I-dependent lupus anticoagulant highly correlates with thrombosis in the antiphospholipid syndrome. Blood 2004; 104(12):3598-3602.
4. Lutters BC, Meijers JC, Derksen RH, Arnout J, de Groot PG. Dimers of beta 2-glycoprotein I mimic the in vitro effects of beta 2-glycoprotein I-anti-beta 2-glycoprotein I antibody complexes. J Biol Chem 2001; 276(5):3060-3067.

3

Prolonged bleeding time and lupus anticoagulant: a second paradox in the antiphospholipid syndrome

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Abstract

Objective: Systemic lupus erythematosus and the antiphospholipid syndrome often go side by side. Nephropathy occurs in both systemic lupus erythematosus and the antiphospholipid syndrome and cannot be distinguished clinically, although etiology and treatment differ greatly. Therefore examination of a renal biopsy is essential in order to provide the correct treatment. We have observed that patients with lupus anticoagulant, a serological marker for the antiphospholipid syndrome, often have a prolonged bleeding time, which is a contraindication for the performance of a percutaneous renal biopsy. Here we report a systematic evaluation of the bleeding time in 27 consecutive patients.

Methods: The bleeding time was measured in 27 patients that were persistently positive for lupus anticoagulant and who were not exposed to aspirin or non-steroid anti-inflammatory drugs. Platelet function and VWF parameters were subsequently assessed in patients with a prolonged bleeding time.

Results: Out of 27 patients, 21 (77%) had a prolonged bleeding time, despite a normal platelet count in the majority of patients (81%). Platelet functioning and Von Willebrand Factor parameters of these 21 patients were normal, apart from one patient with a mild storage pool disease.

Conclusion: With this study we introduce yet another paradox in the field of the antiphospholipid syndrome. Although a prolonged bleeding time is generally accepted to be a sign of defective primary hemostasis, lupus anticoagulant is associated with thrombosis. Further studies are needed to elucidate the mechanism behind this disturbance of primary hemostasis.

Introduction

The antiphospholipid syndrome (APS) is characterized serologically by persistent presence of antiphospholipid antibodies (aPL), notably anticardiolipin antibodies (aCL) and lupus anticoagulant (LAC), and clinically by thrombosis and (recurrent) pregnancy loss.[1] For thrombosis, the presence of LAC carries a higher relative risk than aCL.[2] Of the systemic autoimmune diseases, systemic lupus erythematosus (SLE) is the one with the highest frequency of APS. However, APS is often diagnosed in absence of other manifestations of systemic autoimmunity (primary APS).[3] Apart from thrombosis and complications of pregnancy the presence of aPL has been associated with (mild) thrombocytopenia, heart valve lesions, livedo reticularis, chorea and recently with ischemic microangiopathic nephropathy.[3-5] Clinically this aPL-related nephropathy is manifested by (malignant) hypertension, proteinuria and renal failure. The histopathology of kidney biopsies shows vaso-occlusive lesions of intrarenal vessels associating side-by-side acute thrombosis and intra-arteriolar lesions as well as zones of cortical ischemic atrophy. This pathology is well recognizable in renal biopsies from patients with SLE, both independent of and in addition to the classical pathology of lupus nephritis. As about 40 percent of patients with SLE have aPL and up to 50% of SLE patients develop nephritis, awareness of aPL-related nephropathy is important, especially since immunosupressive drugs are indicated in lupus nephritis whereas anticoagulant or vasoprotective therapy seems appropriate in ischemic nephropathy related to aPL. As lupus nephritis and aPL-related ischemic nephropathy cannot be distinguished clinically, examination of a renal biopsy is essential. Although the results of bleeding time tests are poorly predictive of perioperative bleeding, the bleeding time is still the method of choice for assessment of primary hemostasis in patients with an indicaton for a percutaneous renal biopsy.

We noticed that in some SLE patients with renal manifestations and LAC a percutaneous renal biopsy was not performed due to a prolonged bleeding time. Here we report a systematic evaluation of the bleeding time in 27 consecutive patients with LAC.

Patients, materials and methods

Patients

Patients eligible for inclusion in this study were persistently positive for LAC, did not have a bleeding tendency and did not use aspirin or non-steroid anti-inflammatory drugs (NSAIDs) for at least four weeks prior to measurement of the bleeding time. Twenty-seven patients that are followed at the lupus clinic from the department of Rheumatology and Clinical Immunology and who were persistently positive for LAC gave their informed consent to undergo a bleeding time test and to donate extra blood for additional tests. The medical histories of these 27 patients were taken and their charts were reviewed to classify them as having APS, SLE (at least four American College of Rheumatology (ACR) criteria are fulfilled) or lupus like disease (if one to three

ACR criteria are met).[6] All included patients were female. Their median age was 37 years (range 23-56 years). Sixteen patients had APS secondary to SLE (n=12) or lupus like disease (all had a history of thrombosis), 7 patients had primary APS based on a history of thrombosis and 4 patients had SLE without manifestations of APS.

Lupus anticoagulant

LAC was assessed by measurement of the activated partial thromboplastin time (aPTT) (PTT-LA; Diagnostica Stago, Asnières-sur-Seine, France) and the dilute Russell's Viper Venom Time (dRVVT) (LAC screen/LAC confirm; Gradipore Ltd, North Ryde, Australia). All coagulation assays were performed in a KC-10 coagulometer (Amelung, Lemgo, Germany) with citrated plasma (10.9 mM) obtained by spinning down whole blood at 500*g* for 10 minutes twice. Measurements were performed according to the instructions of the manufacturers.

Bleeding time

The bleeding time was estimated by an experienced technician by making two standardized horizontal incisions in the volar surface of the forearm with the Simplate IIR device (Organon Technica, Durham, NC) . Bleeding time was measured up to 30 minutes. Normal values were obtained by measuring the bleeding time in 61 healthy volunteers (mean bleeding time 5 min 46 sec; standard deviation 1 min 20 sec).

Platelets

Anticoagulated whole blood (1.8 mg mL^{-1} EDTA) was used to estimate platelet count and mean platelet volume (MPV) in a Cell-dyn 1700 (Abbott Laboratories, Abbott Park, IL). Platelet function tests were performed with platelet rich plasma (PRP) obtained from patients with a prolonged bleeding time. PRP was made by spinning down citrated whole blood (10.9 mM) for 10 minutes at 150*g*. Platelet count was subsequently set at $250 \cdot 10^9$ L^{-1} by the addition of platelet poor plasma. Aggregation studies were performed in an optical aggregometer (Chronolog Corporation, Haverport, PA) with the agonists ADP (1 and 5 µM), epinephrine (1 and 5 µM), collagen (1 and 4 µg mL^{-1}) and arachidonic acid (15 mM). Ristocetin (0.5-1.5 mg mL^{-1}) was used to test glycoprotein 1b (GP1b) dependent platelet agglutination.

Platelet ADP and ATP content was measured in a Packard LumiCount luminometer (Packard Bioscience, Meriden, CT) using the commercially available ATPlite kit from the same company. Measurements were performed according to the instructions of the manufacturer. Platelet serotonin levels were determined using a Hitachi F-4500 fluorimeter (HHT corporation, Wokingham, UK). Plasma thrombopoietin levels were determined using the Human Thrombopoietin Quantikine kit (R&D systems, Abingdon, UK). Measurements were performed according to the instructions of the manufacturer.

VWF

VWF antigen levels were determined in an enzyme-linked immunosorbent assay (ELISA) setup, using polyclonal rabbit anti-human VWF IgG (Dako, Glostrup, Denmark). VWF collagen binding was determined as described before.[7] In short, diluted patient plasma was incubated on 96-wells microtiter plates (Costar, Cambridge, MA) coated with fibrillar human collagen type III (Sigma, St. Louis, MO, USA). Horseradish peroxidase-conjugated polyclonal rabbit anti-human VWF (Dako, Glostrup, Denmark) was used for staining. The multimeric distribution of VWF was assessed as described by Lawrie et al.[8] Ristocetin cofactor activity was measured in a BCS coagulation analyzer (Dade Behring, Marburg, Germany) using the Von Willebrand Reagent (Dade Behring, Marburg, Germany).

Statistical analysis

Correlation between variables was determined using Spearman's Rho test.

Results

In figure 1A the relation between bleeding time and platelet count is shown. In 21/27 (77%) LAC-positive patients the bleeding time was prolonged (>8 min 27 sec). Three out of 21 patients with a prolonged bleeding time had SLE with aPL, 10 had SLE with secondary APS, 3 had LLD with secondary APS and 5 had primary APS. Platelet counts were normal in 16/21 (76%) patients with a prolonged bleeding time. Three patients had a platelet count between 100 and $150 \cdot 10^9$ L^{-1} and two had a platelet count below $100 \cdot 10^9$ L^{-1}. Platelet count in relation to MPV and platelet count in relation to TPO levels are depicted in figure 1B and 1C respectively. Both MPV and TPO levels are inversely correlated with the platelet count ($r=-0.7525$; $p<0.001$ and $r=-0.4787$; $p<0.05$, respectively).

In all patients with a prolonged bleeding time the aggregation of platelets on the agonists ADP, collagen, arachidonic acid and epinephrine was normal (table 1). Ristocetin dependent platelet agglutination was normal in these patients as well. Study of platelet granule content revealed mild dense-granule storage pool disease in one patient with LLD and secondary APS (bleeding time >30 min, platelet ADP 1.61 μmol $(10^{11}$ platelets$)^{-1}$ (normal range 1.7-3.8 μmol $(10^{11}$ platelets$)^{-1}$) and platelet serotonin 151 μmol $(10^{11}$ platelets$)^{-1}$ (normal range 200-600 μmol $(10^{11}$ platelets$)^{-1}$).

Normal plasma levels of circulating VWF were found in all patients. The multimeric distribution of VWF was normal in all patients. Furthermore, no defects in VWF-collagen binding were found. VWF was capable of binding to GP1b in all patients as shown by normal ristocetin cofactor activity tests.

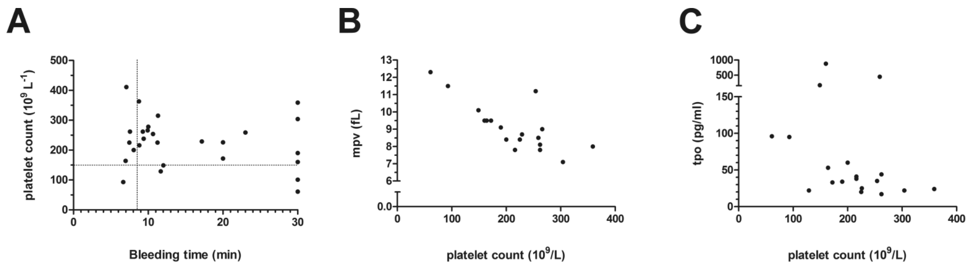

Figure 1. **A** Platelet count in relation to bleeding time. Dotted lines indicate lower level of normality for platelet count ($150 \cdot 10^9$ L^{-1}, horizontal) and upper level of normality for the bleeding time (8 min 27 sec (mean + 2SD), vertical). Twenty-one out of 27 (77%) patients have a prolonged bleeding time. **B** Platelet count in relation to mean platelet volume (MPV), n=24. Mean platelet volume was not obtained in three patients with a normal bleeding time. An inverse correlation was found (r=-.7525, p<0.001). **C** Platelet count in relation to plasma thrombopoietin levels, n=24. An inverse correlation was found (r=-0.4787, p=0.038).

Discussion

The presence of antibodies causing a phospholipid dependent prolongation of plasma clotting assays in vitro, also known as lupus anticoagulant, is a serological hallmark of thrombosis. This association is paradoxical, as most conditions associated with a prolonged in vitro clotting time are associated with bleeding. The elucidation of this paradox is subject of ongoing research, in which the increased affinity of antibody-bound protein cofactors such as β_2-glycoprotein I for phospholipid surfaces plays a central role – both in explaining this in vitro phenomenon and in cellular processes leading to thrombosis.

Our finding of a prolonged bleeding time in a majority of patients with LAC (in this study 77%) introduces a second paradox in the field of APS. A prolonged bleeding time is generally considered to be an indication of disturbed primary hemostasis and therefore a contraindication to perform a percutaneous renal biopsy.

Out of the 27 LAC positive patients we studied, 5 (19%) patients had a platelet count below $150 \cdot 10^9$ L^{-1}, 2 of whom had a platelet count below $100 \cdot 10^9$ L^{-1} (figure 1A). An immune-mediated underlying mechanism for thrombocytopenia is generally accepted, as most authorities agree that the interaction of antibodies with the platelet membrane results in rapid clearance through the reticulo-endothelial system.[9] The inverse correlation we found between platelet count and MPV and between platelet count and thrombopoietin levels (figure 1B-C) is in line with this view.

In general, a platelet count above $100 \cdot 10^9$ L^{-1} does not lead to a prolongation of the bleeding time.[10] As only 2 out of 27 (7%) patients had a platelet count below $100 \cdot 10^9$ L^{-1} (one of whom had a normal bleeding time) and 77% of these 27 patients had a prolonged bleeding time and a normal platelet count, low platelet count alone cannot explain the prolongation of the bleeding time.

To find an explanation for the prolongation of the bleeding time we performed stud-

Table 1. Relevant parameters of primary hemostasis measured in 21 patients with a prolonged bleeding time.

Parameter	Patients – N	Median – Range	Normal range
Haematocrit – L (L blood)$^{-1}$	21	0.39 (0.35-0.41)	*0.36-0.46*
Platelet count – 10^9 L^{-1}	21	216 (61-359)	*150-450*
Mean platelet volume – fL	21	8.8 (7.1-12.3)	*7.0-9.5*
Platelet ADP content – µmol (10^{11} platelets)$^{-1}$	21	3.1 (1.6-4.2)	*1.7-3.8*
Platelet ATP content – µmol (10^{11} platelets)$^{-1}$	21	4.5 (3.4-6.3)	*3.1-7.0*
ATP/ADP ratio	21	1.46 (1.06-2.81)	*<2.0*
Platelet serotonin content – µmol (10^{11} platelets)$^{-1}$	19*	317 (42-911)	*200-600*
Thrombopoietin – pg mL^{-1}	18*	38 (17-879)	*0-131*
vWF antigen – % of control	21	155 (46-477)	*60-130*
vWF ristocetin cofactor activity – % of control	19*	134 (62-250)	*40-150*
vWF multimers	21	normal	
vWF collagen binding	21	normal	
ADP induced aggregation	21	normal	
Epinephrine induced aggregation	21	normal	
Collagen induced aggregation	21	normal	
Arachidonic acid induced aggregation	21	normal	
Ristocetin induced aggregation	20§	normal	

ADP denotes adenosine diphosphate; ATP denotes adenosine triphosphate; vWF denotes von Willebrand Factor.
* Insufficient amount of test material available in some patients.
§ Ristocetin induced aggregation could not be tested in one patient due to a low platelet count ($101 \cdot 10^9$ L^{-1}).

ies on platelet function in the 21 patients with a prolonged bleeding time. Aggregation studies showed that all patients had functional platelets. One patient had a mild dense-granule storage pool disease. This however, cannot explain the extent of the prolongation of the bleeding time in this patient (>30 minutes).

Aside from defects in the functioning of platelets, von Willebrand's disease (VWD) is a common cause of a prolonged bleeding time. However, as plasma levels, multimeric pattern and binding capacity of VWF to collagen and platelet GP1b were normal in all patients, VWD can be excluded.

As the bleeding time is not only influenced by platelet and VWF parameters, but also by properties of the vessel wall and the skin, one might speculate that parameters interfering with the interaction between platelets and the vessel wall cause a prolongation of the bleeding time. In this respect glucocorticoid-induced atrophy of the skin might influence the bleeding time. However, the notion that 15/27 (56%) patients, including 10 with a prolonged bleeding time, were never exposed to glucocorticoids, argues against an important role of skin atrophy as the cause of a prolonged bleeding time in our patients.

Decreased platelet adhesion to collagen in the presence of aPL has been mentioned as

a cause for the prolongation of the bleeding time.[11] However, in an in vitro flow model for thrombosis, we described increased platelet adhesion to collagen or endothelial cell matrix in the presence of aPL.[12;13] We therefore hypothesize that antibodies causing LAC might be capable of activating endothelial cells, causing upregulation of enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). The subsequent localized formation of nitric oxide (NO) and prostacyclin (PGI_2), known inhibitors of platelet functioning, might hamper primary hemostasis.[14;15] The short half life of both NO and PGI_2 might explain the absence of any abnormalities in our laboratory assays. Further studies are needed to elucidate this.

In conclusion, many patients with LAC have a prolonged bleeding time, despite the absence of a bleeding tendency. The prolongation of the bleeding time is disproportionate to platelet count and cannot be explained by any known defects in platelet functioning or abnormalities in VWF. Further studies are needed to elucidate the influence of antibodies causing LAC on the endothelium.

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References

1. Wilson WA, Gharavi AE, Koike T et al. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop. Arthritis Rheum. 1999;42:1309-1311.
2. Horbach DA, van Oort E, Donders RC, Derksen RH, de Groot PG. Lupus anticoagulant is the strongest risk factor for both venous and arterial thrombosis in patients with systemic lupus erythematosus. Comparison between different assays for the detection of antiphospholipid antibodies. Thromb.Haemost. 1996;76:916-924.
3. Cervera R, Piette JC, Font J et al. Antiphospholipid syndrome: clinical and immunologic manifestations and patterns of disease expression in a cohort of 1,000 patients. Arthritis Rheum. 2002;46:1019-1027.
4. Nochy D, Daugas E, Droz D et al. The intrarenal vascular lesions associated with primary antiphospholipid syndrome. J.Am.Soc. Nephrol. 1999;10:507-518.
5. Daugas E, Nochy D, Huong DL et al. Antiphospholipid syndrome nephropathy in systemic lupus erythematosus. J.Am.Soc.Nephrol. 2002;13:42-52.
6. Tan EM, Cohen AS, Fries JF et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1982;25:1271-1277.
7. van der Plas RM, Gomes L, Marquart JA et al. Binding of von Willebrand factor to collagen type III: role of specific amino acids in the collagen binding domain of vWF and effects of neighboring domains. Thromb.Haemost. 2000;84:1005-1011.
8. Lawrie AS, Hoser MJ, Savidge GF. Phast assessment of vWf:Ag multimeric distribution. Thromb. Res. 1990;59:369-373.
9. Macchi L, Rispal P, Clofent-Sanchez G et al. Anti-platelet antibodies in patients with systemic lupus erythematosus and the primary antiphospholipid antibody syndrome: their relationship with the observed thrombocytopenia. Br.J.Haematol. 1997;98:336-341.
10. Harker LA, Slichter SJ. The bleeding time as a screening test for evaluation of platelet function. N.Engl.J.Med. 1972;287:155-159.
11. Orlando E, Cortelazzo S, Marchetti M, Sanfratello R, Barbui T. Prolonged bleeding time in patients with lupus anticoagulant. Thromb.Haemost. 1992;68:495-499.
12. Lutters BC, Derksen RH, Tekelenburg WL et al. Dimers of beta 2-glycoprotein I increase platelet deposition to collagen via interaction with phospholipids and the apolipoprotein E receptor 2'. J.Biol.Chem. 2003;278:33831-33838.
13. Oosting JD, Derksen RH, Blokzijl L, Sixma JJ, de Groot PG. Antiphospholipid antibody positive sera enhance endothelial cell procoagulant activity--studies in a thrombosis model. Thromb. Haemost. 1992;68:278-284.
14. Cahill PA, Redmond EM, Sitzmann JV. Endothelial dysfunction in cirrhosis and portal hypertension. Pharmacol.Ther. 2001;89:273-293.
15. Aiello S, Noris M, Remuzzi G. Nitric oxide/L-arginine in uremia. Miner.Electrolyte Metab 1999;25:384-390.

4

Antiphospholipid antibodies and the risk of myocardial infarction and ischemic stroke in young women

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Submitted

Abstract

Background: Arterial thrombosis is a major clinical manifestation of the antiphospholipid syndrome, an autoimmune disease mainly found in young women. Although the presence of circulating antiphospholipid antibodies is a prerequisite for diagnosis, the thrombotic risk associated with the presence of antiphospholipid antibodies in the general population is unclear.

Methods: We performed a large multi-center population-based case-control study among women aged under 50: 175 women with ischemic stroke, 203 women with myocardial infarction and 628 healthy controls who were frequency matched for age, residence area and index year. We determined antiphospholipid antibody profiles in all women and gathered data on cardiovascular risk factors.

Results: In women with lupus anticoagulant as compared to women without lupus anticoagulant the odds ratio for myocardial infarction was 5.3 (95%-confidence interval, 1.4-20.8) and that for ischemic stroke was 43.1 (95%-confidence interval, 12.2-152). Among women with lupus anticoagulant the odds ratio for myocardial infarction was 21.6 (95%-confidence interval, 1.9-242) in oral contraceptive users and 33.7 (95%-confidence interval, 6.0-189) in smokers. Among women with lupus anticoagulant the odds ratio for ischemic stroke was 201 (95%-confidence interval, 22.1-1828) in oral contraceptive users and 87 (95%-confidence interval, 14.5 -523) in smokers. Neither anticardiolipin nor antiprothrombin antibodies influenced the risk of myocardial infarction or ischemic stroke.

Conclusions. Our study shows that lupus anticoagulant, found in 0.6% of women, is a major risk factor for the development of arterial thrombotic events in young women. The presence of additional cardiovascular risk factors increases the risk even further.

Introduction

Arterial thrombosis is the second most common cause of death in developed countries. Although the incidence below age 50 is low, the burden of disease among young people is large. Arterial thrombosis is usually associated with the presence of multiple risk factors for cardiovascular disease such as smoking, hypertension, diabetes, obesity, hyperlipidemia or a family history of cardiovascular disease. The antiphospholipid syndrome is another acquired risk factor for arterial thrombosis. Mainly diagnosed in young women,[1] the syndrome is characterized by vascular thrombosis or pregnancy morbidity in association with a repeatedly positive laboratory test for antiphospholipid antibodies.[2] There are several subpopulations of antiphospholipid antibodies, of which the coagulation inhibitor known as lupus anticoagulant and antibodies against the phospholipid cardiolipin or the plasma proteins β_2-glycoprotein I and prothrombin, are most frequently encountered.

Given the nonspecific clinical symptoms, the diagnosis of the antiphospholipid syndrome relies completely on the detection of antiphospholipid antibodies. Data on the risk of a first thrombotic event in the presence of antiphospholipid antibodies are inconclusive,[3;4] as most studies are performed in selected patient populations. Over the years, several studies were performed to obtain data on thrombotic risk associated with the presence of antiphospholipid antibodies in the general population.[5-13] Although there is general consensus among these studies that the presence of antiphospholipid antibodies is independently associated with an increased risk of arterial thrombosis, there is still debate on the value of measuring different antiphospholipid antibody subpopulations.[14]

We investigated whether the presence of several antiphospholipid antibodies (lupus anticoagulant, anticardiolipin, anti-β_2-glycoprotein I and antiprothrombin antibodies) influences the risk of myocardial infarction or ischemic stroke in young women. We additionally studied the influence of smoking, the use of oral contraceptives,[15;16] the factor V G1691A (factor V Leiden) mutation,[17;18] the prothrombin G20210A mutation[18-20] and the factor XIII 204Phe allele[21] on the relationship between antiphospholipid antibodies and arterial thrombosis.

Methods

Study design

The Risk of Arterial thrombosis in Relation to Oral Contraceptives (RATIO) study is a multi-center population-based case-control study designed to investigate the risk of three presentations of arterial thrombosis (myocardial infarction, ischemic stroke and peripheral vascular disease). Data collection occurred in two phases; during the first phase (1990-1995) patients were included and the risk of arterial thrombosis in relation to the use of oral contraceptives was investigated. During the second phase (1998-2002), blood samples were drawn and buccal swabs were taken to investigate prothrombotic

risk factors. Details of the study have been published previously.[15;16;22]

Patients

Eligible patients were women, aged 18-49, hospitalized for a first event of myocardial infarction or ischemic stroke in 16 hospitals (8 academic centers and 8 large non-academic hospitals) between January 1990 and October 1995.

Myocardial infarction was confirmed by the presence of symptoms, elevated cardiac enzyme levels and electrocardiographic changes. 218 patients out of 248 patients included during the first phase of the study were willing to participate in the second phase of the study. Samples of venous blood were collected from 203 patients.

Noncardioembolic ischemic stroke was confirmed by medical history and physical examination, as well as by CT or MRI scan evaluated by experienced neurologists in the participating centers. Exclusion criteria were transient ischemic attack (event lasting < 24 hours), hemorrhagic stroke, cerebral sinus venous thrombosis, carotid artery dissection, history of cardiovascular or cerebrovascular disease, aphasia or cognitive impairment interfering with the questionnaire, or not speaking Dutch. Out of 203 women participating in the first phase of the study, 140 women agreed to participate during the second phase (6 patients died, 10 refused to participate, 44 could not be traced and blood sampling failed in 3 patients). Samples of venous blood were obtained from 127 patients. To account for loss of patients we approached an additional 59 cases from the University Medical Center Utrecht in the year 2000. Out of 50 patients willing to participate, 48 donated blood. A total of 175 patients were available for the current

Table 1. Baseline characteristics of control women and women with myocardial infarction or ischemic stroke

Characteristic	Myocardial infarction patients	Ischemic stroke patients	Controls
(N)	203	175	628
Age, mean in years (range)	42 (25-49)	39 (19-49)	39 (18-49)
European ethnicity – N (%)	193 (95)	167 (95)	592 (95)
Hypertension – N (%)	53 (26)	50 (28)	39 (6)
Diabetes Mellitus – N (%)	10 (5)	7 (4)	10 (2)
Hyperlipidemia – N (%)	20 (10)	14 (8)	19 (3)
Oral contraceptive use – N (%)	80 (39)	92 (53)	209 (33)
Smoking at index date – N (%)	167 (82)	105 (60)	266 (42)
Alcohol use – N (%)			
Never	78 (38)	83 (47)	180 (29)
0-15 drinks a week	120 (59)	77 (44)	414 (66)
> 15 drinks a week	5 (3)	2 (1)	29 (5)

Data on ethnicity were missing for 1 ischemic stroke patient and 3 control women, on hypertension for 3 control women, diabetes mellitus for 1 myocardial infarction patient and 3 control women, on hypercholesterolemia for 1 myocardial infarction patient and 3 control women, on oral contraceptive use for 6 control women and on smoking behavior for 5 control women.

analysis.

Control women were identified by random-digit dialing. Eligible for inclusion were women, aged 18-49 years, without a history of myocardial infarction, ischemic stroke or peripheral vascular disease. Controls were frequency-matched to patients for age, residence area and index date of the event (defined as mid-year of the same year as the event). Blood samples were collected from 638 women out of the 925 women included during the first phase of the study. Of these samples, 628 were available for the current analysis.

The study was approved by the local medical ethics committee of all participating hospitals. Informed consent was obtained from all patients and controls in accordance with the declaration of Helsinki.

Data collection

Patients and controls received a standardized questionnaire between 1997 and 2001 on use of oral contraceptives, smoking status, alcohol use, weight, height and physician's diagnosis and medication use for hypertension, diabetes and hyperlipidemia. Questions referred to the period preceding the index date (date of myocardial infarction or ischemic stroke for patients, mid-year of the same year for controls). Blood, obtained through venipuncture, was collected into tubes containing 0.106 M trisodium citrate for coagulation assays. Plasma was obtained by centrifugation of whole anticoagulated blood at 2000*g* for 10 minutes and stored at -80°C until used. EDTA-anticoagulated blood was used for DNA extraction. Hypertension, diabetes and hyperlipidemia were confirmed at the time of blood draw.

Lupus anticoagulant

Lupus anticoagulant was determined with dilute Russell's Viper Venom Time reagents (LA-screen and LA-confirm; Gradipore, Australia) according to the instructions of the manufacturer, with a few modifications. In short, plasma samples thawed for the first time, were diluted 1:1 (vol:vol) with pooled normal plasma of 173 healthy volunteers. Coagulation was initiated by adding an equal volume of LA-screen reagent to mixed plasma. Coagulation times were recorded. In case of a prolonged coagulation time (LA-screen > 99th percentile of normal as determined with 40 healthy volunteers) LA-confirm assays were performed. Normalized ratio's between LA-screen and LA-confirm coagulation times ($Ratio_{s/c}$) were calculated according to the following equation:

$$Ratio_{s/c} = \frac{(\text{LA-screen}^{sample}/\text{LA-screen}^{normal})}{(\text{LA-confirm}^{sample}/\text{LA-confirm}^{normal})}$$

with LA-screen^{normal} the mean LA-screen coagulation time of 40 healthy volunteers and LA-confirm^{normal} the mean LA-confirm coagulation time of 40 healthy volunteers. Samples were deemed positive of Lupus Anticoagulant when $Ratio_{s/c} \geq 1.15$, based on the 99th percentile of 40 healthy volunteers.

Anti-β_2-glycoprotein I antibodies
Human plasma-derived β_2-Glycoprotein I was purified as described before.[23] β_2-glycoprotein I-coated 96-well microtiter plates (Nunc MaxiSorp U96; Nunc, Wiesbaden, Germany) were washed and incubated with plasma samples, diluted 1:100 in PBST (phosphate-buffered saline [10 mM phosphate, 140 mM NaCl, pH 7.35] with 0.1 % Tween-20), at ambient temperature for 1 hour. After washing, plates were incubated with horseradish peroxidase-labelled goat anti-human-IgG antibodies (Southern Biotech, Birmingham, AL, USA) and developed with Amplex Red reagent (Invitrogen, Paisley, UK). Fluorescence was measured in a FluoSTAR OPTIMA reader (BMG labtech, Offenburg, Germany). PBST was used for all washing procedures. Values are expressed as percentage of a positive control. Cut-off was set at the 90th percentile of RATIO controls.

Anticardiolipin antibodies
IgG anticardiolipin antibodies were measured in plasma with a commercially available kit (Corgenix, Broomfield, CO, USA) according to the instructions of the manufacturer. Values are expressed as anticardiolipin immunoglobin G levels (GPL). Cut-off was set at the 90th percentile of RATIO controls.

Antiprothrombin antibodies
IgG antiprothrombin antibodies were measured as described previously.[24] Values are expressed as percentage of a positive control. Cut-off was set at the 90th percentile of RATIO controls.

DNA analyses
The factor V G1691A, prothrombin G20210A and factor XIII 204Phe variants were assessed by polymerase chain reaction as described before.[21;25;26]

Statistical analysis
Relative risks of myocardial infarction and stroke associated with the presence of antiphospholipid antibodies were calculated as odds ratios with 95%-confidence intervals (95%-CI) using logistic regression. Odds Ratios were adjusted for the stratifying variables age (as a continuous variable), residence area (4 categories) and index year (6 categories). The additional ischemic stroke patients were assigned the highest index year of patients included in the first phase of the study. We furthermore calculated the risk of arterial thrombosis in the presence of combinations of antiphospholipid antibody subtypes. Finally, we investigated the contribution of cardiovascular risk factors (smoking, hyperlipidemia, diabetes and the Factor V Leiden, prothrombin G20210A and FXIII 204Phe variants) to the risk of arterial thrombosis in the presence of antiphospholipid antibodies.

Table 2. Distribution of antiphospholipid antibodies and odds ratios for myocardial infarction and ischemic stroke

	Distribution - N			Odds Ratio (95% CI)§	
	Myocardial infarction	Ischemic stroke	Controls	Myocardial infarction	Ischemic stroke
Lupus anticoagulant					
(N)	202	175	627		
$Ratio_{s/c} \geq 1.15$	6	30	4	5.3 (1.4 - 20.8)	43.1 (12.2 - 152)
$Ratio_{s/c} \geq 1.20$	5	19	2	11.7 (2.1 - 65)	48.3 (8.2 - 284)
$Ratio_{s/c} \geq 1.30$	4	10	0	NA	NA
Anticardiolipin IgG – GPL					
(N)	201	169	623		
≥14.6*	26	12	62	1.6 (0.9 - 2.6)	0.8 (0.4 - 1.7)
≥16.9†	16	8	31	1.8 (0.9 - 3.4)	0.9 (0.4 - 2.2)
≥24.5‡	2	6	6	1.5 (0.3 - 8.4)	1.9 (0.5 - 7.6)
Anti-β_2-Glycoprotein I IgG – % of positive control					
(N)	203	175	628		
≥27.9*	18	39	62	0.9 (0.5 - 1.6)	2.3 (1.4 - 3.7)
≥37.7†	11	24	31	1.2 (0.6 - 2.6)	2.8 (1.5 - 5.3)
≥74.3‡	3	4	6	2.2 (0.5 - 10.3)	1.7 (0.4 - 7.9)
Anti-prothrombin IgG – % of positive control					
(N)	203	175	628		
≥31.8*	14	21	62	0.7 (0.4 - 1.2)	1.3 (0.7 - 2.4)
≥47.4†	7	13	31	0.8 (0.3 - 1.9)	1.8 (0.8 - 4.2)
≥85.9‡	3	4	6	1.8 (0.4 - 7.4)	2.6 (0.6 - 11.1)

* Cut-off, value corresponds to the 90th percentile of controls
† Cut-off, value corresponds to the 95th percentile of controls
‡ Cut-off, value corresponds to the 99th percentile of controls
§ Values adjusted for age, residence area and index year
CI denotes confidence interval; NA denotes not applicable.

Results

Table 1 shows characteristics of the study population. As expected, both myocardial infarction and ischemic stroke patients had a higher prevalence than controls of cardiovascular risk factors, such as hypertension, diabetes, hyperlipidemia, oral contraceptive use and smoking. All oral contraceptives were combinations of an estrogen (ethinylestradiol) and a progestin (levonorgestrel, desorgestrel, lynestrenol or cyproterone acetate).

We analyzed the effect of different antiphospholipid antibody subpopulations on the risk of myocardial infarction or ischemic stroke after adjustment for the matching variables (Table 2). Lupus anticoagulant was found in 6 (3%) myocardial infarction patients and 30 (17%) ischemic stroke patients, whereas only 4 (0.6%) controls had lupus anticoagulant. The odds ratio for myocardial infarction was 5.3 (95%-CI, 1.4 to 20.8) and the odds ratio for ischemic stroke was 43.1 (95%-CI, 12.2 to 152) in women

with lupus anticoagulant compared with women without lupus anticoagulant. Twenty-three myocardial infarction patients and 22 ischemic stroke patients received coumarin derivatives. After exclusion of coumarin derivative users the odds ratio for myocardial infarction was 4.6 (95%-CI, 1.1 to 19.5) and that for ischemic stroke 45.7 (95%-CI 12.4 to 169). When we used a more stringent cut-off level for lupus anticoagulant ($Ratio_{s/c} \geq 1.2$) the odds ratio for myocardial infarction and ischemic stroke increased. None of the control women had lupus anticoagulant when $Ratio_{s/c} \geq 1.30$ was used as cut-off level. Neither anticardiolipin nor antiprothrombin antibodies affected the risk of myocardial infarction or ischemic stroke. The risk of ischemic stroke was increased 2-fold (95%-CI, 1.4 to 3.7) in women with anti-β_2-glycoprotein I antibodies compared with women without anti-β_2-glycoprotein I antibodies, whereas anti-β_2-glycoprotein I antibodies did not affect the risk of myocardial infarction. The presence of lupus anticoagulant and any additional antiphospholipid antibody subpopulation did not affect the risk of myocardial infarction or ischemic stroke as compared with the risk in patients with lupus anticoagulant only.

Adjusting for the cardiovascular risk factors hypertension, diabetes and hyperlipidemia did not influence the relative risks for myocardial infarction or ischemic stroke in women with antiphospholipid antibodies.

We analyzed the joint effect of additional cardiovascular risk factors in women with lupus anticoagulant, as compared with women without lupus anticoagulant and each cardiovascular risk factor (Table 3). In women without lupus anticoagulant the risk of myocardial infarction was increased 2-fold (95%-CI, 1.6 to 3.4) in users of oral contraceptives and 6-fold (95%-CI, 4.2 to 9.7) in smokers. Lupus anticoagulant in combination with oral contraceptive use increased the risk of myocardial infarction 20-fold (95%-CI, 1.9 to 242), while the combination of lupus anticoagulant and smoking increased the risk of myocardial infarction 33-fold (95%-CI, 6.0 to 189). Neither the factor V G1691A, the prothrombin G20210A or the factor XIII 204Phe variant affected the risk of myocardial infarction.

In women without lupus anticoagulant the odds ratio for ischemic stroke was 2.9 (95%-CI, 1.8 to 4.6) in oral contraceptive users, 2.2 (95%-CI, 1.5 to 3.4) in smokers and 8.8 (95%-CI, 4.9 to 15.9) in women with the FXIII 204Phe allele. Combined, lupus anticoagulant and oral contraceptive use increased the risk of ischemic stroke 201-fold (95%-CI, 22.1 to 1828). Smoking and lupus anticoagulant increased the risk of ischemic stroke 87-fold (95%-CI, 14.5 to 523) and the combination of lupus anticoagulant and the FXIII 204Phe allele increased the risk of ischemic stroke 81-fold (95%-CI, 8.9 to 739). The factor V G1691A and prothrombin G20210A variants did not affect the risk of ischemic stroke.

Discussion

We found that lupus anticoagulants were associated with an increased risk of both myo-

cardial infarction (5-fold increased risk) and ischemic stroke (43-fold increased risk) in young women. Furthermore, anti-β_2-glycoprotein I antibodies were associated with a 2-fold increased risk of ischemic stroke. Neither anticardiolipin nor antiprothrombin antibodies alone increased the risk of myocardial infarction or ischemic stroke. There were no indications that the presence of more than one antiphospholipid antibody subpopulation affected the risk of myocardial infarction or ischemic stroke. Additional cardiovascular risk factors, such as oral contraceptives use, smoking or the FXIII 204Phe variant, increased the already elevated risk of myocardial infarction or ischemic stroke further in women with lupus anticoagulant.

The RATIO study compared characteristics of a large number of women with either myocardial infarction or ischemic stroke with a large population-based control group obtained through random-digit dialing. Diagnosis and origin of all ischemic strokes was verified with CT or MRI, which reduces the risk of misclassification. The random digit dialing procedure and the high response-rate in the control-group, which was not informed on the determinants of this study such as oral contraceptive use or smoking, minimizes the risk of participation-bias in the control group.

We should address the limitations of our study as well. All patients were hospitalized survivors of a major arterial event. The restriction to surviving cases potentially leads to an attenuation of the true risk of arterial thrombosis associated with antiphospholipid antibodies. It is improbable that this has a major impact on our findings, as ischemic stroke or myocardial infarction is rarely fatal in young women.[27] Correct sample handling is imperative for detection of lupus anticoagulant. Contamination of plasma samples with residual blood cells was avoided as this might mask the presence of lupus anticoagulant. Blood sample handling was standardized to exclude possible effects of sample handling on differences between cases and controls. Another potential source of bias is anticoagulant treatment with coumarin derivatives, which might interfere with the detection of lupus anticoagulant. Exclusion of patients treated with anticoagulants resulted in a slight decrease in the risk of myocardial infarction, but did not affect the risk of ischemic stroke. As blood was sampled once, we cannot exclude that a proportion of detected anticardiolipin antibodies reflects transient infection-related antibodies.

Two other studies compared effects of antiphospholipid antibodies on the risk of ischemic stroke[5] or myocardial infarction[9] in an unselected population of young women. In line with our results, each study reported an increased risk of an arterial thrombotic event associated with antiphospholipid antibodies, although risks were attributed to different antiphospholipid antibody subpopulations. One of these studies[5] reported a 2-fold increased risk for ischemic stroke in the presence of lupus anticoagulant or anticardiolipin antibodies, whereas anticardiolipin antibodies did not increase the risk of ischemic stroke in our study. Inclusion of unconfirmed strokes or cardioembolic strokes combined with the use of the less specific APTT-based lupus anticoagulant assay[28] might explain the low risk of stroke associated with lupus anticoagulant in this particular study. A study on the risk of myocardial infarction in young women[9] reported a 2-fold in-

Table 3. Risk of myocardial infarction or ischemic stroke in relation to the presence of lupus anticoagulant and the presence or absence of cardiovascular risk factors.

	No lupus anticoagulant				
	Distribution, N			Odds Ratio (95% CI)	
	Myocardial infarction	Ischemic stroke	Controls	Myocardial infarction	Ischemic stroke
Oral contraceptives					
No	120	71	409	1†	1†
Yes	76	74	208	2.3 (1.6 – 3.4)	2.9 (1.8 – 4.6)
Smoking					
No	36	57	354	1†	1†
Yes	160	88	264	6.4 (4.2 – 9.7)	2.2 (1.5 – 3.4)
Factor V G1691A					
No	184	126	587	1†	1†
Yes	12	11	33	1.1 (0.5 – 2.2)	1.9 (0.9 – 4.2)
Prothrombin G20210A					
No	192	139	605	1†	1†
Yes	4	3	15	0.7 (0.2 – 2.5)	0.7 (0.2 – 2.8)
FXIII 204Phe allele					
No	186	91	582	1†	1†
Yes	10	44	35	0.9 (0.4 – 1.9)	8.8 (4.9 – 5.9)

	Lupus anticoagulant*				
	Distribution, N			Odds Ratio (95% CI)	
	Myocardial infarction	Ischemic stroke	Controls	Myocardial infarction	Ischemic stroke
Oral contraceptives					
No	2	12	3	3.5 (0.5 – 22.6)	33.6 (6.8 – 167)
Yes	4	18	1	21.6 (1.9 – 242)	201 (22.1 – 1828)
Smoking					
No	0	13	2	NA	47.2 (8.1 – 276)
Yes	6	17	2	33.7 (6.0 – 189)	87 (14.5 – 523)
Factor V G1691A					
No	6	24	3	6.6 (1.5 – 29)	57 (13 – 251)
Yes	0	1	1	NA	11 (0.5 – 225)
Prothrombin G20210A					
No	6	27	4	5.3 (1.4 – 21)	42 (12 – 153)
Yes	0	1	0	NA	NA
FXIII 204Phe allele					
No	6	17	3	6.5 (1.5 – 28)	51.8 (9.9 – 270)
Yes	0	8	1	NA	81.4 (8.9 – 739)

*$Ratio_{s/c} \geq 1.15$
† reference
CI denotes confidence interval; NA denotes not applicable. All odds ratios are adjusted for age, residence area and index year

creased risk in the presence of anti-β_2-glycoprotein I antibodies, but not in the presence of anticardiolipin antibodies. Anti-β_2-glycoprotein I antibodies did not increase the risk of myocardial infarction in our study.

The lupus anticoagulant subpopulation of antiphospholipid antibodies encompasses a heterogeneous pool of antibodies with several antigenic targets. Over the years, insight into the mechanism behind the prothrombotic effects of antiphospholipid antibodies steadily increased. Platelet activation[29] and a pro-coagulant endothelial phenotype[30] is described upon exposure to lupus anticoagulants. Prothrombotic effects on the coagulation system, such as antiphospholipid antibody-related acquired activated protein C resistance,[31] are found in patients with the antiphospholipid syndrome as well. It is therefore not surprising that smoking, which causes endothelial dysfunction[32], and oral contraceptive use, which affects the anticoagulant protein C axis[33], increase the effect of lupus anticoagulant on the risk of ischemic stroke or myocardial infarction. Why the effect of lupus anticoagulant on ischemic stroke is more pronounced than its effect on myocardial infarction remains to be established.

Our study shows that lupus anticoagulant is a major risk factor for arterial thrombotic disease. Despite its low prevalence in the general population[7] (0.6% in this study), a population attributable risk of 20.1% shows that lupus anticoagulant is responsible for a large proportion of ischemic strokes in young women. As secondary thromboprophylaxis of noncardioembolic strokes in antiphospholipid syndrome patients might consist of life-long administration of oral anticoagulants rather than treatment with antiplatelet agents,[34] screening for the presence of lupus anticoagulant in young women with ischemic stroke appears to be warranted.

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References

1. Cervera R, Piette JC, Font J et al. Antiphospholipid syndrome: clinical and immunologic manifestations and patterns of disease expression in a cohort of 1,000 patients. Arthritis Rheum. 2002;46:1019-1027.
2. Miyakis S, Lockshin MD, Atsumi T et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J.Thromb.Haemost. 2006;4:295-306.
3. Urbanus RT, Derksen RH, de Groot PG. Current insight into diagnostics and pathophysiology of the antiphospolipid syndrome. Blood Rev. 2008;22:93-105.
4. Galli M, Luciani D, Bertolini G, Barbui T. Lupus anticoagulants are stronger risk factors for thrombosis than anticardiolipin antibodies in the antiphospholipid syndrome: a systematic review of the literature. Blood 2003;101:1827-1832.
5. Brey RL, Stallworth CL, McGlasson DL et al. Antiphospholipid antibodies and stroke in young women. Stroke 2002;33:2396-2400.
6. Caso V, Parnetti L, Panarelli P et al. Selection of thrombogenetic antiphospholipid antibodies in cerebrovascular disease patients. J.Neurol. 2003;250:593-597.
7. de Groot PG, Lutters B, Derksen RH et al. Lupus anticoagulants and the risk of a first episode of deep venous thrombosis. J.Thromb.Haemost. 2005;3:1993-1997.
8. Kahwa EK, Sargeant LA, Farlane-Anderson N et al. Anticardiolipin antibodies and risk of thromboembolic disease in young Jamaican women. J.Cardiovasc. Risk 2001;8:349-354.
9. Meroni PL, Peyvandi F, Foco L et al. Anti-beta 2 glycoprotein I antibodies and the risk of myocardial infarction in young premenopausal women. J.Thromb. Haemost. 2007;5:2421-2428.
10. Metz LM, Edworthy S, Mydlarski R, Fritzler MJ. The frequency of phospholipid antibodies in an unselected stroke population. Can.J.Neurol.Sci. 1998;25:64-69.
11. Runchey SS, Folsom AR, Tsai MY, Cushman M, McGovern PD. Anticardiolipin antibodies as a risk factor for venous thromboembolism in a population-based prospective study. Br.J.Haematol. 2002;119:1005-1010.
12. Vaarala O, Manttari M, Manninen V et al. Anti-cardiolipin antibodies and risk of myocardial infarction in a prospective cohort of middle-aged men. Circulation 1995;91:23-27.
13. Vaarala O, Puurunen M, Manttari M et al. Antibodies to prothrombin imply a risk of myocardial infarction in middle-aged men. Thromb.Haemost. 1996;75:456-459.
14. Galli M, Reber G, de Moerloose P, de Groot PG. Invitation to a debate on the serological criteria that define the antiphospholipid syndrome. J.Thromb.Haemost. 2008;6:399-401.
15. Kemmeren JM, Tanis BC, van den Bosch MA et al. Risk of Arterial Thrombosis in Relation to Oral Contraceptives (RATIO) study: oral contraceptives and the risk of ischemic stroke. Stroke 2002;33:1202-1208.
16. Tanis BC, van den Bosch MA, Kemmeren JM et al. Oral contraceptives and the risk of myocardial infarction. N.Engl.J.Med. 2001;345:1787-1793.
17. Rosendaal FR, Siscovick DS, Schwartz SM et al. Factor V Leiden (resistance to activated protein C) increases the risk of myocardial infarction in young women. Blood 1997;89:2817-2821.
18. Longstreth WT, Jr., Rosendaal FR, Siscovick DS et al. Risk of stroke in young women and two prothrombotic mutations: factor V Leiden and prothrombin gene variant (G20210A). Stroke 1998;29:577-580.
19. Rosendaal FR, Siscovick DS, Schwartz SM et al. A common prothrombin variant (20210 G to A) increases the risk of myocardial infarction in young women. Blood 1997;90:1747-1750.
20. De Stefano V, Chiusolo P, Paciaroni K et al. Prothrombin G20210A mutant genotype is a risk factor for cerebrovascular ischemic disease in young patients. Blood 1998;91:3562-3565.
21. Pruissen DM, Slooter AJ, Rosendaal FR, van der Graaf Y, Algra A. Coagulation factor XIII gene variation, oral contraceptives, and risk of ischemic stroke. Blood 2008;111:1282-1286.
22. Slooter AJ, Rosendaal FR, Tanis BC et al. Prothrombotic conditions, oral contraceptives, and the risk of ischemic stroke. J.Thromb. Haemost. 2005;3:1213-1217.
23. Horbach DA, van Oort E, Donders RC, Derksen RH, de Groot PG. Lupus anticoagulant is the strongest risk factor for both venous and arterial thrombosis in patients with systemic lupus erythematosus. Comparison between different assays for the detection of antiphospholipid antibodies. Thromb.Haemost. 1996;76:916-924.
24. de Laat HB, Derksen RH, Urbanus RT, Roest M, de Groot PG. beta2-glycoprotein I-dependent lupus anticoagulant highly correlates with thrombosis in the antiphospholipid syndrome. Blood 2004;104:3598-3602.
25. Bertina RM, Koeleman BP, Koster T et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 1994;369:64-67.
26. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3’-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood 1996;88:3698-3703.
27. Sarti C, Stegmayr B, Tolonen H et al. Are changes in mortality from stroke caused by changes in stroke event rates or case fatality? Results from the WHO MONICA Project. Stroke 2003;34:1833-1840.
28. Derksen RH, de Groot PG. Tests for lupus anticoagulant revisited. Thromb.Res. 2004;114:521-526.
29. Urbanus RT, Pennings MT, Derksen RH, de Groot PG. Platelet activation by dimeric beta(2)-glycoprotein I requires signaling via both glycoprotein Ibalpha and Apolipoprotein E Receptor 2’. J.Thromb.Haemost. 2008;6:1405-1412.
30. Vega-Ostertag M, Casper K, Swerlick R et al. Involvement of p38 MAPK in the up-regulation of tissue factor on endothelial cells by antiphospholipid antibodies. Arthritis Rheum. 2005;52:1545-1554.
31. Safa O, Esmon CT, Esmon NL. Inhibition of APC anticoagulant activity on oxidized phospholipid by anti-{beta}2-glycoprotein I monoclonal antibodies. Blood 2005;106:1629-1635.
32. Ambrose JA, Barua RS. The pathophysiology of cigarette smoking and cardiovascular disease: an update. J.Am.Coll. Cardiol. 2004;43:1731-1737.

33. Rosing J, Middeldorp S, Curvers J et al. Low-dose oral contraceptives and acquired resistance to activated protein C: a randomised cross-over study. Lancet 1999;354:2036-2040.

34. Albers GW, Amarenco P, Easton JD, Sacco RL, Teal P. Antithrombotic and thrombolytic therapy for ischemic stroke: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest 2008;133:630S-669S.

5

Lupus anticoagulants are not a risk factor for myocardial infarction in men

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Submitted

Summary

The presence of lupus anticoagulants has been shown to be predictive of a first episode of venous thrombosis in a general population. Data on the predictive value of lupus anticoagulant for arterial thrombosis in a general population is lacking. We have measured the presence of lupus anticoagulants in the Study of Myocardial Infarction Leiden (SMILE), a population-based case-control study in men. Nineteen out of 1199 included individuals tested positive for lupus anticoagulant, nine of whom had experienced a myocardial infarction (OR 1.04; 95% CI 0.42-2.57). In conclusion, the presence of lupus anticoagulants is not an important risk factor for myocardial infarction in elderly men without a history of autoimmunity.

The association of vascular thrombosis or pregnancy loss with circulating antiphospholipid antibodies (aPL) is known as the antiphospholipid syndrome (APS). In a recent international consensus meeting three methods to detect aPL were recommended: an enzyme-linked immunosorbent assay (ELISA) detecting anti-cardiolipin antibodies, an ELISA in which anti-β_2-glycoprotein I antibodies are detected and a coagulation assay in which aPL known as Lupus Anticoagulants (LAC) prolong coagulation time in a phospholipid dependent manner.[1]

A systematic review has shown that in particular the presence of LAC is associated with a high thrombotic risk, irrespective of the type and site of thrombosis.[2] As the studies described in this review were based on small or selected patient populations, data on the risk of thrombosis for the presence of LAC in the general population is limited. Recently, LAC have been shown to predict the occurrence of a first episode of venous thrombosis in a general population.[3] Data on the risk of arterial thrombosis in a general population for the presence of LAC are lacking. Although the Antiphospholipid Antibodies and Stroke Study (APASS) has shown that presence of LAC does not predict an increased risk for subsequent occlusive vascular events in patients with a history of ischemic stroke,[4] the risk of a first arterial thrombotic event for LAC remains to be established. In the present study, we have measured LAC in the "Study of Myocardial Infarctions Leiden" (SMILE), a large population-based case-control study in men.[5]

Patients were 560 men who experienced a first myocardial infarction before the age of 70 years and were hospitalized (in Leiden) between January 1990 and January 1996. Mean age of patients was 56.2 (standard deviation [SD] 9.0 years). Myocardial infarction had to be confirmed by documentation in hospital records or discharge reports. At least two of the following characteristics had to be present: typical chest pain, changes in electrocardiogram typical for myocardial infarction, or an elevation of cardiac enzymes to more then twice the upper limit of a normal value. Control subjects were 646 men who had an orthopaedic intervention between January 1990 and June 1994, for which they routinely received prophylactic anticoagulants for a short period of time. Mean age of control subjects was 57.3 (SD 10.8) years. Excluded from participation were men with renal disease, severe (neuro)psychiatric problems, or men with a life expectancy of less than one year. Full details of the SMILE study have been published elsewhere.[5]

Blood was collected in Sarstedt Monovette tubes containing trisodium citrate (10.6 mM final concentration). Plasma was obtained by centrifugation at 2000*g* for 10 minutes, aliquoted and stored at –70 °C until use. Plasma samples used to measure LAC were thawed once and immediately measured. Seven samples could not be tested for LAC due to shortage of material.

LAC was measured with a dilute Russell's Viper Venom Time reagent (LA screen, Gradipore, Australia). All samples were mixed 1:1 with pooled normal plasma of 150 healthy donors to correct for the effect of anticoagulants. Samples were considered posi-

Table 1. Lupus anticoagulant and the risk of a first myocardial infarction

	Patients – N (%)	Controls – N (%)	Odds Ratio (95% CI)
N	557	642	
Lupus anticoagulant (≥ cut-off)§	9 (1.6)	10 (1.6)	1.04 (0.42 - 2.57)
No lupus anticoagulant (< cut-off)	548 (98.4)	632 (98.4)	1†

§ cut-off value of 1.2 was used
† reference
CI denotes confidence interval

tive if the clotting time was longer than the mean of 40 healthy volunteers +3 SD, i.e. >39.5 seconds. A confirmation test was performed on all positive samples by repeating the assay in the presence of extra phospholipids (LA confirm, Gradipore, Australia). Clotting times reduced more than 20% by the addition of phospholipids, i.e. the ratio LA screen/LA confirm > 1.2, were considered to be positive for LAC. Personnel performing the assay was unaware of the case or control status of the samples.

LAC were detected in 19 persons (1.6%) out of the entire study population of 1199 individuals. Out of 19 LAC positive samples, 18 were weakly positive (LA screen / LA confirm ratio between 1.2 and 1.5), whereas in one individual the LA screen / LA confirm ratio was > 1.5. Nine out of 557 patients with myocardial infarction (1.6%) were LAC positive compared to 10 out of 642 control subjects (1.6%) (table). The odds ratio (OR) for the risk of myocardial infarction in LAC positive individuals was 1.04 with a 95% confidence interval of 0.42-2.57 compared to LAC negative individuals (table), indicating no increased risk. Adjustment for age did not change the odds ratio (OR 1.03; 95% CI 0.41 – 2.55). To test whether raising the cut-off levels for the presence of LAC would increase the risk of myocardial infarction for LAC, we determined the odds ratio for a cut-off value of 1.3. Only 4 persons remained positive, 3 of whom were patients and one a control subject, for an odds ratio of 3.5 (95% CI 0.4-33.5).

The antiphospholipid syndrome is associated with high morbidity and its diagnosis requires rapid and adequate treatment to prevent further episodes of thrombosis. Despite evidence in the literature of an association between the presence of LAC and the occurrence of myocardial infarction in patients with an underlying autoimmune disease,[6] we have not been able to confirm this in a general population. Although we detected an effect of the presence of LAC on the occurrence of myocardial infarction at a cut-off for LAC ≥ 1.3, our study lacks power to solidify this observation.

The discrepancy between our results and other studies[2] could be explained by the populations selected to investigate the relation between LAC and arterial thrombosis. In populations with underlying systemic autoimmune diseases such as Systemic Lupus Erythematosus, the presence of LAC is relatively common, whereas the presence of LAC is rare in the general population. Aside from that, most studies have focused on the relation between the presence of LAC and cerebral stroke, whereas we have focused on myocardial infarction. Furthermore, as approximately 80% of all patients with the

antiphospholipid syndrome are women,[3] a relation between the presence of LAC and arterial thrombosis might still exist in women.

In conclusion, the presence of lupus anticoagulants is not an important risk factor for myocardial infarction in men without a history of autoimmunity. Therefore, routine screening for LAC in men with myocardial infarction does not appear warranted. We cannot, however, exclude a relation between LAC and arterial thrombosis in women.

Acknowledgements

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References

1. Miyakis S, Lockshin MD, Atsumi T et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J.Thromb.Haemost. 2006;4:295-306.
2. Galli M, Luciani D, Bertolini G, Barbui T. Lupus anticoagulants are stronger risk factors for thrombosis than anticardiolipin antibodies in the antiphospholipid syndrome: a systematic review of the literature. Blood 2003;101:1827-1832.
3. de Groot PG, Lutters B, Derksen RH et al. Lupus anticoagulants and the risk of a first episode of deep venous thrombosis. Journal of Thrombosis and Haemostasis. 2005;3:1993-1997.
4. Levine SR, Brey RL, Tilley BC et al. Antiphospholipid antibodies and subsequent thrombo-occlusive events in patients with ischemic stroke. JAMA 2004;291:576-584.
5. Doggen CJ, Cats VM, Bertina RM, Rosendaal FR. Interaction of coagulation defects and cardiovascular risk factors: increased risk of myocardial infarction associated with factor V Leiden or prothrombin 20210A. Circulation 1998;97:1037-1041.
6. Petri M. The lupus anticoagulant is a risk factor for myocardial infarction (but not atherosclerosis): Hopkins Lupus Cohort. Thromb. Res. 2004;114:593-595.

6

Platelet activation by dimeric β_2-glycoprotein I requires signaling via both glycoprotein Ibα and Apolipoprotein E receptor 2'

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Abstract

Background: Dimerization of β_2-Glycoprotein I (β_2-GPI) by auto-antibodies is thought to trigger the clinical manifestations observed in the antiphospholipid syndrome. Arterial thrombosis, a frequently occurring clinical manifestation of the antiphospholipid syndrome, is a process in which platelets play a crucial role. Previous work has shown that binding of dimeric β_2-GPI to the platelet receptors Apolipoprotein E Receptor 2' (ApoER2') and Glycoprotein Ibα (GPIbα) mediates increased platelet activation in an in vitro thrombosis model.

Objective: The individual roles of ApoER2' and GPIbα in mediating platelet activation by dimeric β_2-GPI is hitherto unclear. In this study we have determined the roles of either receptor in platelet activation by dimeric β_2-GPI.

Methods: Platelet activation by dimeric β_2-GPI was studied under conditions of flow. Intracellular signaling induced by dimeric β_2-GPI was subsequently analyzed by means of SDS-PAGE and western blot analysis.

Results: The increase in platelet deposition to a fibronectin surface under conditions of flow by dimeric β_2-GPI was completely abolished by inhibition of the interaction of dimeric β_2-GPI with either GPIbα or ApoER2'. Upon platelet stimulation with dimeric β_2-GPI, GPIbα translocated to the cytoskeleton via the scaffold protein 14-3-3ζ. Concomitantly ApoER2' dissociated from the adapter protein Disabled1, presumably through phosphorylation of the cytoplasmic tail. Inhibition of one process could not inhibit the other.

Conclusion: We show that dimeric β_2-glycoprotein I signals via two distinct pathways in platelets, both of which are required for platelet activation. Abrogation of either signal results in loss of activation.

Introduction

The 45 kDa plasma protein β_2-glycoprotein I (β_2-GPI) is the major antigen in the antiphospholipid syndrome (APS), a non-inflammatory autoimmune disease characterized by the co-occurrence of vascular thrombosis and/or pregnancy morbidity and the presence of antiphospholipid antibodies (aPL) in plasma of affected individuals.[1] Despite its abundance in human plasma, the function of β_2-GPI remains unknown. The general consensus is that dimerization of β_2-GPI by aPL results in a conformational change in the molecule, which greatly increases its affinity for anionic phospholipids and several cellular receptors, enabling it to interact with cells.[2] Platelets are the key players in the pathogenesis of arterial thrombosis, a manifestation that occurs in a large proportion of antiphospholipid syndrome patients. Over the years, several receptors on a wide array of cell types have been reported to interact with dimerized β_2-GPI, but only the interaction between dimeric β_2-GPI and two receptors on the platelet membrane has been studied in more detail: Apolipoprotein E Receptor 2' (ApoER2', also known as LRP8Δ5)[3;4] and Glycoprotein Ibα (GPIbα).[5;6]

Interestingly, a genome wide study has recently linked a mutation in the cytoplasmic tail of ApoER2 to the occurrence of arterial thrombosis at an early age.[7] ApoER2' is the only LDL-receptor family member present on platelets.[8] Although ApoER2' has originally been described to mediate the inhibitory effect of Apolipoprotein E on platelet activation,[8] LDL has been reported to exert an activating effect on platelets through the same receptor.[9] These data suggest that ApoER2' has both inhibitory and activating properties in platelets, depending on the nature of the ligand. Previous work from our group has shown that the interaction between ApoER2' and β_2-GPI/antibody complexes or recombinant dimeric β_2-GPI causes increased platelet adhesion to a collagen surface in an in-vitro model of arterial thrombosis.[3;4;10] The other platelet receptor that has been shown to interact with dimerized β_2-GPI is GPIbα.[5;6] As the receptor for surface-bound Von Willebrand Factor, GPIbα is considered to be the most important adhesive receptor on platelets. Over the years, other functions have been attributed to GPIbα as well. Not merely an adhesive receptor, GPIbα also functions as a co-receptor for the activation of PAR1 by thrombin[11] and has been reported to bind other coagulation factors.[12;13] Furthermore, ligand binding to GPIbα has been shown to lead to intracellular signaling,[14] implying an active participation in overall platelet activation.

The effect of dimeric β_2-GPI on platelet adhesion to collagen suggests the triggering of an intracellular signaling cascade, leading to greater sensitivity for collagen induced activation.[4] Although ligand binding to both GPIbα and ApoER2' is known to lead to intracellular signaling, only little is known about signaling downstream from GPIbα, whereas all of the data obtained on signaling downstream from ApoER2 have been obtained from neurons.[15;16]

This study addresses several issues. We have studied the individual roles of GPIbα and ApoER2' in platelet activation by dimeric β_2-GPI under conditions of flow. To

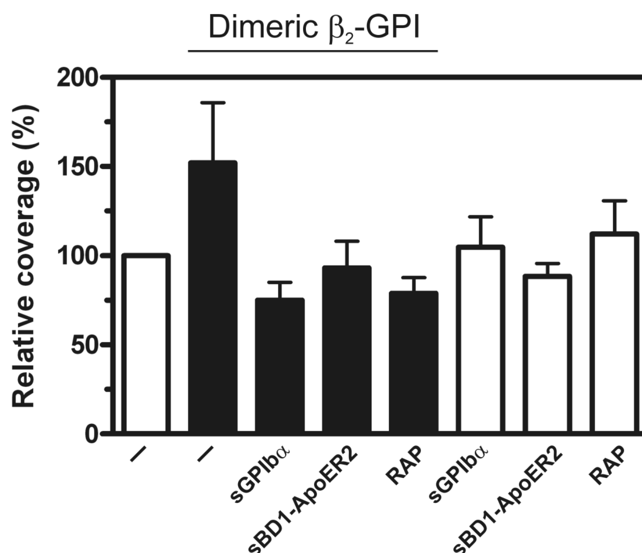

Figure 1. Both GPIbα and ApoER2' are required for the increase in platelet adhesion to fibronectin caused by dimeric β_2-GPI. Reconstituted blood was perfused over a fibronectin surface at a shear rate of 300 s^{-1} for 90 seconds. Platelets were preincubated with dimeric β_2-GPI (50 μg mL^{-1}) with or without inhibitors (sGPIbα [65 μg mL^{-1}], sBD1-ApoER2 [125 μg mL^{-1}], RAP [50 μg mL^{-1}]) at 37 ^{0}C for 5 minutes prior to perfusions. The buffer control was set at 100 %. Data is depicted as mean ± SEM and represents at least three experiments. * $p < 0.01$

discern whether platelet activation by dimeric β_2-GPI is caused by either GPIbα or ApoER2', we have subsequently studied the signaling processes originating from GPIbα and ApoER2' by studying the interaction of GPIbα with the adapter molecule 14-3-3ζ and of ApoER2' with the adapter molecule Disabled1 (Dab1). Both signaling molecules have been shown to be of crucial importance in the generation of intracellular signaling upon binding of relevant ligands.[17;18]

Materials and methods

Reagents

Monoclonal antibody (MAb) MP4.3, directed against human ApoER2, was produced in our laboratory by immunization of mice with the peptide WRCDEDDDCLDHS-DED. Fibronectin was purchased from Sigma (Zwijndrecht, the Netherlands). Goat anti-Dab1 antibodies, goat anti-ApoER2 antibodies, normal rabbit IgG and normal goat IgG were purchased from Santa Cruz (Santa Cruz, California). Rabbit anti-14-3-3ζ antibody was purchased from Bio-connect (Huissen, the Netherlands). Anti-phosphotyrosine MAb 4G10 was from Cell Signaling Technology (Danvers, MA, USA). GST-RAP was produced as described elsewhere.[19] Src-family tyrosine kinase inhibitor PP1 was purchased from Alexis Biochemicals (Carlsbad, CA, USA). Ristocetin was purchased from DiaMed (Cressier sur Morat, Switzerland).

Purified proteins

Recombinant dimeric β_2-GPI was constructed and expressed as described previously.[10]

Recombinant soluble LDL-binding domain 1 of ApoER2 (sBD1-ApoER2) was constructed and purified as described before.[20] Purified protein was analyzed by SDS-PAGE. Only 1 band was observed at 25 kDa.

Recombinant wild-type human soluble GPIbα, residues 1-290 (sGPIbα), containing a C-terminal $(His)_6$ tag, was cloned, expressed and purified as previously described.[21] Only highly sulfated sGPIbα (2 or 3 sulfated tyrosine residues) was used. Purified protein was analyzed by SDS-PAGE. Only 1 band was observed at 35kD.

Plasma-derived human β_2-GPI was purified as described before.[22] Von Willebrand Factor was purified from FVIII concentrates as described before.[23] Normal human IgG and anti-β_2-GPI IgG from an antiphospholipid syndrome patient positive for lupus anticoagulant were purified as described before.[24]

Platelet and red blood cell preparation

Whole blood was collected in 0.129 M trisodium citrate (w/v) and platelet-rich plasma (PRP) was prepared. After acidification by adding 10% (v/v) ACD (2.5% trisodium citrate, 1.5% citric acid, and 2% D-glucose) platelets were pelleted and washed by centrifugation as described before.[25] Platelets were resuspended in Hepes-Tyrode buffer (HT; 10 mM HEPES [N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid], 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1.7 mM $MgCl_2$, 5 mM D-glucose) at pH 7.35 to a platelet count of 220.000 μL^{-1} for aggregation studies. Reconstituted blood for perfusion studies was prepared as described before.[25] For perfusion studies, platelets were resuspended in a human albumin solution (HAS; 4% human albumin, 4 mM KCl, 124 mM NaCl, 20 mM $NaHCO_3$, 2 mM Na_2SO_4, 1.5 mM $MgCl_2$, 5 mM $CaCl_2$, 5 mM D-glucose, pH 7.35) to a platelet count of 333.000 μL^{-1}. Packed red blood cells were mixed with platelets in a 40:60 (v/v) ratio to obtain reconstituted blood with a platelet count of 200.000 μL^{-1} and a hematocrit of 40%.

Perfusion studies

Perfusion experiments were performed in a single pass triplo perfusion chamber as described before.[26;27] Coverslips were coated with fibronectin (100 $\mu g\ mL^{-1}$) at 40°C for 18 hours and blocked in 1% HAS at room temperature for 1 hour. To test the inhibitory capacity of different proteins, proteins were added to platelets and mixed carefully to avoid platelet activation, followed by addition of the red blood cells. TBS or dimeric β_2-GPI was preincubated with inhibitors for 5 minutes at 37°C prior to addition to platelets. Dimeric β_2-GPI, RAP, sGPIbα and sBD1-ApoER2 were added at 50 $\mu g\ mL^{-1}$, 50 $\mu g\ mL^{-1}$, 65 $\mu g\ mL^{-1}$ and 125 $\mu g\ mL^{-1}$ respectively. Reconstituted, unmixed blood was incubated for 5 minutes at 37°C and was then mixed gently to homogeneity and used directly for perfusions at a shear rate of 300 s^{-1} for 90 seconds. Coverslips were subsequently stained with May-Grünwald/Giemsa as described previously.[28] Platelet de-

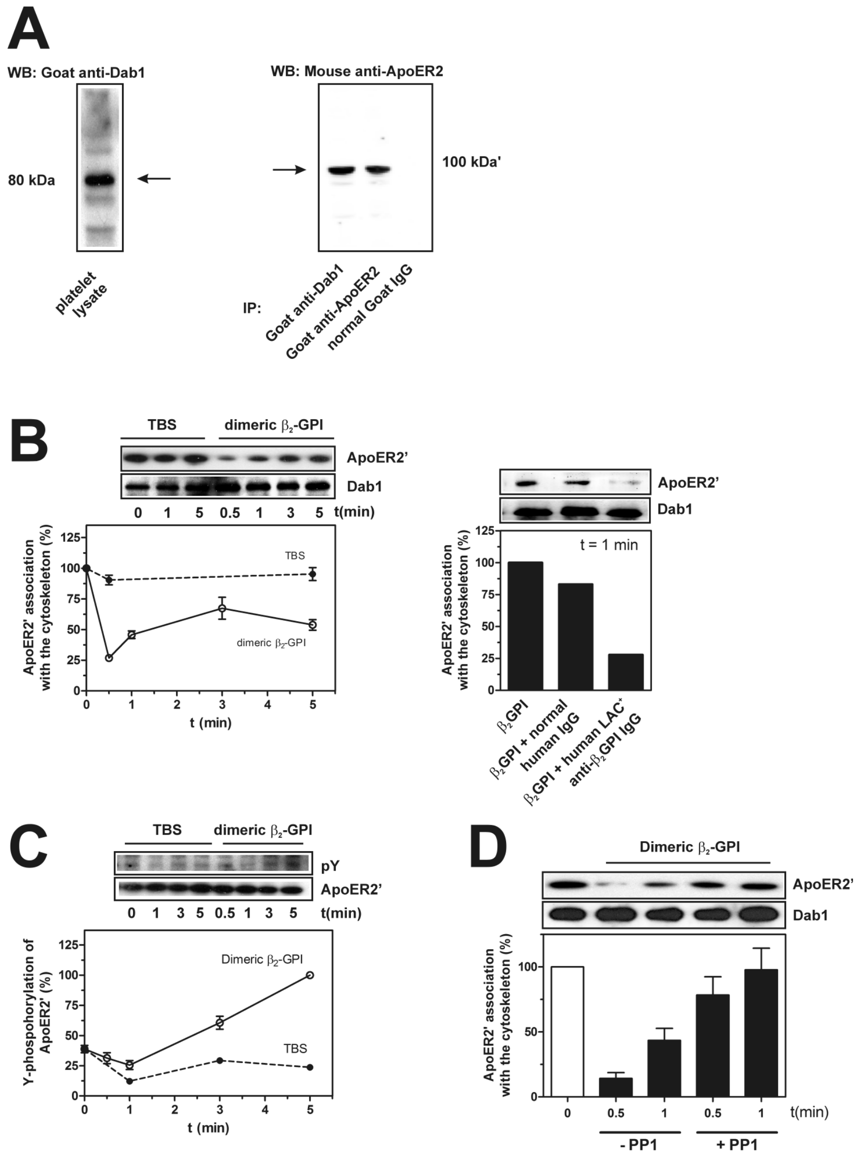

Figure 2. ApoER2' dissociates from the adapter molecule Disabled1 upon platelet stimulation with dimeric β_2-GPI. **A** Whole platelet lysate was probed for Dab1 using goat anti-Dab1 antibodies. Immunoprecipitations were performed on whole platelet lysate and probed for ApoER2' with MP4.3. **B** Platelets were incubated with dimeric β_2-GPI (50 μg mL^{-1}), plasma-derived β_2-GPI (37 μg mL^{-1}) with or without normal human IgG (10 μg mL^{-1}) or human anti-β_2-GPI antibodies (10 μg mL^{-1}), or vehicle for indicated time periods. Immunoprecipitations were performed on cytoskeletal fractions. Western blots were probed for ApoER2' using MP4.3. Dab1 was used as a lane loading control. **C** Platelets were stimulated with dimeric β_2-GPI (50 μg mL^{-1}) or vehicle. ApoER2' was immunoprecipated from whole lysate using goat anti-ApoER2 and probed for phospho-tyrosine (pY) with MAb 4G10. **D** Dimeric β_2-GPI (50 μg mL^{-1}) was added to platelets in the presence or absence of PP1 where indicated. Dab1 was immunoprecipitated from cytoskeletal fractions. Blots were probed with MP4.3. Dab1 was used as a lane loading control. Data depicted as mean ApoER2' density ± SD relative to the buffer control. Immunoblots shown are representative images. At least three experiments were performed.

position was evaluated as described before.[5] Three independent flow experiments were performed for every donor. Platelet adhesion was expressed as the percentage of the surface covered with platelets relative to adhesion to fibronectin in the presence of dimeric β_2-GPI. Results are expressed as mean relative coverage (mean ± SEM, n = 3). Statistical analysis was performed using the Mann-Whitney test for nonparametric correlation.

Translocation of GPIbα and ApoER2'

500 µL aliquots of washed platelets (220,000 μL^{-1}) in HT (pH 7.35) were incubated with TBS or dimeric β_2-GPI at 50 µg/ml for various time points in the presence or absence of inhibitors in an aggregometer while stirring (900 RPM) at 37°C. TBS or dimeric β_2-GPI was preincubated with inhibitors for 5 minutes at 37°C prior to addition to platelets. Platelets were preincubated with Src-family tyrosine kinase inhibitor PP1 (15 µM) for 15 minutes at 37°C prior to addition of dimeric β_2-GPI. Von Willebrand Factor (vWF), ristocetin, plasma derived β_2-GPI, normal human IgG and human anti-β_2-GPI IgG were added at 10 µg mL^{-1}, 1.2 mg/ml, 37 µg mL^{-1}, 10 µg mL^{-1} and 10 µg mL^{-1} respectively. sGPIbα, RAP and sBD1-ApoER2 were used at 65 µg mL^{-1}, 50 µg mL^{-1} and 125 µg mL^{-1}, respectively. Platelets were lysed by adding 10% (v/v) of a 10x concentrated Triton lysis buffer (TxLB; 10% Triton-100, 200mM Tris, 50mM EGTA supplemented with Complete Mini EDTA free proteinase inhibitor cocktail tablets (Roche) according to instructions of the manufacturer). Triton-100 insoluble fractions were spun down at 20.000*g* for 30 minutes and the supernatant was discarded. Pellets were washed twice with 1x TxLB and resuspended in 500 µL RIPA buffer (PBS with 1% Nonidet P40, 0.5% octylglucoside, 0.1% SDS, 0.186% EDTA, 1mM $NaVO_3$, supplemented with Complete Mini EDTA free protease inhibitor cocktail tablets (Roche)). RIPA insoluble fractions were pelleted at 20,000*g* for 10 minutes and the supernatant (i.e. cytoskeletal fraction) was used in immunoprecipitations with a polyclonal anti-14-3-3-ζ antibody or a polyclonal anti-Dab1 antibody to co-immunoprecipitate GPIbα or ApoER2. Samples were separated by means of SDS-PAGE and immunoblotted using mouse anti-human GPIbα (6.30), rabbit anti-14-3-3ζ, mouse anti-human ApoER2 (MP4-3) or goat anti-Dab1 antibodies. Blots were developed using enhanced chemiluminescence reagent plus (Perkin Elmer Life Sciences, Boston USA). Quantification of western blots was done with ImageJ software.

Results

Increased platelet adhesion to fibronectin under influence of dimeric β_2-GPI is mediated by both ApoER2' and GPIbα.

To study the effect of dimeric β_2-GPI on platelet activation in a GPIbα-independent system, we performed perfusion experiments with reconstituted blood over a fibronectin surface. Stimulation of platelets with dimeric β_2-GPI resulted in increased platelet adhesion (relative coverage 152 ± 33%, $p<0.05$) compared to the buffer control (Fig-

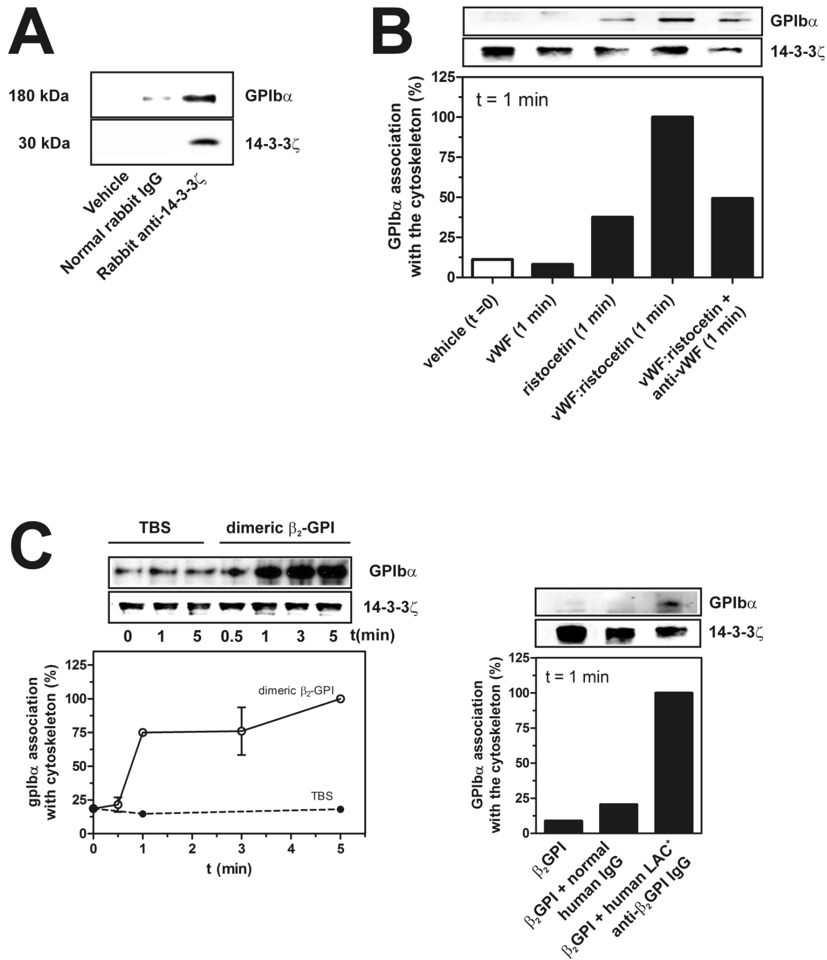

Figure 3. Glycoprotein Ibα associates with the cytoskeleton via the adapter molecule 14-3-3ζ upon platelet stimulation with dimeric β_2-GPI. A Whole platelet lysate was immunoprecipitated using vehicle, rabbit anti-14-3-3ζ antibodies or normal rabbit IgG. Westerns were probed for GPIbα or 14-3-3ζ using MAb 6.30 or rabbit anti-14-3-3ζ, respectively. **B** Platelets were stimulated with vehicle, vWF (10 μg mL^{-1}), ristocetin (1.2 mg mL^{-1}) or ristocetin-activated vWF with or without anti-vWF antibodies (2 μg mL^{-1}) for 1 minute. 14-3-3ζ was immunoprecipitated from cytoskeletal fractions. Western blots were probed with 6.30 or rabbit anti-14-3-3ζ. **C** Platelets were stimulated with dimeric β_2-GPI (50 μg mL^{-1}), plasma derived β_2-GPI (37 μg mL^{-1}) with or without normal human IgG (10 μg mL^{-1}) or human anti-β_2-GPI antibodies (10 μg mL^{-1}) or vehicle for indicated time periods. 14-3-3ζ was immunoprecipitated from cytoskeletal fractions. GPIbα (6.30) and 14-3-3ζ (rabbit anti-14-3-3ζ) were detected on western blot. Data depicted as mean GPIbα density ± SD. GPIbα at t = 5 minutes was set at 100%. Immunoblots shown are representative images. At least three experiments were performed.

ure 1). Blocking the binding site on dimeric β_2-GPI for GPIbα by competition with soluble GPIbα completely abrogated the increase in adhesion caused by dimeric β_2-GPI (relative platelet coverage 75 ± 10%). Likewise, competition with the soluble first LDL-binding domain of ApoER2 (sBD1-ApoER2) completely inhibited the effect of dimeric β_2-GPI (relative platelet coverage 93 ± 15%). Blockage of the binding site on

ApoER2' for dimeric β_2-GPI by the addition of Receptor Associated Protein (RAP), an inhibitor of ligand binding to members of the LDL-receptor family, completely blocked the effect of dimeric β_2-GPI as well (relative coverage 78 ± 8%). Addition of sGPIbα, sBD1-ApoER2 or RAP in the absence of dimeric β_2-GPI had no effect on adhesion to fibronectin.

ApoER2' dissociates from Disabled1 upon binding dimeric β_2-GPI.

To study the signaling processes originating from platelet ApoER2' we investigated the interaction of ApoER2' with the adapter protein Disabled 1 (Dab1), a protein known to mediate the effects of ApoER2 ligands in neurons. Probing for Dab1 in human whole platelet lysate yielded a band at approximately 80 kDa (Figure 2A), corresponding to the molecular weight of Dab1. ApoER2' specifically co-immunoprecipitated with Dab1 in platelets. As our previous work has shown ApoER2' dissociates from the cytoskeleton upon stimulation with dimeric β_2-GPI,[20] we investigated the interaction between ApoER2' and Dab1 in the cytoskeletal fraction. Stimulation of platelets with dimeric β_2-GPI caused the dissociation of ApoER2' from Dab1 in the cytoskeletal fraction, with maximal dissociation occurring after 30 seconds (Figure 2B). Human anti-β_2-GPI IgG from a patient with APS in complex with plasma derived β_2-GPI had the same effect. Plasma derived β_2-GPI or normal human IgG alone did not cause ApoER2' dissociation. As Dab1 only interacts with non-phosphorylated NPxY motifs,[29] we investigated whether platelet stimulation with dimeric β_2-GPI influenced tyrosine (Y)-phosphorylation of ApoER2'. ApoER2' already showed Y-phosphorylation at t=0, although an increase in Y-phosphorylation upon stimulation with dimeric β_2-GPI was observed (Figure 2C). As ApoER2' has no intrinsic kinase activity, we hypothesized that Y-phoshorylation would likely be caused by a downstream kinase. Indeed, inhibition of src-family kinases by preincubation of platelets with PP1 completely inhibited the dissociation of ApoER2' from cytoskeleton bound Dab1 induced by dimeric β_2-GPI (Figure 2C).

GPIbα translocates to the cytoskeleton via 14-3-3ζ upon stimulation with dimeric β_2-GPI.

The interaction between GPIbα and the adapter protein 14-3-3ζ has been shown to be essential for vWF-induced signaling processes.[30] Figure 3A shows that GPIbα co-immunoprecipitates with 14-3-3ζ, yielding a band at approximately 180 kDa under non-reducing conditions. Stimulation of platelets with ristocetin-activated vWF caused a translocation of the GPIb:14-3-3ζ complex to the cytoskeletal fraction (Figure 3B). Whereas vWF alone had no effect on GPIbα translocation, ristocetin itself resulted in a minor shift to the cytoskeletal fraction. Blocking the interaction between ristocetin-activated vWF and GPIbα by addition of an antibody against the vWF A1 domain reduced the translocation of the complex to that induced by ristocetin alone. Incubation of platelets with dimeric β_2-GPI resulted in a time dependent association of the GPIbα complex with the cytoskeleton (Figure 3C). Addition of a complex of plasma derived

β_2-GPI and human anti-β_2-GPI IgG caused translocation of GPIbα to the cytoskeleton as well, whereas β_2-GPI alone or normal human IgG had no effect on GPIbα translocation.

GPIbα translocation and ApoER2' dissociation are two independent processes.
To discern whether translocation of GPIbα and dissociation of ApoER2' are two distinct and separate processes we performed inhibition studies on ApoER2' dissociation from Dab1 and GPIbα association with the cytoskeleton (Figure 4). All experiments were performed at t = 1 minute. Blocking the binding site on dimeric β_2-GPI for platelet ApoER2' by pre-incubating dimeric β_2-GPI with sBD1-ApoER2, completely inhibited ApoER2' dissociation from Dab1 (Figure 4A), although it had no effect on the association of GPIbα with the cytoskeleton (Figure 4B). Likewise, addition of sGPIbα fully inhibited association of GPIbα with the cytoskeleton, whereas no effect on ApoER2' dissociation was observed. Addition of RAP to platelets prior to stimulation with dimeric β_2-GPI fully inhibited ApoER2' dissociation from Dab1 and had no effect on GPIbα translocation. Neither sGPIbα, sBD1-ApoER2 nor RAP alone had an effect on GPIbα translocation or ApoER2' dissociation.

Discussion

Dimerization of the plasma protein β_2-GPI by patient auto-antibodies is thought to be the trigger for the thrombo-embolic manifestations of the antiphospolipid syndrome.[10] Previous studies have identified the platelet receptors ApoER2'[4] and GPIbα[5;6] as binding sites for dimeric β_2-GPI on platelets. The interaction of dimeric β_2-GPI with these receptors was shown to be mediated by domain V of β_2-GPI,[3;5;6] which also contains the phospholipid binding site. Although the importance of ApoER2' in platelet activation by dimeric β_2-GPI has been shown by our group in the past,[4] the role of GPIbα remains unclear.

We have studied the roles of both GPIbα and ApoER2' in platelet activation induced by dimeric β_2-GPI. To be able to distinguish between effects mediated by the association of dimeric β_2-GPI with ApoER2' and those mediated by an interaction with GPIbα, we have studied platelet adhesion under conditions of flow to a surface that can support platelet adhesion independently of GPIbα. Although the vWF-GPIbα interaction has been reported to contribute to the initial adhesion to fibronectin at higher shear rates,[31] platelet adhesion to fibronectin is mostly dependent on the $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ integrins. To exclude the influence of GPIbα on initial platelet adhesion we performed all experiments in the absence of vWF and at a low shear rate. Similar to what has been described for collagen,[4] dimeric β_2-GPI caused an increase in platelet adhesion to fibronectin, which was abrogated by inhibiting the interaction of dimeric β_2-GPI with either GPIbα or ApoER2'. Although indicative of the involvement of both GPIbα and ApoER2' in platelet activation by dimeric β_2-GPI, one might speculate that one of the receptors may

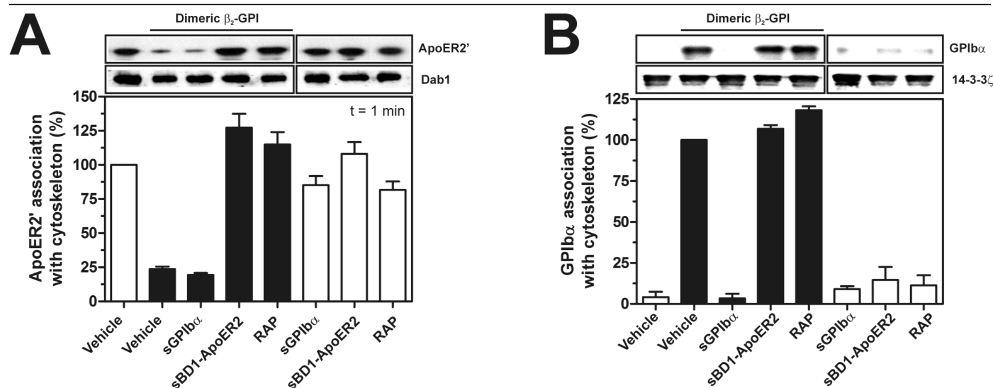

Figure 4. Dimeric β$_2$-GPI induces two independent signaling pathways in platelets. Platelets were stimulated with dimeric β$_2$-GPI (50 μg mL^{-1}) with or without inhibitors (sGPIbα [65 μg mL^{-1}]; sBD1-ApoER2 [125 μg mL^{-1}]; RAP [50 μg mL^{-1}]) for 1 minute. After lysis, cytoskeletal fractions were separated. **A** Dab1 was immunoprecipitated from cytoskeletal fractions. Western blots were probed with MP4.3. Dab1 was used a lane loading control. **B** 14-3-3ζ was immunoprecipitated from cytoskeletal fractions. Presence of GPIbα was shown with 6.30. 14-3-3ζ was used a lane loading control. Data is depicted as mean band density ± SD relative to the buffer control. Immunoblots are representative images. At least three experiments were performed.

play serve as a docking site for dimeric β$_2$-GPI, whereas the other is involved in intracellular signaling. In order to test this hypothesis we have studied the effects of dimeric β$_2$-GPI on signal transduction via both ApoER2’ and GPIbα.

We have focused on the association of ApoER2’ with the adapter molecule Dab1 and of GPIbα with the scaffold protein 14-3-3ζ. Both Dab1 and 14-3-3ζ are of crucial importance in the signaling pathways via ApoER2 in neuronal cells[18] and GPIbα in platelets,[17;30] respectively. We are the first to describe the presence of Dab1 in platelets, linking the molecule to platelet activation via ApoER2’. In neurons, binding of reelin to ApoER2 results in increased binding of the receptor to the adapter molecule Dab1 and it is argued that dissociation of Dab1 from ApoER2 might be a regulatory step in the signaling cascade.[15] Stimulation of platelets with dimeric β$_2$-GPI caused a dissociation of ApoER2’ from Dab1 in the cytoskeletal fraction, the opposite of what was described in neurons. As Dab1 is known to associate with the non-phosphorylated NPxY motif on the intracellular tail of ApoER2’,[32] these results suggest that the interaction between dimeric β$_2$-GPI and ApoER2’ results in phosphorylation of the tyrosine residue in the NPxY motif. Although ApoER2’ already appeared to be phosphorylated in resting platelets, we observed an increase in Y-phosphorylation upon incubation with dimeric β$_2$-GPI. Our data shows this process is probably mediated by src-family kinases, as inhibition of src-family kinases significantly reduced the dissociation of ApoER2’. Interestingly, activation of src-family kinases has been described in neurons upon binding of Reelin to ApoER2’.[33]

Concomitant with Dab1 dissociation, the complex between 14-3-3ζ and GPIbα associated with the cytoskeleton, implying signaling via GPIbα as well. To be able to differentiate between direct and indirect effects of dimeric β$_2$-GPI on signaling via either

ApoER2' or GPIbα, we have performed competition studies with soluble GPIbα, the soluble first binding domain of ApoER2 and RAP. Interestingly, signaling via ApoER2' could only be inhibited by competition with sBD1-ApoER2 and RAP, whereas ApoER2' dissociation could not be inhibited by the addition of sGPIbα. Similarly, only the addition of sGPIbα could inhibit GPIbα translocation. Neither sBD1-ApoER2 nor RAP had an effect. Thus, both GPIbα and ApoER2' elicit intracellular signaling independent of the interaction of dimeric β_2-GPI with the other receptor. As blockage of the interaction between dimeric β_2-GPI and ApoER2' with the soluble first LDL-binding domain of ApoER2 could not prevent the translocation of GPIbα, these results also suggest that dimeric β_2-GPI interacts with ApoER2' and GPIbα with two separate regions on its fifth domain.

While platelet activation by dimeric β_2-GPI was clear under conditions of flow, addition of dimeric β_2-GPI to platelets in an aggregometer did not result in aggregation or $\alpha_{IIb}\beta_3$ activation as assessed using the anti-$\alpha_{IIb}\beta_3$ antibody PAC-1 (data not shown). This suggests that signaling induced by dimeric-β_2-GPI via both ApoER2' and GPIbα leads to sensitization of platelets for a more potent activator, such as an adhesive surface. As blockage of either pathway is enough to abrogate the effect of dimeric β_2-GPI on platelet activation under conditions of flow, the signaling pathways originating from ApoER2' and GPIbα are likely to converge on a downstream level, with input from both pathways crucial for platelet sensitization.

We propose a model for platelet activation in the antiphospholipid syndrome in which both GPIbα and ApoER2' are of equal importance in the transmission of an activation signal. Upon binding of patient antibodies to β_2-GPI, the molecule is dimerized.[34] Dimerized β_2-GPI subsequently binds to both GPIbα and ApoER2' via two different epitopes on its fifth domain. Signaling ensues via the adapter proteins Dab1 and 14-3-3ζ, associated with ApoER2' and GPIbα, respectively. As neither process on its own is enough to sensitize platelets for adhesive surfaces such as fibronectin or collagen, it is highly likely both processes join together in lowering the threshold for platelet activation. The exact mechanism of platelet sensitization remains to be elucidated.

In conclusion, we have shown that platelet activation by dimeric β_2-GPI is mediated by both GPIbα and ApoER2'. Binding of dimeric β_2-GPI to each of these receptors elicits distinct independent signaling pathways, both of which are required for platelet sensitization. Insight in the mechanism by which dimerized β_2-GPI causes a prothrombotic platelet phenotype might provide clues for future anti-thrombotic therapies.

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References

1. Miyakis S, Lockshin MD, Atsumi T et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J.Thromb.Haemost. 2006;4:295-306.
2. de Groot PG, Derksen RH. Pathophysiology of the antiphospholipid syndrome. J.Thromb. Haemost. 2005;3:1854-1860.
3. van Lummel M, Pennings MT, Derksen RH et al. The binding site in {beta}2-glycoprotein I for ApoER2' on platelets is located in domain V. J.Biol.Chem. 2005;280:36729-36736.
4. Lutters BC, Derksen RH, Tekelenburg WL et al. Dimers of beta 2-glycoprotein I increase platelet deposition to collagen via interaction with phospholipids and the apolipoprotein E receptor 2'. J.Biol.Chem. 2003;278:33831-33838.
5. Pennings MT, Derksen RH, van Lummel M et al. Platelet adhesion to dimeric beta2-Glycoprotein-I under conditions of flow is mediated by at least two receptors: glycoprotein Ibalpha and APOER2'. J.Thromb.Haemost. 2006
6. Shi T, Giannakopoulos B, Yan X et al. Anti-beta2-glycoprotein I antibodies in complex with beta2-glycoprotein I can activate platelets in a dysregulated manner via glycoprotein Ib-IX-V. Arthritis Rheum. 2006;54:2558-2567.
7. Shen GQ, Li L, Girelli D et al. An LRP8 variant is associated with familial and premature coronary artery disease and myocardial infarction. Am.J.Hum.Genet. 2007;81:780-791.
8. Riddell DR, Vinogradov DV, Stannard AK, Chadwick N, Owen JS. Identification and characterization of LRP8 (apoER2) in human blood platelets. J.Lipid Res. 1999;40:1925-1930.
9. Korporaal SJ, Relou IA, van Eck M et al. Binding of low density lipoprotein to platelet apolipoprotein E receptor 2' results in phosphorylation of p38MAPK. J.Biol.Chem. 2004;279:52526-52534.
10. Lutters BC, Meijers JC, Derksen RH, Arnout J, de Groot PG. Dimers of beta 2-glycoprotein I mimic the in vitro effects of beta 2-glycoprotein I-anti-beta 2-glycoprotein I antibody complexes. J.Biol. Chem. 2001;276:3060-3067.
11. De Candia E, Hall SW, Rutella S et al. Binding of thrombin to glycoprotein Ib accelerates the hydrolysis of Par-1 on intact platelets. J.Biol.Chem. 2001;276:4692-4698.
12. Baglia FA, Shrimpton CN, Emsley J et al. Factor XI interacts with the leucine-rich repeats of glycoprotein Ibalpha on the activated platelet. J.Biol.Chem. 2004;279:49323-49329.
13. Bradford HN, Pixley RA, Colman RW. Human factor XII binding to the glycoprotein Ib-IX-V complex inhibits thrombin-induced platelet aggregation. J.Biol.Chem. 2000;275:22756-22763.
14. Du X. Signaling and regulation of the platelet glycoprotein Ib-IX-V complex. Curr.Opin.Hematol. 2007;14:262-269.
15. Hoe HS, Tran TS, Matsuoka Y, Howell BW, Rebeck GW. DAB1 and Reelin effects on amyloid precursor protein and ApoE receptor 2 trafficking and processing. J.Biol. Chem. 2006;281:35176-35185.
16. Hiesberger T, Trommsdorff M, Howell BW et al. Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation. Neuron 1999;24:481-489.
17. Munday AD, Berndt MC, Mitchell CA. Phosphoinositide 3-kinase forms a complex with platelet membrane glycoprotein Ib-IX-V complex and 14-3-3zeta. Blood 2000;96:577-584.
18. Trommsdorff M, Gotthardt M, Hiesberger T et al. Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. Cell 1999;97:689-701.
19. Bu G, Schwartz AL. RAP, a novel type of ER chaperone. Trends Cell Biol. 1998;8:272-276.
20. Pennings MT, Derksen RH, Urbanus RT et al. Platelets express three different splice variants of ApoER2 that are all involved in signaling. J.Thromb.Haemost. 2007;5:1538-1544.
21. Huizinga EG, Tsuji S, Romijn RA et al. Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. Science 2002;297:1176-1179.
22. Horbach DA, van Oort E, Donders RC, Derksen RH, de Groot PG. Lupus anticoagulant is the strongest risk factor for both venous and arterial thrombosis in patients with systemic lupus erythematosus. Comparison between different assays for the detection of antiphospholipid antibodies. Thromb.Haemost. 1996;76:916-924.
23. Sodetz JM, Pizzo SV, McKee PA. Relationship of sialic acid to function and in vivo survival of human factor VIII/von Willebrand factor protein. J.Biol.Chem. 1977;252:5538-5546.
24. Simmelink MJ, Derksen RH, Arnout J, de Groot PG. A simple method to discriminate between beta2-glycoprotein I- and prothrombin-dependent lupus anticoagulants. J.Thromb.Haemost. 2003;1:740-747.
25. Weeterings C, Adelmeijer J, Myles T, de Groot PG, Lisman T. Glycoprotein Ibalpha-mediated platelet adhesion and aggregation to immobilized thrombin under conditions of flow. Arterioscler. Thromb.Vasc.Biol. 2006;26:670-675.
26. Remijn JA, Wu YP, IJsseldijk MJ et al. Absence of fibrinogen in afibrinogenemia results in large but loosely packed thrombi under flow conditions. Thromb. Haemost. 2001;85:736-742.
27. van Os E, Wu YP, Pouwels JG et al. Thrombopoietin increases platelet adhesion under flow and decreases rolling. Br.J.Haematol. 2003;121:482-490.
28. Sakariassen KS, Aarts PA, de Groot PG, Houdijk WP, Sixma JJ. A perfusion chamber developed to investigate platelet interaction in flowing blood with human vessel wall cells, their extracellular matrix, and purified components. J.Lab Clin.Med. 1983;102:522-535.
29. Stolt PC, Bock HH. Modulation of lipoprotein receptor functions by intracellular adaptor proteins. Cell Signal. 2006;18:1560-1571.
30. Dai K, Bodnar R, Berndt MC, Du X. A critical role for 14-3-3zeta protein in regulating the VWF binding function of platelet glycoprotein Ib-IX and its therapeutic implications. Blood 2005;106:1975-1981.
31. Beumer S, Heijnen HF, IJsseldijk MJ et al. Platelet adhesion to fibronectin in flow: the importance of von Willebrand factor and glycoprotein Ib. Blood 1995;86:3452-3460.
32. Keshvara L, Benhayon D, Magdaleno S, Curran T. Identification of reelin-induced sites of tyrosyl phosphoryla-

tion on disabled 1. J.Biol.Chem. 2001;276:16008-16014.

33. Bock HH, Herz J. Reelin activates SRC family tyrosine kinases in neurons. Curr.Biol. 2003;13:18-26.

34. de Laat B, Derksen RH, Urbanus RT, de Groot PG. IgG antibodies that recognize epitope Gly40-Arg43 in domain I of beta 2-glycoprotein I cause LAC, and their presence correlates strongly with thrombosis. Blood 2005;105:1540-1545.

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Apolipoprotein E receptor 2 is responsible for the thrombotic complications in a murine model of the antiphospholipid syndrome

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In preparation

Abstract

Background: The antiphospholipid syndrome (APS) is characterized by episodes of vascular thrombosis or pregnancy morbidity in patients with circulating antiphospholipid antibodies. The major antigen in the syndrome is the plasma protein β_2-glycoprotein I. Anti-β_2-glycoprotein I antibodies induce endothelial and monocyte tissue factor (TF) expression and cause increased platelet deposition in in-vitro flow models. Several cellular receptors have been proposed to mediate these effects, but so far no convincing evidence for the involvement of a specific receptor has been provided.

Methods: We studied the effect of patient-derived antiphospholipid antibodies (IgG-APS) and dimeric β_2-glycoprotein I, which mimics β_2-glycoprotein I-antibody immune complexes, on the thrombotic manifestations of APS and specifically investigated the role of ApoER2 therein. We first investigated endothelial TF expression and platelet activation in vitro. We then assessed the role of ApoER2 in the induction of a prothrombotic state in a murine model of APS, by comparing wild-type and ApoER2-deficient mice.

Results: Dimeric β_2-glycoprotein I induced endothelial TF expression and caused increased platelet aggregation. These effects were attenuated upon inhibition of the interaction between dimeric β_2-glycoprotein I and ApoER2 with a soluble ApoER2 receptor fragment. In wild type mice, IgG-APS and dimeric β_2-glycoprotein I increased thrombus formation as well as carotid artery and peritoneal macrophage TF expression. These effects were all inhibited by addition of a soluble ApoER2 receptor fragment. In ApoER2-deficient mice, dimeric β_2-glycoprotein I had no effect on thrombus formation or carotid artery and peritoneal macrophage TF activity.

Conclusions: Our study shows ApoER2 plays a crucial role in the thrombotic manifestations of the antiphospholipid syndrome.

Introduction

The association between persistently present antiphospholipid antibodies and the clinical manifestations of thrombosis or pregnancy morbidity is known as the antiphospholipid syndrome (APS).[1] Antiphospholipid antibodies are heterogeneous and recognize a wide variety of plasma proteins with phospholipid-binding properties, such as prothrombin[2] and β_2-glycoprotein I (β_2-GPI).[3;4] Antiphospholipid antibodies directed against β_2-GPI, a plasma protein without known physiological function, are considered the most pathologically relevant antibodies.

There is strong experimental evidence that anti-β_2-GPI antibodies have thrombogenic properties. Studies on endothelial cell activation show the induction of a prothrombotic and proinflammatory phenotype upon exposure to anti-β_2-GPI antibodies,[5-8] indicated by expression of tissue factor (TF) and increased surface expression of adhesion molecules such as intercellular adhesion molecule (ICAM)-1, vascular-cell adhesion molecule (VCAM)-1 and E-selectin. Activation of monocytes by anti-β_2-GPI antibodies leads to TF expression as well.[9] Furthermore, anti-β_2-GPI antibodies, or recombinant dimers of β_2-GPI that mimic β_2-GPI-antibody immune complexes, increase platelet deposition to extracellular matrix components in in-vitro flow models.[10] Injection of anti-β_2-GPI antibodies in murine[11] or hamster[12] thrombosis models leads to increased thrombus formation.

Several receptors were postulated to mediate the prothrombotic cellular effects of anti-β_2-GPI antibodies. The interaction between annexin A2 and β_2-GPI-antibody immune complexes has been reported to lead to endothelial cell activation.[13] It seems unlikely, however, that annexin A2 is able to convey activation signals across the cell membrane, since this phospholipid binding protein lacks a transmembrane domain. Toll-like receptor (TLR)-4 is another candidate receptor for antiphospholipid antibodies, as TLR-4-like signaling was reported in endothelial cells upon incubation with antiphospholipid antibodies.[14] Furthermore, a mutation in murine TLR4 known to disrupt lipopolysaccharide-binding, attenuated the increased prothrombotic state observed in wild-type mice injected with antiphospholipid antibodies.[15] A direct interaction between Toll-like receptor 4 and β_2-GPI-antibody immune complexes, however, remains to be confirmed to this date.

Members of the low density lipoprotein (LDL)-receptor family do bind β_2-GPI-antibody immune complexes.[16] The interaction between β_2-GPI-antibody immune complexes and both Apolipoprotein E Receptor 2' (ApoER2'), the only LDL-receptor family member present on platelets,[17] and the platelet adhesive receptor glycoprotein Ibα was shown to lead to increased thrombus formation in vitro.[18;19] Platelet activation could be attenuated by inhibition of the interaction between either receptor and β_2-GPI.[20]

We investigated whether ApoER2, which is present on both platelets and endothelial cells,[21] mediates the prothrombotic effects of antiphospholipid antibodies in a murine

thrombosis model. Here we present evidence that antiphospholipid antibodies and dimeric β_2-GPI enhance in vivo thrombus formation through ApoER2 expressed on both endothelial cells, monocytes and platelets.

Material and Methods

Reagents

Recombinant apple4-C321S-β_2-GPI (dimeric β_2-GPI) and apple2-β_2-GPI (monomer β_2-GPI) were expressed and purified as described before.[22] The soluble first LDL-binding domain of ApoER2 (sBD1-ApoER2) was expressed and purified as described before.[23] Purity of all recombinant proteins was assessed with SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Plasma-derived β_2-GPI was purified as described before.[24] Patient-derived antiphospholipid antibodies (IgG-APS) were purified as described before.[5] IgG-APS were obtained from a patient with lupus anticoagulant and high titer anticardiolipin and anti-β_2-GPI antibodies. Serum samples were obtained with informed consent and the study design was approved by the local institutional review board. Protein preparations used in animal or cell-based experiments were LPS free (<0.05 IU mL^{-1}) as determined with a limulus amoebocyte assay (E-toxate; Sigma-Aldritch, St. Louis, MO, USA).

Coagulation factor X (FX) was purified as described before.[25] Recombinant factor VIIa (rFVIIa) was purchased from Novo Nordisk (Måløv, Denmark). Recombinant TF (Innovin) was from Dade Behring (Marburg, Germany). Chromogenic substrate Pefachrome Xa was obtained from Kordia (Leiden, the Netherlands). NFκB-inhibitor MG132 was from EMD Biosciences (Gibbstown, NJ, USA). Human fibronectin and recombinant human tumor necrosis factor α (TNFα) were purchased from Bio-connect (Huissen, the Netherlands). Monoclonal antibody (MoAb) 21B2 against human β_2-GPI was kindly provided by dr. J. Arnout (Leuven, Belgium).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated as described previously.[26] Isolated HUVECs were grown to confluence in EBM-2 medium (Lonza, Walkerville, MD, USA) supplemented with 5% fetal bovine serum and endothelial cell growth factors (EGM-2 bullitkit; Lonza, Walkersville, USA). HUVECs from the first passage were used for experiments.

β_2-GPI binding to HUVECs

HUVECs were seeded in 96-well flatbottom microtiter plates (Nunc, Wiesbaden, Germany) coated with human fibronectin. Confluent monolayers were incubated with 10 pM TNFα for 16 hours at 37°C. Cells were then put on ice and incubated with phosphate buffered saline (PBS; 10 mM phosphate, 140 mM NaCl, pH 7.4) with 3% Bovine serum albumin for 2 hours. Dimeric β_2-GPI, monomer β_2-GPI or monoclonal

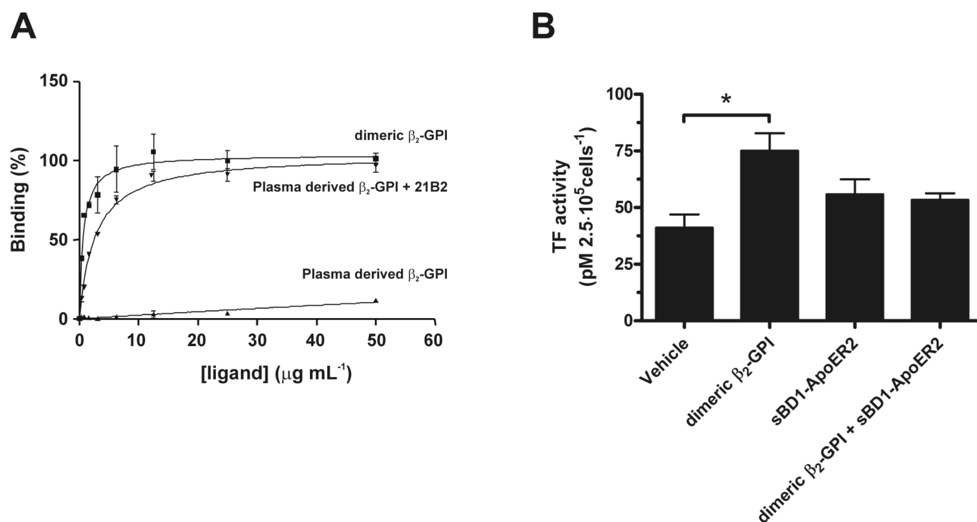

Figure 1. Dimeric β_2-GPI interacts with HUVECs and induces TF expression. A HUVECs were grown to confluence in 96-well microtiter plates and activated with 10 pM TNFα for 16 hours. Binding of dimeric β_2-GPI, plasma derived β_2-GPI or plasma derived β_2-GPI complexed with anti-β_2-GPI MoAb 21B2 was detected with an affinity purified goat anti-human β_2-GPI antibody. Binding is expressed as percentage of maximum binding. Graph represents data of at least three experiments. **B** HUVECs were grown to 90% confluence in 6-well plates and incubated with dimeric β_2-GPI (50 μg mL^{-1}) or sBD1-ApoER2 (50 μg mL^{-1}) supplemented with 10 pM TNFα for 16 hours. Cells were harvested and FXa generation was determined with a chromogenic substrate as a measure of TF activity. Recombinant TF (Innovin) with a known concentration was used as a reference. Results are expressed as pM per $2.5 \cdot 10^5$ cells TF activity. Data represent three experiments. Asterisk (*) indicates $p < 0.05$.

anti-β_2-GPI 21B2 were incubated for 1 hour. Cells were subsequently washed with PBS and bound β_2-GPI was detected with goat anti-human β_2-GPI antibodies. Results are expressed as percentage of maximum binding.

Tissue Factor activity in HUVECs

HUVECs were seeded in 6-well plates coated with human fibronectin and grown to 90% confluence. After rinsing with PBS, cells were incubated with EBM-2 medium supplemented with 10 pM TNFα and dimeric β_2-GPI (50 μg mL^{-1}) or sBD1-ApoER2 (50 μg mL^{-1}) at 37°C for 16 hours. Cells were collected with a non-enzymatic cell dissociation solution (Sigma-Aldritch, St. Louis, MO, USA). After thorough washing, cells were pelleted and adjusted to a concentration of $4 \cdot 10^6$ mL^{-1} in hepes buffered saline (HBS; 25 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 125 mM NaCl, pH 7.3). Aliquots of 50 μL cell suspension were supplemented with human FX (final concentration 10 μg mL^{-1}), rFVIIa (5 units mL^{-1}) and 5 mM $CaCl_2$ and incubated at 37°C for 45 minutes. The chromogenic substrate for FXa (Pefachrome Xa) was then added and absorbance was read at 405 nm. TF activity was determined by comparing values with a reference obtained from a standard dilution of recombinant tissue factor (Innovin) with known concentration. Values are expressed in pM per $2.5 \cdot 10^5$ cells.

Animals
C57BL/6 wild-type and B6;129S6-Lrp8^{tm1Her}/J (ApoER2$^{-/-}$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were genotyped with the following primers: (1) GAT TGG GAA GAC AAT AGC AGG CAT GC, (2) GCT TGT TGG AAT TCA GCC AGT TAC C, (3) ACG ATG ACC CCA ATG ACA GCA GCG and (4) CCA CAG TGT CAC ACA GGT AAT GTG. Animals were housed in the Animal Care facilities of the University of Texas Medical Branch (UTMB) at Galveston (AALAC-Approved). Animals were handled by trained personnel according to Institutional Animal Care and Use Committee guidelines.

Platelet aggregation
Blood from C57BL/6 wild-type mice was obtained by cardiac puncture and collected in 1:9 (v:v) ACD (2.5% trisodium citrate, 1.5% citric acid and 2% D-glucose). Blood of 5 mice was pooled per condition. Platelet rich plasma (PRP) was obtained by centrifugation for 20 min at 120*g*. Aggregation of platelets in PRP was measure turbidimetrically with a dual channel aggregometer (Minigator II) following calibration with platelet poor plasma at a stirring speed of 800 rpm. PRP was adjusted to 240,000 platelets μL^{-1} with platelet poor plasma. Platelet aggregation was induced by addition of thrombin (0.005 U mL^{-1}) in the presence of dimeric β_2-GPI (50 $\mu g\ mL^{-1}$), monomer β_2-GPI (50 $\mu g\ mL^{-1}$) or sBD1-ApoER2 (40 $\mu g\ mL^{-1}$). Light transmission was recorded for 5 min. Results are expressed as percentage of maximum aggregation.

Analysis of thrombus dynamics
Mice (7-9 animals per group) were injected intra peritoneally with IgG-APS (500 µg), dimeric β_2-GPI (50 µg), monomer β_2-GPI (50 µg), or an equivalent volume of PBS twice, with an interval of 48 hours. Some animals were injected additionally with sBD1-ApoER2 (50 µg) or 0.1 mL of MG132 (10 µM in 50% dimethyl sulfoxide). In all cases, surgical procedures were performed to study thrombus dynamics at 72 hours after the first reagent injections, as described previously.[5] Thrombus size was measured in μm^2. Mice were subsequently used to determine TF activity in carotid arteries and peritoneal macrophages.

TF activity in murine carotid artery homogenates
Pieces of approximately 5 mm of uninjured carotid arteries were dissected from both sides in each animal and were collected in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4) with 0.1% Triton X-100, containing heparin as anticoagulant. Samples were subsequently homogenized, as described elsewhere.[27] TF activity in carotid artery homogenates was determined using a commercial chromogenic assay (Actichrome TF; American Diagnostica, Stamford, CT, USA). TF activity was expressed in $pM\ mg^{-1}$. Each pooled sample was assayed in duplicate and experiments were repeated

three times.

TF activity in murine peritoneal macrophages

Peritoneal macrophages were collected immediately after euthanasia by flushing the peritoneal cavity of each mouse with 5 mL of PBS. TF activity was determined as described previously.[8] TF activity was expressed in pM mg^{-1}.

Statistical analysis

Data was analysed with the Mann-Whitney test for non-parametric correlation.

Results

Dimeric β_2-GPI binds to and activates endothelial cells

We first determined whether dimeric β_2-GPI is able to interact with endothelial cells. Neither the recombinant apple4-C321S-β_2-GPI (dimeric β_2-GPI), which contains the dimerization domain of coagulation factor XI, nor plasma-derived β_2-GPI interacted with quiescent cultured HUVECs (data not shown). Dimeric β_2-GPI did interact with HUVECs (apparent K_D of 5.8 nM) upon preactivation with a low dose of TNFα (10 pM). Plasma derived β_2-GPI hardly interacted with activated HUVECs (figure 1A). Complexes of plasma derived β_2-GPI and monoclonal anti-β_2-GPI antibody 21B2 interacted with HUVECs with high affinity as well (apparent K_D 18 nM), which indicates dimeric β_2-GPI mimics the function of β_2-GPI-antibody complexes.

To determine whether dimeric β_2-GPI is able to induce a prothrombotic endothelial phenotype, we assessed TF activity in HUVECs. Figure 1B shows incubation of HUVECs with dimeric β_2-GPI (50 µg mL^{-1}) leads to significantly increased TF activity ($p < 0.05$). Patient-derived anti-β_2-GPI antibodies and monoclonal anti-β_2-GPI antibodies had a similar effect on endothelial TF expression (data not shown). Incubation with IgG-APS or dimeric β_2-GPI also resulted in upregulation of endothelial ICAM-1 expression (data not not shown). We subsequently investigated the influence of ApoER2 in dimeric β_2-GPI related endothelial TF expression. The presence of ApoER2 in HUVECs was confirmed on both mRNA and protein level (data not shown). Previous work showed that the interaction of dimeric β_2-GPI with ApoER2 could be inhibited by addition of the recombinant soluble first LDL-binding domain of ApoER2 (sBD1-ApoER2).[20] Indeed, incubation of HUVECs with both dimeric β_2-GPI (50 µg mL^{-1}) and sBD1-ApoER2 (40 µg mL^{-1}) completely abrogated the effects of dimeric β_2-GPI on TF activity. sBD1-ApoER2 itself had no effect on endothelial TF expression.

Dimeric β_2-GPI sensitizes murine platelets for activation by suboptimal doses of thrombin

We studied the influence of dimeric β_2-GPI on murine platelet activation. We therefore performed aggregation experiments with a suboptimal dose of thrombin (0.005 U mL^{-1}),

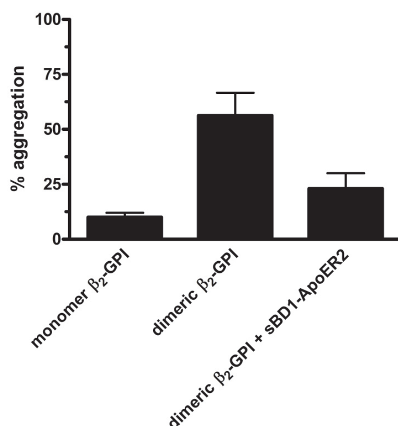

Figure 2. Dimeric β_2-GPI increases platelet sensitivity for agonists. Platelet rich plasma was obtained from C57BL/6 mice and platelet concentration was adjusted to $100 \cdot 10^9$ L^{-1}. Platelet aggregation was recorded turbidimetrically in an aggregometer upon stimulation with 0.005 U mL^{-1} thrombin, in the presence of dimeric β_2-GPI (50 μg mL^{-1}), monomer β_2-GPI (50 μg mL^{-1}) or sBD1-ApoER2 (40 μg mL^{-1}). Results are expressed as percentage aggregation. Data are representative of three experiments.

a model that proved successful in human platelets.[28] As shown in figure 2, incubation of murine platelets with dimeric β_2-GPI resulted in significantly more aggregation than incubation of platelets with its monomer control, recombinant apple2-β_2-GPI, which contains a domain of coagulation factor XI not involved in dimerization. Addition of sBD1-ApoER2 significantly attenuated the effects of dimeric β_2-GPI. These studies show human β_2-GPI can interact with murine ApoER2.

ApoER2 mediates the prothrombotic effects of dimeric β_2-GPI and antiphospholipid antibodies in vivo

To study the effects of dimeric β_2-GPI on the induction of a prothrombotic state in vivo, we investigated thrombus formation in C57BL/6 wild-type and ApoER2$^{-/-}$ mice. Wild-type mice injected with dimeric β_2-GPI showed significantly larger thrombi compared to mice injected with monomer β_2-GPI (figure 3A). Injection of IgG-APS also resulted in significantly larger thrombi. Treatment with sBD1-ApoER2 inhibited the effects of dimeric β_2-GPI by 86%. Injection of MG132, an inhibitor of the NFκB pathway, inhibited the effects of dimeric β_2-GPI by 67%. Compared with the monomer β_2-GPI, dimeric β_2-GPI did not lead to increased thrombus formation in ApoER2$^{-/-}$ mice, nor did IgG-APS (Figure 3B).

We studied the effects of dimeric β_2-GPI on vascular TF activity in carotid artery homogenates. Treatment of wild-type C57BL/6 mice with dimeric β_2-GPI caused a significant increase in carotid artery TF activity compared with treatment with the monomer control (figure 3C). Addition of sBD1-ApoER2 and MG132 completely attenuated the effects of dimeric β_2-GPI. Dimeric β_2-GPI did not cause increased TF activity in carotid artery homogenates of ApoER2$^{-/-}$ mice (figure 3D).

We investigated the influence of dimeric β_2-GPI on monocyte activation in macrophages derived from the peritoneal cavity. Macrophages derived from wild-type C57BL/6 mice treated with dimeric β_2-GPI displayed significantly increased TF activity compared with mice treated with monomer β_2-GPI (figure 3E). Treatment of mice with sBD1-ApoER2 or MG132 completely abrogated the effects of dimeric β_2-GPI on peritoneal macrophage TF activity. ApoER2$^{-/-}$ mice treated with dimeric β_2-GPI showed similar peritoneal macrophage TF activity as ApoER2$^{-/-}$ mice treated with monomer β_2-GPI (figure 3F).

Discussion

We have shown that injection of antiphospholipid antibodies or dimeric β_2-GPI caused a significant increase in thrombus formation, as well as increased vascular TF expression and monocyte activation in a murine thrombosis model. Inhibition of the interaction between dimeric β_2-GPI and ApoER2 with the soluble first LDL-binding domain of ApoER2 attenuated the in vivo effects of dimeric β_2-GPI completely. The importance of ApoER2 in the induction of a prothrombotic state by antiphospholipid antibodies or dimeric β_2-GPI in vivo was confirmed in ApoER2 deficient mice, in which injection of antiphospholipid antibodies or dimeric β_2-GPI did not result in additional thrombus formation, vessel wall TF expression or monocyte activation. In vitro, dimeric β_2-GPI induced a prothrombotic endothelial phenotype, as evidenced by increased TF expression in endothelial cells, and primed murine platelets for aggregation by suboptimal doses of agonist. These effects could be inhibited by addition of the soluble first LDL-binding domain of ApoER2, indicative of the importance of ApoER2 in both endothelial cell and platelet activation by antiphospholipid antibodies.

ApoER2 is a member of the LDL-receptor family, a multiligand receptor family with a wide tissue distribution.[29] Originally identified in neurons, in which it mediates neuronal plasticity[30;31] and is involved in neuronal migration during brain development,[30] ApoER2 is expressed on platelets, endothelial cells and monocytes as well (Dr. JH Griffin, La Jolla, personal communication). Studies on the role of ApoER2 in platelets showed ApoER2 mediates both Apolipoprotein E-dependent platelet inhibition[17] and LDL-dependent platelet activation.[32] The platelet activating properties of ApoER2-derived signaling were supported by a genome wide linkage-scan that linked the onset of premature cardiovascular disease to a polymorphism in the ApoER2 gene.[33] This polymorphism results in increased platelet sensitivity for ligands such as LDL.[33] These observations point to ApoER2 as a key regulator of thrombus formation, not only under pathological conditions such as in the antiphospholipid syndrome, but also under physiological conditions.

Our data indicate a major role for ApoER2 in thrombus formation in the antiphospholipid syndrome. We cannot, however, exclude a potential role for additional receptors. Antiphospholipid antibody or dimeric β_2-GPI binding to both the platelet specific

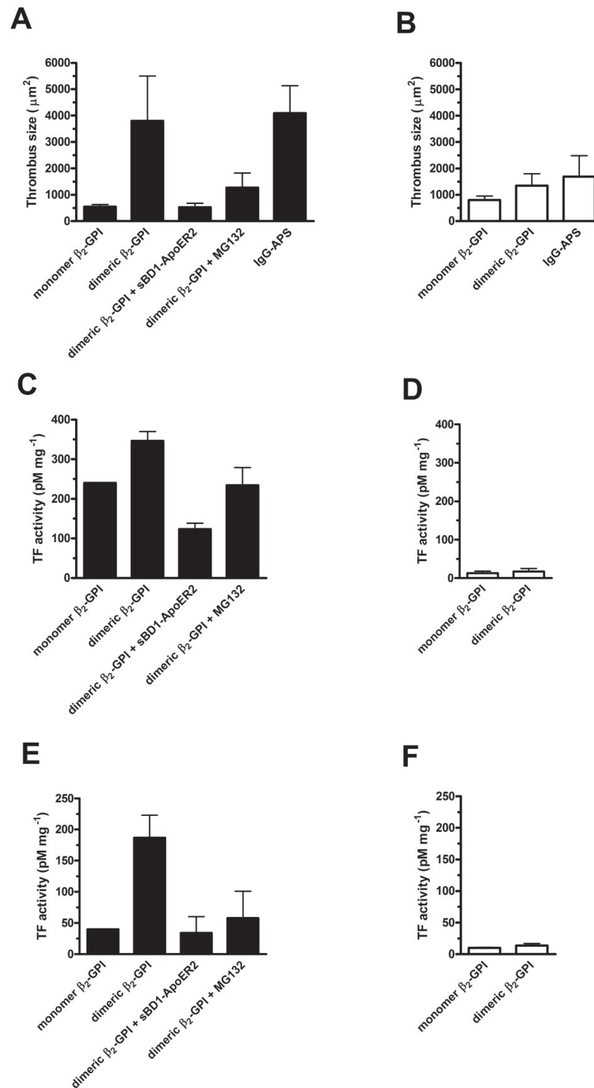

Figure 3. Dimeric β_2-GPI induces a prothrombotic state in vivo through an interaction with ApoER2. C57BL/6 wild-type or ApoER2 deficient mice were injected with dimeric β_2-GPI (50 μg), monomer β_2-GPI (50 μg), APS-IgG (500 μg) twice with an interval of 48 hours. Some mice were additionally treated with sBD1-ApoER2 (40 μg) or the NFκB inhibitor MG132 (10 μM in 0.1 mL). Mice were subjected to experiments 72 hours after the first injection. Thrombus dynamics were assessed in wild-type mice (**A**) or ApoER2-deficient mice (**B**) after induction of tissue damage in the femoral vein. Results are expressed in μm^2. Carotid arteries were harvested from C57BL/6 wild type mice (**C**) or ApoER2-deficient mice (**D**) and TF activity in homogenates was determined with a commercial chromogenic assay for Xa formation. Results are expressed as pM mg^{-1}. Peritoneal macrophages were obtained from C57BL/6 (**E**) or ApoER2-deficient (**F**) mice by rinsing the peritoneal cavity. Cells were collected and TF activity was determined in lysate with a commercial chromogenic assay for Xa formation. Results are expressed as pM mg^{-1}. At least three animals were used per condition.

receptor glycoprotein Ibα and ApoER2, for instance, is crucial to convey activation signals across the platelet membrane, as inhibition of the interaction between dimeric β_2-GPI and either glycoprotein Ibα or ApoER2' completely abrogates the platelet sensitizing effects of dimeric β_2-GPI.[20] This two receptor mechanism could be responsible for the effects of antiphospholipid antibodies on other cells as well. Since glycoprotein Ibα is restricted to platelets, a potential second receptor for antiphospholipid antibodies on other cells remains to be identified. TLR-4 seems a likely candidate, as it is already implicated in antiphospholipid antibody induced signaling in endothelial cells[14] and there are structural similarities with glycoprotein Ibα; both glycoprotein Ibα and TLR-4 contain Leusine-rich repeats.[34] Moreover, dysfunctional TLR-4 was shown to abrogate the prothrombotic effects of antiphospholipid antibodies in a murine thrombosis model.[15] Annexin A2 might play a role in cellular activation by antiphospholipid antibodies, although it most likely functions as a docking site. It is difficult to envision intracellular signaling upon binding to a protein without a transmembrane domain.

Antibody binding to a cellular surface has been shown to lead to cellular activation via Fc-receptors. Nevertheless, there seems to be no role for Fc-receptor-mediated cellular activation in the thrombotic complications of APS. $F(ab')_2$ fragments of antiphospholipid antibodies were shown to have the same in-vitro[28] and in-vivo[12] effects in models of APS as whole IgG. Our data does not support involvement of Fc-receptors either, as dimeric β_2-GPI does not contain the Fc-domains of IgG.

Involvement of the complement system in the prothrombotic effects of dimeric β_2-GPI seems questionable. Complement components C3 and C5a are reported to mediate antiphospholipid antibody-induced fetal loss[35;36] and are suggested to mediate the thrombotic complications of the syndrome as well.[37;38] Dimeric β_2-GPI, however, does not support antibody-mediated complement activation. The classical complement activation pathway is therefore unlikely to play a major role in our system. On the other hand, thrombin can also generate C5a.[39] We cannot exclude that dimeric β_2-GPI, which causes increased vessel wall TF expression and therefore thrombin formation, causes activation of complement components indirectly.

Our data indicate a substantial role for the TF-pathway in antiphospholipid antibody-mediated thrombus formation. Inhibition of the NFκB pathway, which mediates de-novo TF synthesis,[40] attenuated thrombus formation in our murine thrombosis model. As expected, inhibition of the NFκB pathway fully inhibited the effects of dimeric β_2-GPI on vessel wall and peritoneal macrophage TF activity. Thrombus formation itself, however, could not be fully inhibited by inhibition of the NFκB pathway, indicating an additional role for platelet activation by antiphospholipid antibodies.

Thrombotic events occur only occasionally in patients with the antiphospholipid syndrome, despite the continuous presence of circulating antiphospholipid antibodies. A possible explanation is that antiphospholipid antibodies can only exert their prothrombotic influence when the local environment is primed for further activation. This second-hit mechanism was shown in an animal model of the antiphospholipid

syndrome, in which injection of antiphospholipid antibodies in rats only resulted in increased thrombus formation when rats were pretreated with lipopolysaccharide, not when rats were injected with buffer.[41] Our results are in line with these data, as both in-vitro endothelial cell activation and in-vitro platelet activation by dimeric β_2-GPI required cellular preactivation with a suboptimal dose of another agonist.

We propose the following sequence of events that leads to the thrombotic manifestations of the antiphospholipid syndrome: β_2-GPI binds to anionic phospholipids exposed on the surface of cells that become activated as a result of a small injury. Subsequently, β_2-GPI undergoes a conformational change. This conformational change leads to exposure of the binding site for pathological anti-β_2-GPI antibodies.[42] β_2-GPI is dimerized upon antibody binding, which stabilizes the interaction with cells and allows interaction with ApoER2. Interaction with ApoER2 results in cell activation and the induction of a prothrombotic cellular phenotype. The pivotal role of ApoER2 in this thrombotic mechanism is a potential therapeutic target for treatment of the thrombotic manifestations of the antiphospholipid syndrome.

References

1. Miyakis S, Lockshin MD, Atsumi T et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J.Thromb.Haemost. 2006;4:295-306.
2. Bevers EM, Galli M, Barbui T, Comfurius P, Zwaal RF. Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin. Thromb. Haemost. 1991;66:629-632.
3. Galli M, Comfurius P, Maassen C et al. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. Lancet 1990;335:1544-1547.
4. McNeil HP, Simpson RJ, Chesterman CN, Krilis SA. Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2-glycoprotein I (apolipoprotein H). Proc.Natl.Acad.Sci.U.S.A 1990;87:4120-4124.
5. Pierangeli SS, Colden-Stanfield M, Liu X et al. Antiphospholipid antibodies from antiphospholipid syndrome patients activate endothelial cells in vitro and in vivo. Circulation 1999;99:1997-2002.
6. Del Papa N, Guidali L, Sala A et al. Endothelial cells as target for antiphospholipid antibodies. Human polyclonal and monoclonal anti-beta 2-glycoprotein I antibodies react in vitro with endothelial cells through adherent beta 2-glycoprotein I and induce endothelial activation. Arthritis Rheum. 1997;40:551-561.
7. Simantov R, LaSala JM, Lo SK et al. Activation of cultured vascular endothelial cells by antiphospholipid antibodies. J.Clin.Invest 1995;96:2211-2219.
8. Vega-Ostertag M, Casper K, Swerlick R et al. Involvement of p38 MAPK in the up-regulation of tissue factor on endothelial cells by antiphospholipid antibodies. Arthritis Rheum. 2005;52:1545-1554.
9. Sorice M, Longo A, Capozzi A et al. Anti-beta2-glycoprotein I antibodies induce monocyte release of tumor necrosis factor alpha and tissue factor by signal transduction pathways involving lipid rafts. Arthritis Rheum. 2007;56:2687-2697.
10. Lutters BC, Derksen RH, Tekelenburg WL et al. Dimers of beta 2-glycoprotein I increase platelet deposition to collagen via interaction with phospholipids and the apolipoprotein E receptor 2'. J.Biol.Chem. 2003;278:33831-33838.
11. Montiel-Manzano G, Romay-Penabad Z, Papalardo de Martinez E et al. In vivo effects of an inhibitor of nuclear factor-kappa B on thrombogenic properties of antiphospholipid antibodies. Ann.N.Y.Acad.Sci. 2007;1108:540-553.
12. Jankowski M, Vreys I, Wittevrongel C et al. Thrombogenicity of beta 2-glycoprotein I-dependent antiphospholipid antibodies in a photochemically induced thrombosis model in the hamster. Blood 2003;101:157-162.
13. Zhang J, McCrae KR. Annexin A2 mediates endothelial cell activation by antiphospholipid/anti-beta2 glycoprotein I antibodies. Blood 2005;105:1964-1969.
14. Raschi E, Testoni C, Bosisio D et al. Role of the MyD88 transduction signaling pathway in endothelial activation by antiphospholipid antibodies. Blood 2003;101:3495-3500.
15. Pierangeli SS, Vega-Ostertag ME, Raschi E et al. Toll-like receptor and antiphospholipid mediated thrombosis: in vivo studies. Ann. Rheum.Dis. 2007;66:1327-1333.
16. Pennings MT, van Lummel M, Derksen RH et al. Interaction of beta2-glycoprotein I with members of the low density lipoprotein receptor family. J.Thromb.Haemost. 2006;4:1680-1690.
17. Riddell DR, Vinogradov DV, Stannard AK, Chadwick N, Owen JS. Identification and characterization of LRP8 (apoER2) in human blood platelets. J.Lipid Res. 1999;40:1925-1930.
18. Pennings MT, Derksen RH, van Lummel M et al. Platelet adhesion to dimeric beta-glycoprotein I under conditions of flow is mediated by at least two receptors: glycoprotein Ibalpha and apolipoprotein E receptor 2'. J.Thromb. Haemost. 2007;5:369-377.
19. Shi T, Giannakopoulos B, Yan X et al. Anti-beta2-glycoprotein I antibodies in complex with beta2-glycoprotein I can activate platelets in a dysregulated manner via glycoprotein Ib-IX-V. Arthritis Rheum. 2006;54:2558-2567.
20. Urbanus RT, Pennings MT, Derksen RH, de Groot PG. Platelet activation by dimeric beta(2)-glycoprotein I requires signaling via both glycoprotein Ibalpha and Apolipoprotein E Receptor 2'. J.Thromb.Haemost. 2008;6:1405-1412.
21. Sacre SM, Stannard AK, Owen JS. Apolipoprotein E (apoE) isoforms differentially induce nitric oxide production in endothelial cells. FEBS Lett. 2003;540:181-187.
22. Lutters BC, Meijers JC, Derksen RH, Arnout J, de Groot PG. Dimers of beta 2-glycoprotein I mimic the in vitro effects of beta 2-glycoprotein I-anti-beta 2-glycoprotein I antibody complexes. J.Biol. Chem. 2001;276:3060-3067.
23. Pennings MT, Derksen RH, Urbanus RT et al. Platelets express three different splice variants of ApoER2 that are all involved in signaling. J.Thromb.Haemost. 2007;5:1538-1544.
24. Horbach DA, van Oort E, Donders RC, Derksen RH, de Groot PG. Lupus anticoagulant is the strongest risk factor for both venous and arterial thrombosis in patients with systemic lupus erythematosus. Comparison between different assays for the detection of antiphospholipid antibodies. Thromb.Haemost. 1996;76:916-924.
25. Hackeng TM, van 't Veer C, Meijers JC, Bouma BN. Human protein S inhibits prothrombinase complex activity on endothelial cells and platelets via direct interactions with factors Va and Xa. J.Biol.Chem. 1994;269:21051-21058.
26. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J.Clin.Invest 1973;52:2745-2756.
27. Day SM, Reeve JL, Pedersen B et al. Macrovascular thrombosis is driven by tissue factor derived primarily from the blood vessel wall. Blood 2005;105:192-198.
28. Vega-Ostertag M, Harris EN, Pierangeli SS. Intracellular events in platelet activation induced by antiphospholipid antibodies in the presence of low doses of thrombin. Arthritis Rheum. 2004;50:2911-2919.
29. Herz J, Gotthardt M, Willnow TE. Cellular signalling by lipoprotein receptors. Curr.Opin.Lipidol. 2000;11:161-166.
30. Beffert U, Durudas A, Weeber EJ et al. Functional dissection of Reelin signaling by site-directed disruption of Disabled-1 adaptor

binding to apolipoprotein E receptor 2: distinct roles in development and synaptic plasticity. J.Neurosci. 2006;26:2041-2052.

31. Weeber EJ, Beffert U, Jones C et al. Reelin and ApoE receptors cooperate to enhance hippocampal synaptic plasticity and learning. J.Biol.Chem. 2002;277:39944-39952.
32. Korporaal SJ, Relou IA, van Eck M et al. Binding of low density lipoprotein to platelet apolipoprotein E receptor 2' results in phosphorylation of p38MAPK. J.Biol.Chem. 2004;279:52526-52534.
33. Shen GQ, Li L, Girelli D et al. An LRP8 variant is associated with familial and premature coronary artery disease and myocardial infarction. Am.J.Hum.Genet. 2007;81:780-791.
34. Bell JK, Mullen GE, Leifer CA et al. Leucine-rich repeats and pathogen recognition in Toll-like receptors. Trends Immunol. 2003;24:528-533.
35. Holers VM, Girardi G, Mo L et al. Complement C3 activation is required for antiphospholipid antibody-induced fetal loss. J.Exp.Med. 2002;195:211-220.
36. Girardi G, Berman J, Redecha P et al. Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. J.Clin.Invest 2003;112:1644-1654.
37. Redecha P, Tilley R, Tencati M et al. Tissue factor: a link between C5a and neutrophil activation in antiphospholipid antibody induced fetal injury. Blood 2007;110:2423-2431.
38. Pierangeli SS, Girardi G, Vega-Ostertag M et al. Requirement of activation of complement C3 and C5 for antiphospholipid antibody-mediated thrombophilia. Arthritis Rheum. 2005;52:2120-2124.
39. Huber-Lang M, Sarma JV, Zetoune FS et al. Generation of C5a in the absence of C3: a new complement activation pathway. Nat.Med. 2006;12:682-687.
40. Hall AJ, Vos HL, Bertina RM. Lipopolysaccharide induction of tissue factor in THP-1 cells involves Jun protein phosphorylation and nuclear factor kappaB nuclear translocation. J.Biol. Chem. 1999;274:376-383.
41. Fischetti F, Durigutto P, Pellis V et al. Thrombus formation induced by antibodies to beta2-glycoprotein I is complement dependent and requires a priming factor. Blood 2005;106:2340-2346.
42. de Laat B, Derksen RH, van Lummel M, Pennings MT, de Groot PG. Pathogenic anti-beta2-glycoprotein I antibodies recognize domain I of beta2-glycoprotein I only after a conformational change. Blood 2006;107:1916-1924.

8

General discussion

Introduction

The antiphospholipid syndrome is characterized by vascular thrombosis or pregnancy morbidity combined with the persistent presence of antiphospholipid antibodies in blood of patients.[1] The term "antiphospholipid" is a misnomer, as antiphospholipid antibodies that are relevant in the syndrome recognize plasma proteins with affinity for phospholipids such as β_2-glycoprotein I[2] and prothrombin,[3] not phospholipids per se. Antiphospholipid antibodies are categorized according to the antigen used in the assay with which they are detected, e.g. anticardiolipin, anti-β_2-glycoprotein I or antiprothrombin antibodies. Further distinction can be made, regardless of antigen specificity, between antibodies that inhibit coagulation assays in a phospholipid-dependent manner, so-called lupus anticoagulants, and antibodies that do not affect coagulation assays.

The definition of the syndrome is based on the assumption that antiphospholipid antibodies are causally related to the thrombotic manifestations of the syndrome. The role of antiphospholipid antibodies in the etiology of thrombotic disease, however, is far from clear. Although our understanding of the molecular mechanisms responsible for the thrombotic tendency caused by antiphospholipid antibodies in general, and anti-β_2-glycoprotein I antibodies in particular, slowly increases, we have a long way to go before we can say we understand the pathophysiology of the syndrome. To complicate matters further, current data on the risk of developing a thrombotic event in the presence of antiphospholipid antibodies are inconclusive, because most studies on antiphospholipid antibody-related thrombotic risk were performed in selected patient populations, such as patients with underlying autoimmune disease, and prospective studies in untreated patients are rare and small. Efforts are made to provide data on antiphospholipid antibody-related thrombotic risk in the general population, but the currently available data does not provide the necessary insights.

This thesis aims to contribute to our knowledge of antiphospholipid antibody-related risk of arterial thrombosis in the general population and to provide a possible molecular mechanism behind antiphospholipid antibody-mediated activation of platelets, key players in the process of arterial thrombosis. In this chapter the implications of the findings reported in the previous chapters will be discussed in light of current knowledge.

Antiphospholipid antibodies and thrombotic risk in the general population

The updated consensus criteria for classification of the antiphospholipid syndrome state that the anticardiolipin, the anti-β_2-glycoprotein I or the lupus anticoagulant assay should be used to diagnose the syndrome.[1] Although antiprothrombin antibodies can induce a lupus anticoagulant effect,[3] data on their association with thrombosis and pregnancy morbidity were deemed insufficient to justify inclusion in the classification criteria of the syndrome. Diagnosis of the antiphospholipid syndrome requires a repeatedly positive test result, with an interval of at least 12 weeks, in either of the three official

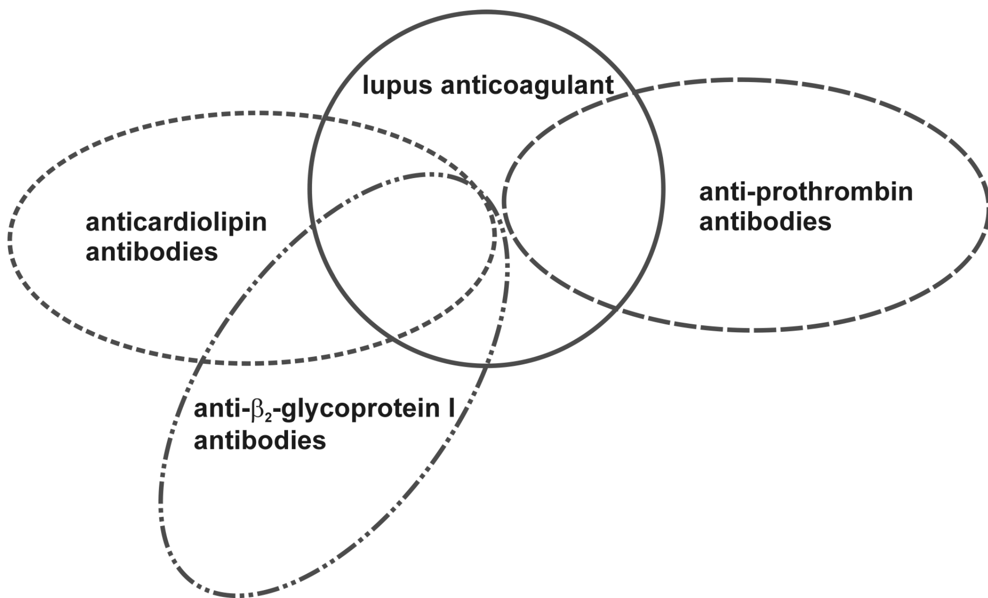

Figure 1. Assays for antiphospholipid antibodies detect overlapping, but not necessarily identical antibody populations.

assays. Each assay, however, detects overlapping, but not necessarily identical antibody populations (Figure 1).

The anticardiolipin assay for example, was developed after it was noted that systemic lupus erythematosus patients with lupus anticoagulant often have antibodies against the main antigen in the Venereal Disease Research Laboratory (VDRL) test for syphilis; cardiolipin. Nevertheless, plasma with high titer anticardiolipin antibodies does not necessarily display the lupus anticoagulant phenomenon in a coagulation assay.[4] Likewise, even though the anti-β_2-glycoprotein I assay was developed after cardiolipin-bound β_2-glycoprotein I was identified as the antigen in the anticardiolipin assay,[2;5] it does not detect the same antibody population as the anticardiolipin assay. Furthermore, although anti-β_2-glycoprotein I antibodies can have lupus anticoagulant activity,[6] not all lupus anticoagulants are caused by anti-β_2-glycoprotein I antibodies.

Systematic reviews of data on antiphospholipid antibody-related thrombotic risk show that the presence of lupus anticoagulants correlate best with overall thrombosis.[7;8] In fact, lupus anticoagulants are the only antiphospholipid antibody subpopulation that is consistently associated with all types of thrombosis in the studies that were reviewed.[8]

The majority of studies on the risk of antiphospholipid antibody-mediated thrombosis in the general population investigated the influence of only 1 or 2 antiphospholipid

antibody subpopulations, with an emphasis on the role of anticardiolipin antibodies.[9-20] Table 1 shows a summary of a MEDLINE search on currently available data on antiphospholipid-antibody related thrombotic risk in the general (unselected) population. The following search terms were used: antiphospholipid, anti-β_2-glycoprotein antibodies, antiprothrombin antibodies, anticardiolipin antibodies, lupus anticoagulant, deep vein thrombosis, arterial thrombosis, ischemic stroke and myocardial infarction. Only case-control and prospective cohort studies were included. Studies in systemic lupus erythematosus (SLE)-cohorts or groups of patients with known antiphosholipid antibodies were not included in table 1. No attempts were made to calculate relative risks when they were not provided; these studies were left out of table 1 as well.

Although the majority of the studies listed in table 1 conclude that anticardiolipin antibodies increase the risk of either a venous[10;17] or arterial thrombotic event,[13-16;18;20] others contradict these results.[9-12;19] All studies on the role of antiprothrombin antibodies[21-24] show an increased risk of arterial[22] or venous[21;24] thrombosis. Anti-β_2-glycoprotein I antibodies are associated with an increased risk of venous[24] and arterial thrombosis[19] in two studies, which is contradicted by others.[23] Even though lupus anticoagulant is considered the most relevant antiphospholipid antibody category, its relation with thrombotic risk in the general population was investigated in two studies only. Both of these studies showed lupus anticoagulant is associated with an increased risk of venous[24] or arterial[18] thrombosis. We could not confirm that either anticardiolipin or antiprothrombin antibodies increase the risk of arterial thrombosis in a population of young women (chapter 4). Whereas others reported that anti-β_2-glycoprotein I antibodies increase the risk of myocardial infarction,[19] we could not confirm these data in either young women (chapter 4) or middle aged men (chapter 5). However, the presence of anti-β_2-glycoprotein I antibodies is associated with an increased risk of ischemic stroke in young women (chapter 4). Lupus anticoagulant is associated with a significantly increased risk of both myocardial infarction and ischemic stroke in young women (chapter 4), but is not associated with increased risk of myocardial infarction in men (chapter 5).

Antiphospholipid antibodies are often encountered in patients with underlying autoimmune disease, such as SLE. Patients with SLE are already at higher risk of cardiovascular disease than healthy individuals[25] and it is difficult to assess the contribution of antiphospholipid antibodies to that risk. Nevertheless, most studies on the risk of arterial thrombosis in SLE patients with antiphospholipid antibodies report lupus anticoagulants are associated with an increased risk.[26;27]

Thrombotic risk determined by antiphospholipid antibody assay?

There are several explanations for the observed differences in thrombotic risk in the general population associated with the presence of antiphospholipid antibodies. Pitfalls of currently available antiphospholipid antibody assays are listed in table 2. A major reason is poor assay standardization, which is true especially for the anticardiolipin assay.[28-30] Despite guidelines for standardization of the anticardiolipin assay,[31-33] several

TABLE 1. Antiphospholipid antibody-related thrombotic risk in the general population.

	Cases (N)	Controls (N)	Sex (M/F)	Odds Ratio (95-% Confidence interval)			
				anti-cardiolipin antibodies	anti-prothrombin antibodies	anti-β_2-glycoprotein I antibodies	Lupus anticoagulant
PE / DVT							
de Groot et al.[24]	473	472	M/F		1.4 (1.0-2.1)	2.4 (1.3-4.2)	3.6 (1.2-10.9)
Ginsburg et al.[10]	90	90	M	5.5 (1.2-24.8)			
Kahwa et al.[17]	29	148	F	3.32 (1.5-9.54)			
Naess et al.[9]	508	1464	M/F	1.11 (0.71-1.74)			
Paluoso et al.[21]	265	265	M		6.56 (1.73-25)		
Runchey et al.[11]	317	655	M/F	0.88 (0.43-1.78)			
Myocardial infarction							
Brey et al.[13]	374	1360	M	1.5 (1.1-2.1)			
Meroni et al.[19]	172	172	F	NA		2.47 (1.81-3.38)	
Vaarala et al.[22]	106	106	M		2.5 (1.2-5.3)		
Chapter 4	*203*	*628*	*F*	*1.6 (0.9-2.6)*	*0.7 (0.4-1.2)*	*0.9 (0.5-1.6)*	*5.3 (1.4-20.8)*
Chapter 5	*560*	*646*	*M*				*1.03 (0.41-2.55)*
Stroke							
Ahmed et al.[12]	123	241	M/F	1 (0.79-1.26)			
APASS[16]	248	250	M/F	2.34 (1.12-4.91)			
Brey et al.[13]	259	1360	M	1.5 (1-2.3)			
Brey et al.[18]	160	340	F	1.65 (1.06-2.57			1.8 (1.06-3.06)
Camerlingo et al.[20]	100	100	M/F	4.9 (1.05-22.9)			
Ginsburg et al.[10]	100	100	M	NA			
Janardhan et al.[14]	106	2156	F	1.3 (0.7-2.4)			
Janardhan et al.[14]	116	2596	M	2.6 (1.3-5.4)			
Kahwa et al.[17]	21	148	F	1.76 (0.46-6.73)			
Tuhrim et al.[15]	524	1020	M/F	3.9 (2.8-5.5)			
Chapter 4	*175*	*628*	*F*	*0.8 (0.4-1.7)*	*1.3 (0.7-2.4)*	*2.3 (1.4-3.7)*	*43 (12-152)*

PE / DVT denotes Pulmonary embolism / deep venous thrombosis. NA denotes not applicable

comparisons of commercially available anticardiolipin assays report differences when measuring the same sample twice, or report that two different commercial assays do not generate the same results in a substantial amount of samples.[28-30] Discordant results were especially seen with low titer antibodies. This probably reflects differences in the phospholipids used by each manufacturer (purified cardiolipin or a phospholipid mixture), problems with the immobilization of phospholipids onto microtiter plates, differences in blocking agents and different sources of the β_2-glycoprotein I (human or bovine) with which most of the microtiter plates are saturated. The lack of consensus on the cut-off between a positive and a negative test result complicates matters further. The use of a home-made assay with unconfirmed reproducibility, as in most of the studies listed in table 1, will probably only increase this effect.

The Sapporo criteria for the antiphospholipid syndrome state that anticardiolipin antibodies should be β_2-glycoprotein I dependent.[34] This was omitted from the criteria in the Sydney update, presumably because a β_2-glycoprotein I-dependency is virtually impossible to prove: In order to prove dependency on β_2-glycoprotein I, assays should

be performed in the absence of β_2-glycoprotein I, which can only be achieved by β_2-glycoprotein I-depletion of plasma, an impossibility for most routine diagnostic laboratories. A substantial amount of anticardiolipin antibodies will therefore reflect transient infection-related "true" anticardiolipin antibodies. Although the chance of detecting transient antibodies in a diagnostic setting is minimized by the mandatory 12-week interval between initial and confirmation measurements,[1] all studies on thrombotic risk in the general population, including ours, determined antiphospholipid antibody levels in one sample only and are therefore unable to differentiate between infection-related and persistent anticardiolipin antibodies.

No guidelines are provided for correct determination of antiprothrombin antibodies. Major differences that might interfere with outcome are the source of the prothrombin (human or bovine), the presence or absence of calcium ions[35] and either direct prothrombin immobilization onto a microtiter plate or the saturation of an immobilized phospholipid layer with prothrombin.[36]

As the anti-β_2-glycoprotein I assay was only recently added to the criteria, there is little data on assay-reproducibility as of yet. Nevertheless, assay standardization guidelines were proposed.[37] Some studies state that assay performance has improved compared to the anticardiolipin assay,[38;39] whereas others report comparable inter- and intra-assay variability between anticardiolipin and anti-β_2-glycoprotein I assays.[40] Anticardiolipin antibodies are known to occur transiently in infectious disease, but whether the same is true for anti-β_2-glycoprotein I antibodies is unclear. Anti-β_2-glycoprotein I antibodies are reported in leprosy,[41] leishmania, leptospirosis and syphilis,[42] although more data are necessary to confirm the occurrence of infection-related antibodies against β_2-glycoprotein I. Approximately half of all positive samples in an anti-β_2-glycoprotein I antibody-assay seems to reflect low affinity charge-dependent antibodies against β_2-glycoprotein I,[43] detection of which might be decreased by increasing ionic strength during assay procedures.

Correct ligand immobilization is an issue for the anti-β_2-glycoprotein I assay, just as it is for the anticardiolipin asay. Differences in protein density and net charge of plastic microtiter plates onto which β_2-glycoprotein is immobilized will influence test results.[44;45] All studies on anti-β_2-glycoprotein I-related thrombotic risk in the general population used different home-made assays, which further complicates the comparison of results from different studies.

Lupus anticoagulant can be detected in several ways.[46] Each coagulation assay has a different sensitivity and specificity for lupus anticoagulant. The only other study on risk of stroke in women with antiphospholipid antibodies in which lupus anticoagulant was measured, used both an activated partial thromboplastin time (APTT)-based assay and the dilute Russell's Viper Venom Time (dRVVT).[18] Lupus anticoagulant-associated thrombotic risk was assessed for samples positive in either assay. We determined lupus anticoagulant with the dRVVT only (chapter 5). As the dRVVT is generally considered the assay with the greatest specificity, this might partly explain the differences in relative

Table 2. Pitfalls in determination of antiphospholipid antibody profile.

	Anticardiolipin antibody assays	**Antiprothrombin antibody assays**	**Anti-β_2-glycoprotein I antibody assays**	**Lupus anticoagulant**
Ligand immobilization	No consensus on phospholipid(s) to immobilize, source of β_2-glycoprotein I or blocking agent.	No consensus. Either direct prothrombin immobilization or binding to immobilized phospholipids.	No consensus on source of β_2-glycoprotein I or blocking agent.	Not applicable.
Inter- / intra-assay variability	Poor.	No data available.	Poor.	Poor.
Standardization	Guidelines for improving comparability between studies with general calibrators.[30-32]	No guidelines.	Guidelines were proposed.[37]	Guidelines of a standardization subcommittee of the International Society of Thrombosis and Haemostasis.[47-49]
Cut-off value	No consensus.	No consensus.	No consensus.	No consensus.

risks for ischemic stroke between our studies.

Testing for lupus anticoagulant is not easy and should be performed by specialized laboratories with experience in coagulation. Several factors influence assay-outcome. Sample quality is imperative in lupus anticoagulant testing. Too many residual platelets in test plasma might mask a phospholipid-dependent coagulant inhibitor. Use of vitamin K antagonists or heparin might further interfere with correct lupus anticoagulant testing. Therefore, strict guidelines were set for proper assay performance by a scientific standardization committee of the International Society for Thrombosis and Haemostasis.[47-49] Nevertheless, inter and intra-assay variability of lupus anticoagulant assays remain poor,[46] especially when laboratories disregard the proposed guidelines.[50] Although the available guidelines address many issues, the issue of determining a proper cut-off for sample positivity remains unresolved.

Not surprisingly, the serological criteria for definition of the antiphospholipid syndrome are currently under debate.[51] The outcome of this international discussion will hopefully improve assessment of antiphospholipid antibody related thrombotic risk, which is an urgent need for clinicians. Amongst the things that should be addressed in this discussion are assay standardization, the determination of proper cut-off values for positive and negative test results and the specificity and sensitivity of the available assays. The low antiphospholipid antibody-associated relative risk of thrombosis reported in most of the studies listed in table 1, certainly suggest the currently available assays to detect antiphospholipid antibodies are not specific enough. That, or we have not identified the true antigen responsible for the thrombotic manifestations of the syndrome.

Lupus anticoagulant; the link with sex and age

The study described in chapter 4 shows that lupus anticoagulant is a major risk factor for both myocardial infarction and ischemic stroke. This study was performed in a population of young women and the obtained relative risks are substantial (5-fold increased risk of myocardial infarction and a 43-fold increased risk of ischemic stroke). We could not, however, confirm these results in a population of elderly men with myocardial infarction (chapter 5).

Two major differences between the two study populations were sex and age: chapter 4 describes a study amongst women below age 50 and chapter 5 a study amongst men below age 70. Like most autoimmune diseases, the antiphospholipid syndrome mainly occurs in young women, with a ratio of about 5 women to 1 man.[52] In itself, this might be an explanation for the discrepancy in results between chapters 4 and 5, as more power is needed to show the same effects in a population with a lower prevalence. In this case, approximately 5 times as many cases would be needed to show the same effect of antiphospholipid antibodies on risk of myocardial infarction.

The reason for the skewed distribution of autoimmune disease incidence amongst men and women is unknown, although several theories exist. One possible explanation lies in the role of estrogens as immune-response enhancing hormones, which was shown to occur in vitro.[53] Clinical practice does not always support these data,[54] as exacerbation of existing systemic lupus erythematosus is not observed in women undergoing hormone therapy.[55;56] Aberrant patterns of X-chromosomal gene silencing by DNA methylation, a process also known as X-chromosome inactivation, is another possible explanation. Skewed X-chromosome inactivation may lead to insufficient thymic deletion of T-cells and subsequent loss of tolerance.[57] A severely skewed X-chromosomal inactivation pattern is indeed observed in peripheral blood cells of patients with systemic scleroderma[58] and autoimmune thyroid disease.[59;60]

The thrombotic risk associated with antiphospholipid antibodies seems to be greatest in the young, probably because in an older population the influence of an accumulation of standard cardiovascular risk factors on the incidence of thrombotic disease is greater than the influence of antiphospholipid antibodies. Our study on the risk of arterial thrombosis in women with antiphospholipid antibodies allowed us to study a population in which the influence of classic cardiovascular risk factors is low; women are less susceptible to atherosclerosis during their reproductive years. Age, however, is not the only determinant in the differences between the two studies described in chapters 4 and 5, as lupus anticoagulant was not associated with an increased risk of myocardial infarction in a subgroup analysis amongst men below age 50 (data not shown).

Mechanisms of arterial thrombosis

The relation between in vitro prolongation of coagulation time and a prothrombotic state is the central paradox of the antiphospholipid syndrome. Although we know lupus

anticoagulant inhibits coagulation in an in vitro setting by competing with phospholipid-dependent coagulation factors for binding sites on anionic phospholipids, the precise mechanisms through which these antibodies exert their prothrombotic effects are still obscure. Antiphospholipid antibodies can bind to and activate the majority of cells involved in the process of thrombosis, i.e. platelets, endothelial cells and monocytes, and have both anticoagulant and procoagulant properties. As reviewed in Chapter 1, antiphospholipid antibodies are described to interact with these cells via a multitude of different receptors, such as Toll-like receptors 2 and 4, members of the low density lipoprotein (LDL) receptor family, annexin A2 and glycoprotein Ibα.

Platelet activation by antiphospholipid antibodies

Patients with the antiphospholipid syndrome have more activated platelets in their circulation than healthy individuals, as is shown by the increased levels of thromboxane A2 metabolites[61] and β-thromboglobulin in their urine,[62] both of which are secreted by activated platelets. The search for the molecular mechanism behind the platelet activation observed in the antiphospholipid syndrome led to the discovery that anti-β_2-glycoprotein I antibodies dimerize β_2-glycoprotein I[63] and that such antibody-antigen immune complexes activate platelets in an Fc-receptor independent manner.[64] The search for the responsible receptor led to the identification of two receptors involved in the activation of platelets by antiphospholipid antibodies: Apolipoprotein E receptor 2' (ApoER2'),[65] a truncated version of a member of the LDL-receptor family also known as LRP-8, and the platelet adhesive receptor glycoprotein Ibα.[66;67] Sensitization of platelets by antiphospholipid antibodies, or a recombinant dimer of β_2-glycoprotein I that mimics the effects of antiphospholipid antibodies, requires an interaction with both glycoprotein Ibα and ApoER2'. Inhibition of the interaction of the recombinant dimer with each of these receptors completely abrogates its sensitizing effects, despite continued signaling through the other, non-inhibited receptor (Chapter 6). The importance of ApoER2' in transmitting prothrombotic signals to platelets was confirmed in Chapter 7, in which massive in vivo thrombus formation induced by dimeric β_2-glycoprotein I in normal mice was abolished in ApoER2 deficient mice.

Toll-like receptor 4 is implicated in cellular activation in the antiphospholipid syndrome as well.[68] Although present on platelets, currently available data indicate this lipopolysaccharide receptor is not involved in platelet activation.[69]

The ApoER2'-glycoprotein Ibα axis

Both ApoER2' (which binds LDL, apolipoprotein E and the extracellular matrix protein reelin) and glycoprotein Ibα (which binds Von Willebrand Factor (VWF) and the coagulation factors thrombin, FXI and FXII) are multiligand receptors. Both receptors are also known to cooperate with other receptors in transmitting transmembrane activation signals. ApoER2 mediates the effects of reelin on neuronal positioning by clustering with the very low density lipoprotein (VLDL) receptor[70] and binding of thrombin to

glycoprotein Ibα results in signaling through protease activated receptor (PAR)-1[71] and glycoprotein Ibα[72;73] itself. Why two different signaling pathways are necessary to convert the interaction of a ligand with its receptor(s) into an activation signal remains to be elucidated. Perhaps the combination of signals emitted from two separate, unrelated multiligand receptors binding the same ligand determines the specificity of the cellular response, not the ligand-receptor interaction itself.

The dimeric form of β_2-glycoprotein I is not the only protein that binds to platelets via the ApoER2'-glycoprotein Ibα receptor-combination. Recently, both the zymogen protein C and its counterpart activated protein C were shown to bind to platelets and subsequently activate them.[74] Very much like what was described for the binding of platelets to dimeric β_2-glycoprotein I under conditions of flow,[66] platelet adhesion to protein C requires both glycoprotein Ibα and ApoER2'. Furthermore, both dimeric β_2-glycoprotein I and (activated) protein C bind to the first LDL-binding domain of ApoER2' and the sulfated tyrosine residues on glycoprotein Ibα. Perhaps we have identified a pathway of platelet activation that is not only relevant under the pathological conditions of the antiphospholipid syndrome (chapter 6), but may play a role in normal hemostasis. Furthermore, as protein C not only functions as a circulating anticoagulant, but also has cytoprotective properties,[75] ApoER2 might play be role in mediating these effects as well.

Activation of other cells involved in arterial thrombosis

Endothelial cells can be activated by antiphospholipid antibodies. Tissue factor expression[76] and upregulation of various adhesion molecules[77;78] upon incubation with antiphospholipid antibodies is described. As described in chapter 4, smoking greatly increased the risk of an arterial thrombotic event in young women. The joint effect between smoking and antiphospholipid antibodies is not surprising, as smoking, like antiphospholipid antibodies, is known to cause endothelial dysfunction.[79;80]

Several different receptors are deemed responsible for transmission of activation signals across the endothelial cell membrane upon binding of antiphospholipid antibodies. Annexin A2,[81] a phospholipid binding protein without transmembrane domain, is proposed to be a receptor for antiphospholipid antibodies that mediates cellular activation. It seems unlikely, however, that annexin A2 is involved in translation of a signal due to its inability to convey signals across the cell membrane. It might, however, still serve as a docking site for β_2-glycoprotein I-antibody immune complexes. Toll-like receptor 4-like signaling is shown in endothelial cells upon stimulation with antiphospholipid antibodies,[68] but until the actual interaction between antiphospholipid antibodies and the receptor is shown all evidence regarding TLR4 involvement remains circumstantial.

ApoER2 is present on endothelial cells as well.[82] The data described in chapter 7 suggests there is a role for ApoER2 in endothelial cell activation, as the endothelial tissue factor upregulation induced by dimeric β_2-glycoprotein I in vivo is attenuated both in ApoER2-deficient mice and after blocking the interaction between ApoER2(') and

dimeric β_2-glycoprotein I with the soluble first LDL-binding domain of ApoER2.

The protein C axis

The anticoagulant activated protein C pathway is responsible for the degradation of the cofactors Factor Va and VIIIa, which results in inhibition of the coagulation reaction. Activated protein C resistance is most often caused by an Arginine to Glutamine substitution in coagulation factor V at position 506,[83] also known as the factor V Leiden mutation. This mutation is associated with an increased risk of venous thrombosis, especially in young women who use oral contraceptives.[84] The association of Factor V Leiden with an increased risk of arterial thrombosis is more controversial.[85] Antiphospholipid antibodies are a possible cause of acquired activated protein C resistance.[86;87] Although an increased risk of both arterial and venous thrombosis is suggested in patients with antiphospholipid antibody induced acquired protein C resistance,[88] sufficient data are lacking on this hypothesis. Oral contraceptive use also results in acquired activated protein C resistance.[89] Although the effects of antiphospholipid antibodies on the increased risk of myocardial infarction or ischemic stroke in young women might be fully explained by platelet and endothelial cell activation, the joint effect between oral contraceptive use and lupus anticoagulant suggests a role for the protein C pathway as well. Perhaps the combination of two different causes of activated protein C resistance is responsible for at least a proportion of the increase in risk we observed. As dimeric β_2-glycoprotein I can compete with (activated) protein C for a binding site on ApoER2 (unpublished data), there might be a role for endothelial cells or platelets in this system as well.

A second paradox in the antiphospholipid syndrome

The relation between an in vitro clotting defect and a thrombotic tendency is known as the great paradox of the antiphospholipid syndrome. In chapter 3 we described a second paradox in the syndrome. Patients with lupus anticoagulant not only have abnormal coagulation test results, but also have abnormal results in a diagnostic test of primary hemostasis. The bleeding time, which is considered to be a test for (ex-vivo) platelet function, is prolonged in the majority of patients with lupus anticoagulant. Explanations for this phenomenon could not be found in well-known defects in platelet or Von Willebrand Factor function in the majority of these patients.

During the bleeding time test, a standardized incision is made in the skin of patients. The time it takes for the wound to stop bleeding is recorded. The process responsible for cessation of bleeding is the formation of a platelet plug.[90;91] The central role of platelets in this proces is shown by a prolonged bleeding time in patients with defects of platelet function[92] and in patients with Von Willebrand Factor deficiency (Von Willebrand's Disease).[93] Secondary hemostasis serves to stabilize formed platelet plugs. Bleeding times are normal in patients deficient in coagulation Factor VIII, a disease known as hemophilia A, as platelet plug formation in these patients is normal. However, the wounds will re-bleed due to a lack of stabilization by a fibrin network.[94]

Why, then, do patients with lupus anticoagulant exhibit a prolonged bleeding time? All experimental data on platelet adhesion suggests the reverse should happen: addition of antiphospholipid antibodies to platelets results in increased platelet adhesion to collagen[65] and results in excessive thrombus formation in murine (chapter 7) or hamster[64] vascular injury models.

As suggested in chapter 3, the endothelium could play a role here, as endothelial cell activation by antiphospholipid antibodies results in an increased production of prostacyclin,[95] a potent platelet inhibitor, and expression of inducible nitric oxide synthase, also known as iNOS,[76] which can generate the vasodilatory nitric oxide. Increased platelet reactivity towards prostacyclin is known to lead to a prolonged bleeding time.[96] It is therefore likely that increased platelet exposure to prostacyclin would lead to an inhibition of platelet function as well.

On the other hand, the prolonged bleeding time could also be caused by the interaction of antiphospholipid antibodies with platelets. The bleeding time is highly dependent on the presence of Von Willebrand factor, as is shown by the prolongation of the bleeding time in patients with Von Willebrand's disease.[93] Anti-β_2-glycoprotein I antibodies are suggested to bind to glycoprotein Ibα at the Von Willebrand factor binding site,[67] thereby potentially interfering with adhesion under high shear stress conditions. This hypothesis does not seem likely for two reasons: (1) If this hypothesis were true, platelet adhesion to immobilized collagen under conditions of high shear stress , which is dependent upon Von Willebrand factor-glycoprotein Ibα interactions, would be lower in the presence of antiphospholipid antibodies, contrary to what was reported.[65] (2) β_2-glycoprotein I was reported to serve as a natural inhibitor of the Von Willebrand Factor-glycoprotein Ibα interaction. Antiphospholipid antibodies attenuate rather than strengthen the inhibitory function of β_2-glycoprotein I, allowing a stronger interaction between glycoprotein Ibα and Von Willebrand Factor.[97]

Although the end-product of coagulation, fibrin, does not play a role in the processes leading to the initial cessation of bleeding, thrombin also plays a role in the process of platelet activation. Thrombin, the key enzyme in fibrin formation, is able to activate platelets by cleavage of PAR-1 and -4, which leads to receptor auto-activation. Binding of thrombin to glycoprotein Ibα facilitates this process.[71] As we mapped the binding site of β_2-glycoprotein I-antibody complexes to the thrombin binding site on glycoprotein Ibα,[66] this might lead to impaired platelet activation via the PAR-1 pathway. Recently, platelet plug formation was proposed to occur in three stages: primary adhesion of platelets that spread over the exposed subendothelium, formation of a loose platelet aggregate, which contains a large amount of discoid platelets, followed by secretion-dependent plug stabilization, leading to full activation of the loosely packed platelets caught in the aggregate.[98] Antiphospholipid antibodies might interfere with this final stage, partly preventing the formation of a stable platelet aggregate, but allowing (excessive) primary adhesion to exposed subendothelial layers. Perhaps these unstable platelet plugs embolize and wash away, which might explain the prolongation of the bleeding

time.

The combination of excessive platelet adhesion to sites of vascular injury and antiphospholipid antibody-induced inhibition of the activated protein C system, which results in excessive thrombin formation, might still result in the formation of a vessel-occluding thrombus as observed in the animal models.

Clinical implications

Data described in this thesis show that women with lupus anticoagulant have a greater risk of developing a first episode of myocardial infarction or ischemic stroke than women without lupus anticoagulant. Nevertheless, otherwise healthy individuals with lupus anticoagulant are not treated with thromboprophylaxis to prevent the occurrence of a first thrombotic event, simply because such treatment does not seem effective.[99] Whether or not the secondary prophylaxis given to patients with a history of myocardial infarction or ischemic stroke alters when they have circulating lupus anticoagulants is unclear as well. According to the current ACCP guidelines for treatment of ischemic stroke, non-cardioembolic stroke should be treated with aspirin combined with dipyramidole for an undefined period of time.[100] Myocardial infarction is treated by administration of platelet inhibitors as well.[101] The presence of antiphospholipid syndrome might justify life-long treatment with (even high dose) coumarin-derivatives according to some authors,[102] but long-term treatment with low-dose aspirin seems to be just as effective.[103;104]

The risk of a first event of ischemic stroke or myocardial infarction is increased even further in women with lupus anticoagulant who smoke or use oral contraceptives compared to non-smokers and non-users without lupus anticoagulant. It would therefore seem reasonable to advice women with lupus anticoagulant who have not experienced an arterial thrombotic event yet to stop smoking and find a different means of birth control.

Data described in chapters 6 and 7 suggest that inhibition of the interaction of antibody-β_2-glycoprotein I complexes with either glycoprotein Ibα or ApoER2' might be a potential therapeutic option. As glycoprotein Ibα is essential for platelet function, inhibition of this receptor is not the method of choice. Inhibition of the interaction of β_2-glycoprotein I with its receptors by administration of the fifth domain of β_2-glycoprotein I does not seem to be the best option either, as this would potentially lead to the generation of new anti-β_2-glycoprotein I antibodies. Furthermore, domain V of β_2-glycoprotein I contains the phospholipid binding site and might therefore interfere with coagulation when administered in a high concentration. Capture of pathogenic antiphospholipid antibodies[44] by administration of the first domain of β_2-glycoprotein I does not seem very attractive either, as this will potentially lead to the development of a new pool of pathological antiphospholipid antibodies as well. Treatment with the soluble first LDL-binding domain of ApoER2, or the peptides thereof that correspond to the β_2-glycoprotein I-binding site, could be an option. Administration of these proteins would not interfere with platelet function. However, (activated) protein C binds to

the same epitope on ApoER2 as dimeric β_2-glycoprotein I. If ApoER2-bound activated protein C functions as an anticoagulant, treatment with the soluble first LDL-binding domain of ApoER2 might result in the induction of a new prothrombotic state. If the protein C-ApoER2 interaction also has cytoprotective properties, administration of the soluble first LDL-binding domain of ApoER2 might furthermore be detrimental to activated protein C function during infection. Moreover, ApoER2 mediates neuronal plasticity,[105;106] so interfering with the interaction between ApoER2 and reelin might have neurological implications as well. Although murine models show a redundancy in the ApoER2-reelin pathway of neuronal plasticity,[107] the same is not guaranteed in humans.

Future directions

The increased risk of arterial thrombosis in women with lupus anticoagulant as described in this thesis (chapter 4) should be confirmed in large prospective cohort studies. These studies should include measurement of antiphospholipid antibodies in two samples from each included individual, taken at least 12 weeks apart. That leaves, however, the issue of the assays with which to determine these antiphospholipid antibody profiles. There are many pitfalls that need to be resolved before the currently available assays can be implemented in large-scale studies; inter- and intra-assay variability should be minimized, assay standardization should be improved and proper cut-off levels should be determined. As currently available data shows only a mildly increased risk of a thrombotic event in the presence of antiphospholipid antibodies, this might indicate we have not yet identified the relevant antiphospholipid antibody subpopulation(s). Future research should therefore focus on the development of new antiphospholipid antibody assays as well.

Now that the activation of endothelial cells, monocytes and platelets by antiphospholipid antibodies has been demonstrated, the complicated interplay between these cells in the process of thrombosis should be investigated in vivo. Here lies a great challenge, as it is difficult to differentiate between separate processes in in-vivo systems.

References

1. Miyakis S, Lockshin MD, Atsumi T et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J.Thromb.Haemost. 2006;4:295-306.
2. Galli M, Comfurius P, Maassen C et al. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. Lancet 1990;335:1544-1547.
3. Bevers EM, Galli M, Barbui T, Comfurius P, Zwaal RF. Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin. Thromb. Haemost. 1991;66:629-632.
4. McNeil HP, Chesterman CN, Krilis SA. Anticardiolipin antibodies and lupus anticoagulants comprise separate antibody subgroups with different phospholipid binding characteristics. Br.J.Haematol. 1989;73:506-513.
5. McNeil HP, Simpson RJ, Chesterman CN, Krilis SA. Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2-glycoprotein I (apolipoprotein H). Proc.Natl.Acad.Sci.U.S.A 1990;87:4120-4124.
6. Roubey RA, Pratt CW, Buyon JP, Winfield JB. Lupus anticoagulant activity of autoimmune antiphospholipid antibodies is dependent upon beta 2-glycoprotein I. J.Clin.Invest 1992;90:1100-1104.
7. Galli M, Luciani D, Bertolini G, Barbui T. Anti-beta 2-glycoprotein I, antiprothrombin antibodies, and the risk of thrombosis in the antiphospholipid syndrome. Blood 2003;102:2717-2723.
8. Galli M, Luciani D, Bertolini G, Barbui T. Lupus anticoagulants are stronger risk factors for thrombosis than anticardiolipin antibodies in the antiphospholipid syndrome: a systematic review of the literature. Blood 2003;101:1827-1832.
9. Naess IA, Christiansen SC, Cannegieter SC, Rosendaal FR, Hammerstroem J. A prospective study of anticardiolipin antibodies as a risk factor for venous thrombosis in a general population (the HUNT study). J.Thromb.Haemost. 2006;4:44-49.
10. Ginsburg KS, Liang MH, Newcomer L et al. Anticardiolipin antibodies and the risk for ischemic stroke and venous thrombosis. Ann.Intern.Med. 1992;117:997-1002.
11. Runchey SS, Folsom AR, Tsai MY, Cushman M, McGovern PD. Anticardiolipin antibodies as a risk factor for venous thromboembolism in a population-based prospective study. Br.J.Haematol. 2002;119:1005-1010.
12. Ahmed E, Stegmayr B, Trifunovic J et al. Anticardiolipin antibodies are not an independent risk factor for stroke: an incident case-referent study nested within the MONICA and Vasterbotten cohort project. Stroke 2000;31:1289-1293.
13. Brey RL, Abbott RD, Curb JD et al. beta(2)-Glycoprotein 1-dependent anticardiolipin antibodies and risk of ischemic stroke and myocardial infarction: the honolulu heart program. Stroke 2001;32:1701-1706.
14. Janardhan V, Wolf PA, Kase CS et al. Anticardiolipin antibodies and risk of ischemic stroke and transient ischemic attack: the Framingham cohort and offspring study. Stroke 2004;35:736-741.
15. Tuhrim S, Rand JH, Wu XX et al. Elevated anticardiolipin antibody titer is a stroke risk factor in a multiethnic population independent of isotype or degree of positivity. Stroke 1999;30:1561-1565.
16. Anticardiolipin antibodies are an independent risk factor for first ischemic stroke. The Antiphospholipid Antibodies in Stroke Study (APASS) Group. Neurology 1993;43:2069-2073.
17. Kahwa EK, Sargeant LA, Farlane-Anderson N et al. Anticardiolipin antibodies and risk of thromboembolic disease in young Jamaican women. J.Cardiovasc. Risk 2001;8:349-354.
18. Brey RL, Stallworth CL, McGlasson DL et al. Antiphospholipid antibodies and stroke in young women. Stroke 2002;33:2396-2400.
19. Meroni PL, Peyvandi F, Foco L et al. Anti-beta 2 glycoprotein I antibodies and the risk of myocardial infarction in young premenopausal women. J.Thromb. Haemost. 2007;5:2421-2428.
20. Camerlingo M, Casto L, Censori B et al. Anticardiolipin antibodies in acute non-hemorrhagic stroke seen within six hours after onset. Acta Neurol.Scand. 1995;92:69-71.
21. Palosuo T, Virtamo J, Haukka J et al. High antibody levels to prothrombin imply a risk of deep venous thrombosis and pulmonary embolism in middle-aged men--a nested case-control study. Thromb.Haemost. 1997;78:1178-1182.
22. Vaarala O, Puurunen M, Manttari M et al. Antibodies to prothrombin imply a risk of myocardial infarction in middle-aged men. Thromb.Haemost. 1996;75:456-459.
23. Previtali S, Barbui T, Galli M. Anti-beta2-glycoprotein I and anti-prothrombin antibodies in antiphospholipid-negative patients with thrombosis: a case control study. Thromb.Haemost. 2002;88:729-732.
24. de Groot PG, Lutters B, Derksen RH et al. Lupus anticoagulants and the risk of a first episode of deep venous thrombosis. J.Thromb.Haemost. 2005;3:1993-1997.
25. Ward MM. Premature morbidity from cardiovascular and cerebrovascular diseases in women with systemic lupus erythematosus. Arthritis Rheum. 1999;42:338-346.
26. Abu-Shakra M, Gladman DD, Urowitz MB, Farewell V. Anticardiolipin antibodies in systemic lupus erythematosus: clinical and laboratory correlations. Am.J.Med. 1995;99:624-628.
27. De Bandt M, Benali K, Guillevin L et al. Longitudinal determination of antiphospholipid antibodies in lupus patients without previous manifestations of antiphospholipid syndrome. A prospective study. J.Rheumatol. 1999;26:91-96.
28. Audrain MA, Colonna F, Morio F, Hamidou MA, Muller JY. Comparison of different kits in the detection of autoantibodies to cardiolipin and beta2glycoprotein 1. Rheumatology.(Oxford) 2004;43:181-185.
29. Kutteh WH, Franklin RD. Assessing the variation in antiphospholipid antibody (APA) assays: comparison of results from 10 centers. Am.J.Obstet.Gynecol. 2004;191:440-448.
30. Reber G, Arvieux J, Comby E et al. Multicenter evaluation of nine commercial kits for the quantitation of anticardiolipin antibodies. The Working Group on Methodologies in Haemostasis from the GEHT (Groupe d'Etudes sur l'Hemostase et la Thrombose). Thromb.Haemost. 1995;73:444-452.

31. Harris EN, Pierangeli SS. Revisiting the anticardiolipin test and its standardization. Lupus 2002;11:269-275.
32. Wong RC, Gillis D, Adelstein S et al. Consensus guidelines on anticardiolipin antibody testing and reporting. Pathology 2004;36:63-68.
33. Tincani A, Allegri F, Sanmarco M et al. Anticardiolipin antibody assay: a methodological analysis for a better consensus in routine determinations--a cooperative project of the European Antiphospholipid Forum. Thromb. Haemost. 2001;86:575-583.
34. Wilson WA, Gharavi AE, Koike T et al. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop. Arthritis Rheum. 1999;42:1309-1311.
35. Horbach DA, van Oort E, Donders RC, Derksen RH, de Groot PG. Lupus anticoagulant is the strongest risk factor for both venous and arterial thrombosis in patients with systemic lupus erythematosus. Comparison between different assays for the detection of antiphospholipid antibodies. Thromb.Haemost. 1996;76:916-924.
36. Atsumi T, Ieko M, Bertolaccini ML et al. Association of autoantibodies against the phosphatidylserine-prothrombin complex with manifestations of the antiphospholipid syndrome and with the presence of lupus anticoagulant. Arthritis Rheum. 2000;43:1982-1993.
37. Reber G, Tincani A, Sanmarco M, de Moerloose P, Boffa MC. Proposals for the measurement of anti-beta2-glycoprotein I antibodies. Standardization group of the European Forum on Antiphospholipid Antibodies. J.Thromb. Haemost. 2004;2:1860-1862.
38. Reber G, Schousboe I, Tincani A et al. Inter-laboratory variability of anti-beta2-glycoprotein I measurement. A collaborative study in the frame of the European Forum on Antiphospholipid Antibodies Standardization Group. Thromb.Haemost. 2002;88:66-73.
39. Reber G, Tincani A, Sanmarco M, de Moerloose P, Boffa MC. Variability of anti-beta2 glycoprotein I antibodies measurement by commercial assays. Thromb.Haemost. 2005;94:665-672.
40. Pengo V, Biasiolo A, Bison E, Chantarangkul V, Tripodi A. Antiphospholipid antibody ELISAs: survey on the performance of clinical laboratories assessed by using lyophilized affinity-purified IgG with anticardiolipin and anti-beta2-Glycoprotein I activity. Thromb.Res. 2007;120:127-133.
41. Arvieux J, Renaudineau Y, Mane I et al. Distinguishing features of anti-beta2 glycoprotein I antibodies between patients with leprosy and the antiphospholipid syndrome. Thromb.Haemost. 2002;87:599-605.
42. Santiago M, Martinelli R, Ko A et al. Anti-beta2 glycoprotein I and anticardiolipin antibodies in leptospirosis, syphilis and Kala-azar. Clin.Exp.Rheumatol. 2001;19:425-430.
43. de Laat B, Derksen RH, de Groot PG. High-avidity anti-beta glycoprotein I antibodies highly correlate with thrombosis in contrast to low-avidity anti-beta glycoprotein I antibodies. J.Thromb. Haemost. 2006;4:1619-1621.
44. de Laat B, Derksen RH, Urbanus RT, de Groot PG. IgG antibodies that recognize epitope Gly40-Arg43 in domain I of beta 2-glycoprotein I cause LAC, and their presence correlates strongly with thrombosis. Blood 2005;105:1540-1545.
45. Reddel SW, Wang YX, Krilis SA. Anti-beta2-glycoprotein I autoantibodies require an antigen density threshold, consistent with divalent binding. Lupus 2003;12:37-45.
46. Derksen RH, de Groot PG. Tests for lupus anticoagulant revisited. Thromb.Res. 2004;114:521-526.
47. Brandt JT, Barna LK, Triplett DA. Laboratory identification of lupus anticoagulants: results of the Second International Workshop for Identification of Lupus Anticoagulants. On behalf of the Subcommittee on Lupus Anticoagulants/Antiphospholipid Antibodies of the ISTH. Thromb. Haemost. 1995;74:1597-1603.
48. Brandt JT, Triplett DA, Alving B, Scharrer I. Criteria for the diagnosis of lupus anticoagulants: an update. On behalf of the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the ISTH. Thromb. Haemost. 1995;74:1185-1190.
49. Wisloff F, Jacobsen EM, Liestol S. Laboratory diagnosis of the antiphospholipid syndrome. Thromb.Res. 2002;108:263-271.
50. Jennings I, Greaves M, Mackie IJ et al. Lupus anticoagulant testing: improvements in performance in a UK NEQAS proficiency testing exercise after dissemination of national guidelines on laboratory methods. Br.J.Haematol. 2002;119:364-369.
51. Galli M, Reber G, de Moerloose P, de Groot PG. Invitation to a debate on the serological criteria that define the antiphospholipid syndrome. J.Thromb.Haemost. 2008;6:399-401.
52. Cervera R, Piette JC, Font J et al. Antiphospholipid syndrome: clinical and immunologic manifestations and patterns of disease expression in a cohort of 1,000 patients. Arthritis Rheum. 2002;46:1019-1027.
53. Li J, McMurray RW. Effects of estrogen receptor subtype-selective agonists on autoimmune disease in lupus-prone NZB/NZW F1 mouse model. Clin.Immunol. 2007;123:219-226.
54. Lockshin MD. Sex differences in autoimmune disease. Lupus 2006;15:753-756.
55. Guballa N, Sammaritano L, Schwartzman S, Buyon J, Lockshin MD. Ovulation induction and in vitro fertilization in systemic lupus erythematosus and antiphospholipid syndrome. Arthritis Rheum. 2000;43:550-556.
56. Petri M, Kim MY, Kalunian KC et al. Combined oral contraceptives in women with systemic lupus erythematosus. N.Engl.J.Med. 2005;353:2550-2558.
57. Chitnis S, Monteiro J, Glass D et al. The role of X-chromosome inactivation in female predisposition to autoimmunity. Arthritis Res. 2000;2:399-406.
58. Ozbalkan Z, Bagislar S, Kiraz S et al. Skewed X chromosome inactivation in blood cells of women with scleroderma. Arthritis Rheum. 2005;52:1564-1570.
59. Ozcelik T, Uz E, Akyerli CB et al. Evidence from autoimmune thyroiditis of skewed X-chromosome inactivation in female predisposition to autoimmunity. Eur.J.Hum. Genet. 2006;14:791-797.
60. Brix TH, Knudsen GP, Kristiansen M et al. High frequency of skewed X-chromosome inactivation in females with autoimmune thyroid disease: a possible explanation for the female predisposition to thyroid autoimmunity. J.Clin. Endocrinol.Metab 2005;90:5949-5953.
61. Forastiero R, Martinuzzo M, Carreras LO, Maclouf J. Anti-beta2 glycoprotein I antibodies and platelet activation in patients with antiphospholipid antibodies: association with increased excretion of platelet-derived thromboxane

urinary metabolites. Thromb. Haemost. 1998;79:42-45.

62. Galli M, Cortelazzo S, Viero P et al. Interaction between platelets and lupus anticoagulant. Eur.J.Haematol. 1988;41:88-94.
63. Lutters BC, Meijers JC, Derksen RH, Arnout J, de Groot PG. Dimers of beta 2-glycoprotein I mimic the in vitro effects of beta 2-glycoprotein I-anti-beta 2-glycoprotein I antibody complexes. J.Biol.Chem. 2001;276:3060-3067.
64. Jankowski M, Vreys I, Wittevrongel C et al. Thrombogenicity of beta 2-glycoprotein I-dependent antiphospholipid antibodies in a photochemically induced thrombosis model in the hamster. Blood 2003;101:157-162.
65. Lutters BC, Derksen RH, Tekelenburg WL et al. Dimers of beta 2-glycoprotein I increase platelet deposition to collagen via interaction with phospholipids and the apolipoprotein E receptor 2'. J.Biol.Chem. 2003;278:33831-33838.
66. Pennings MT, Derksen RH, van Lummel M et al. Platelet adhesion to dimeric beta-glycoprotein I under conditions of flow is mediated by at least two receptors: glycoprotein Ibalpha and apolipoprotein E receptor 2'. J.Thromb.Haemost. 2007;5:369-377.
67. Shi T, Giannakopoulos B, Yan X et al. Anti-beta2-glycoprotein I antibodies in complex with beta2-glycoprotein I can activate platelets in a dysregulated manner via glycoprotein Ib-IX-V. Arthritis Rheum. 2006;54:2558-2567.
68. Raschi E, Testoni C, Bosisio D et al. Role of the MyD88 transduction signaling pathway in endothelial activation by antiphospholipid antibodies. Blood 2003;101:3495-3500.
69. Clark SR, Ma AC, Tavener SA et al. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. Nat.Med. 2007;13:463-469.
70. Strasser V, Fasching D, Hauser C et al. Receptor clustering is involved in Reelin signaling. Mol.Cell Biol. 2004;24:1378-1386.
71. De Candia E, Hall SW, Rutella S et al. Binding of thrombin to glycoprotein Ib accelerates the hydrolysis of Par-1 on intact platelets. J.Biol.Chem. 2001;276:4692-4698.
72. Weeterings C, Adelmeijer J, Myles T, de Groot PG, Lisman T. Glycoprotein Ibalpha-mediated platelet adhesion and aggregation to immobilized thrombin under conditions of flow. Arterioscler.Thromb.Vasc.Biol. 2006;26:670-675.
73. Adam F, Guillin MC, Jandrot-Perrus M. Glycoprotein Ib-mediated platelet activation. A signalling pathway triggered by thrombin. Eur.J.Biochem. 2003;270:2959-2970.
74. White TC, Berny MA, Tucker EI et al. Protein C supports platelet binding and activation under flow: role of glycoprotein Ib and apolipoprotein E receptor 2. J.Thromb.Haemost. 2008;6:995-1002.
75. Mosnier LO, Zlokovic BV, Griffin JH. The cytoprotective protein C pathway. Blood 2007;109:3161-3172.
76. Vega-Ostertag M, Casper K, Swerlick R et al. Involvement of p38 MAPK in the up-regulation of tissue factor on endothelial cells by antiphospholipid antibodies. Arthritis Rheum. 2005;52:1545-1554.
77. Pierangeli SS, Colden-Stanfield M, Liu X et al. Antiphospholipid antibodies from antiphospholipid syndrome patients activate endothelial cells in vitro and in vivo. Circulation 1999;99:1997-2002.
78. Meroni PL, Raschi E, Testoni C et al. Statins prevent endothelial cell activation induced by antiphospholipid (anti-beta2-glycoprotein I) antibodies: effect on the proadhesive and proinflammatory phenotype. Arthritis Rheum. 2001;44:2870-2878.
79. Ambrose JA, Barua RS. The pathophysiology of cigarette smoking and cardiovascular disease: an update. J.Am.Coll.Cardiol. 2004;43:1731-1737.
80. Reinders JH, Brinkman HJ, van Mourik JA, de Groot PG. Cigarette smoke impairs endothelial cell prostacyclin production. Arteriosclerosis 1986;6:15-23.
81. Zhang J, McCrae KR. Annexin A2 mediates endothelial cell activation by antiphospholipid/anti-beta2 glycoprotein I antibodies. Blood 2005;105:1964-1969.
82. Sacre SM, Stannard AK, Owen JS. Apolipoprotein E (apoE) isoforms differentially induce nitric oxide production in endothelial cells. FEBS Lett. 2003;540:181-187.
83. Bertina RM, Koeleman BP, Koster T et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 1994;369:64-67.
84. Vandenbroucke JP, Koster T, Briet E et al. Increased risk of venous thrombosis in oral-contraceptive users who are carriers of factor V Leiden mutation. Lancet 1994;344:1453-1457.
85. Juul K, Tybjaerg-Hansen A, Steffensen R et al. Factor V Leiden: The Copenhagen City Heart Study and 2 meta-analyses. Blood 2002;100:3-10.
86. Galli M, Ruggeri L, Barbui T. Differential effects of anti-beta2-glycoprotein I and antiprothrombin antibodies on the anticoagulant activity of activated protein C. Blood 1998;91:1999-2004.
87. Martinuzzo M, Forastiero R, Adamczuk Y, Cerrato G, Carreras LO. Activated protein C resistance in patients with anti-beta 2 glycoprotein I antibodies. Blood Coagul.Fibrinolysis 1996;7:702-704.
88. Liestol S, Sandset PM, Mowinckel MC, Wisloff F. Activated protein C resistance determined with a thrombin generation-based test is associated with thrombotic events in patients with lupus anticoagulants. J.Thromb.Haemost. 2007;5:2204-2210.
89. Rosing J, Middeldorp S, Curvers J et al. Low-dose oral contraceptives and acquired resistance to activated protein C: a randomised cross-over study. Lancet 1999;354:2036-2040.
90. Wester J, Sixma JJ, Geuze JJ, van der Veen J. Morphology of the early hemostasis in human skin wounds: influence of acetylsalicylic acid. Lab Invest 1978;39:298-311.
91. Wester J, Sixma JJ, Geuze JJ, Heijnen HF. Morphology of the hemostatic plug in human skin wounds: transformation of the plug. Lab Invest 1979;41:182-192.
92. Nieuwenhuis HK, Akkerman JW, Sixma JJ. Patients with a prolonged bleeding time and normal aggregation tests may have storage pool deficiency: studies on one hundred six patients. Blood 1987;70:620-623.
93. Sixma JJ, Sakariassen KS, Beeser-Visser NH, Ottenhof-Rovers M, Bolhuis PA. Adhesion of platelets to human artery subendothelium: effect of factor VIII-von Willebrand factor of various multimeric composition. Blood 1984;63:128-139.
94. Sixma JJ, van den Berg A. The haemostatic plug in haemophilia A: a morphological study of haemostatic plug formation in bleeding time skin wounds of patients with severe haemophilia A. Br.J.Haematol. 1984;58:741-753.
95. Del Papa N, Guidali L, Sala A et al. Endothelial cells as target for antiphospholipid antibodies.

Human polyclonal and monoclonal anti-beta 2-glycoprotein I antibodies react in vitro with endothelial cells through adherent beta 2-glycoprotein I and induce endothelial activation. Arthritis Rheum. 1997;40:551-561.

96. Freson K, Hoylaerts MF, Jaeken J et al. Genetic variation of the extra-large stimulatory G protein alpha-subunit leads to Gs hyperfunction in platelets and is a risk factor for bleeding. Thromb. Haemost. 2001;86:733-738.

97. Hulstein JJ, Lenting PJ, de Laat B et al. beta2-Glycoprotein I inhibits von Willebrand factor dependent platelet adhesion and aggregation. Blood 2007;110:1483-1491.

98. Maxwell MJ, Westein E, Nesbitt WS et al. Identification of a 2-stage platelet aggregation process mediating shear-dependent thrombus formation. Blood 2007;109:566-576.

99. Erkan D, Harrison MJ, Levy R et al. Aspirin for primary thrombosis prevention in the antiphospholipid syndrome: a randomized, double-blind, placebo-controlled trial in asymptomatic antiphospholipid antibody-positive individuals. Arthritis Rheum. 2007;56:2382-2391.

100. Albers GW, Amarenco P, Easton JD, Sacco RL, Teal P. Antithrombotic and thrombolytic therapy for ischemic stroke: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest 2008;133:630S-669S.

101. Becker RC, Meade TW, Berger PB et al. The primary and secondary prevention of coronary artery disease: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest 2008;133:776S-814S.

102. Khamashta MA, Cuadrado MJ, Mujic F et al. The management of thrombosis in the antiphospholipid-antibody syndrome. N.Engl.J.Med. 1995;332:993-997.

103. Derksen RH, de Groot PG, Kappelle LJ. Low dose aspirin after ischemic stroke associated with antiphospholipid syndrome. Neurology 2003;61:111-114.

104. Levine SR, Brey RL, Tilley BC et al. Antiphospholipid antibodies and subsequent thrombo-occlusive events in patients with ischemic stroke. JAMA 2004;291:576-584.

105. Beffert U, Durudas A, Weeber EJ et al. Functional dissection of Reelin signaling by site-directed disruption of Disabled-1 adaptor binding to apolipoprotein E receptor 2: distinct roles in development and synaptic plasticity. J.Neurosci. 2006;26:2041-2052.

106. Weeber EJ, Beffert U, Jones C et al. Reelin and ApoE receptors cooperate to enhance hippocampal synaptic plasticity and learning. J.Biol.Chem. 2002;277:39944-39952.

107. Trommsdorff M, Gotthardt M, Hiesberger T et al. Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. Cell 1999;97:689-701.

9

Nederlandse samenvatting

Inleiding

Hemostase

Hemostase (bloedstelping) is het mechanisme dat voorkomt dat een organisme leegbloedt op het moment dat de continuïteit van een bloedvat doorbroken wordt, de "stolling" van bloed. Tevens zorgt dit mechanisme ervoor dat het bloed vloeibaar blijft door stolling tegen te gaan en gevormde stolsels op te ruimen, zodat voedings- en afvalstoffen vervoerd kunnen blijven worden door het lichaam. De stolling en de processen die stolling tegengaan bevinden zich in een delicaat evenwicht dat voortdurend bijgesteld wordt om doorstroming van de bloedvaten te garanderen.

Een aantal bloedbestanddelen zijn betrokken bij de bloedstelping, waaronder de kernloze celfragmenten die bloedplaatjes worden genoemd, de in het bloedplasma aanwezige stolfactoren en de cellen die de vaatwand bekleden (endotheel). Wanneer de balans van het hemostatisch systeem doorslaat naar de kant van de stolling spreekt men van trombose. Als het doorslaat naar de andere kant spreekt men van een bloedingsneiging.

Bloedplaatjes spelen de hoofdrol bij de bloedstelping in slagaders. In slagaders is de stroomsnelheid van het bloed zo hoog dat stolling moeilijk plaats kan vinden; de gevormde stollingsfactoren worden door de hoge stroomsnelheid van het bloed weggespoeld. Bloedplaatjes zijn wel in staat onder hoge snelheid vast te hechten aan de beschadigde vaatwand. Deze celfragmenten hebben een eiwit op het celoppervlak (glycoproteïne Ibα) dat ze in staat stelt een interactie aan te gaan met de beschadigde vaatwand. Denk hierbij aan een straaljager die op een vliegdekschip moet landen. Hierbij maakt de piloot gebruik van een haak aan de onderkant van het vliegtuig en een kabel die over het dek van het vliegtuig gespannen is. Glycoproteïne Ibα fungeert als de haak onder het vliegtuig (het bloedplaatje), terwijl de beschadigde vaatwand de rol van de kabel op het schip uitoefent.

De aan de vaatwand gehechte bloedplaatjes vormen een prop die de beschadiging volledig bedekt. Rondom deze plaatjesprop is de stromingssnelheid van het bloed een stuk lager, waardoor er wel stolling op kan treden. Hierdoor wordt de plaatjesprop op zijn plek verankerd en kan de beschadigde vaatwand genezen. In het geval van trombose in een slagader is de vorming van de plaatjesprop excessief en leidt deze tot de afsluiting van het gehele bloedvat, niet alleen tot het bedekken van de beschadigde vaatwand.

Het anti-fosfolipiden syndroom

Het afweersysteem van het lichaam is erop gericht om lichaamsvreemde stoffen of objecten die het lichaam zijn binnengedrongen onschadelijk te maken. Denk hierbij aan virussen of bacteriën. Het eigen lichaam wordt doorgaans niet aangevallen door het afweersysteem. Als dat wel gebeurt spreekt men van een auto-immuunziekte. Het anti-fosfolipiden syndroom is een auto-immuunziekte die vooral voor komt bij jonge vrouwen (slechts 1 op de 5 patienten is man). De klinische verschijnselen van het anti-

fosfolipiden syndroom zijn trombose en zwangerschapsproblematiek zoals herhaalde miskramen, foetale sterfte en pre-eclampsie. Het syndroom wordt verder gekenmerkt door de persistente aanwezigheid van antistoffen in het bloed die gericht zijn tegen lichaamseigen eiwitten uit het bloedplasma. De eiwitten waartegen anti-fosfolipide antistoffen gericht zijn hebben als gemeenschappelijke deler dat ze kunnen binden aan negatief geladen fosfolipiden (de belangrijkste bouwstenen van de celmembraan).

Anti-fosfolipide antistoffen herkennen vele verschillende eiwitten. Naar algemeen wordt aangenomen is β_2-glycoproteïne I het eiwit waartegen de pathologische "anti-fosfolipide" antistoffen gericht zijn. De functie van dit plasma-eiwit is tot op heden onbekend.

De diagnose "anti-fosfolipiden syndroom" wordt gesteld bij patiënten met trombose of zwangerschapsproblematiek waarbij ten minste twee maal anti-fosfolipide antistoffen zijn aangetoond in het bloed. Deze metingen dienen met een interval van ten minste 12 weken te zijn uitgevoerd. Anti-fosfolipide antistoffen kunnen op een aantal verschillende manieren in een diagnostisch ziekenhuislaboratorium worden aangetoond: (1) Een test die gebaseerd is op de detectie van antistoffen tegen β_2-glycoproteïne I dat gebonden is aan een negatief geladen fosfolipide genaamd cardiolipine, de anticardiolipine test, of (2) een test die antistoffen tegen β_2-glycoproteïne direct meet, de zogenaamde anti-β_2-glycoproteïne I test. Ook kan een stoltest gedaan worden om de zogenaamde "lupus anticoagulans" antistoffen (3) op te sporen. Met een stoltest wordt het tijdsbestek waarbinnen stolling optreedt in een bloedmonster gemeten. De lupus anticoagulans antistoffen veroorzaken een verlengde stoltijd onder laboratoriumcondities. Een vierde mogelijkheid is de detectie van antistoffen tegen het eiwit protrombine, de antiprotrombine test, hoewel deze test geen deel uitmaakt van de officiële inclusiecriteria van het anti-fosfolipiden syndroom.

Bevindingen

Een verhoogd risico op een herseninfarct of een hartinfarct

Bij het stellen van de diagnose "anti-fosfolipiden syndroom" gaat men uit van een causaal verband tussen de gedetecteerde antistoffen en het optreden van trombose. Hoewel er duidelijke experimentele aanwijzingen zijn dat anti-fosfolipide antistoffen in staat zijn een protrombotische staat te induceren, is de toename van het risico op trombose bij individuen met anti-fosfolipide antistoffen vergeleken met mensen zonder deze antistoffen niet duidelijk. De meeste gegevens over deze risicotoename zijn verkregen door anti-fosfolipide antistofprofielen te vergelijken in groepen mensen met een andere auto-immuunziekte, zoals systemische lupus erythematosus. Bij deze ziekte komen anti-fosfolipide antistoffen significant vaker voor dan in de algemene bevolking. Ook is er sprake van een basaal verhoogd risico op trombose, onafhankelijk van de aanwezigheid van anti-fosfolipide antistoffen. Hierdoor zijn de resultaten van deze studies niet zonder meer te extrapoleren naar de algemene bevolking.

Om meer inzicht in te verkrijgen in het risico op trombose in de aanwezigheid van anti-fosfolipide antistoffen is het anti-fosfolipide antistofprofiel bepaald in twee populatiestudies: een studie onder vrouwen jonger dan 50 jaar, waarbij vrouwen met een hartinfarct of een herseninfarct zijn vergeleken met gezonde vrouwen (hoofdstuk 4), en een studie onder mannen, waarbij mannen met een hartinfarct zijn vergeleken met mannen zonder hartinfarct (hoofdstuk 5).

De studie onder vrouwen liet zien dat alleen de aanwezigheid van anti-β_2-glycoproteïne I antistoffen of het lupus anticoagulans geassocieerd was met een verhoogd risico op een herseninfarct of een hartinfarct. Anti-β_2-glycoproteïne I antistoffen waren geassocieerd met een 2 maal zo hoog risico op een herseninfarct, niet met een verhoogd risico op een hartinfarct. De aanwezigheid van het lupus anticoagulans was sterk geassocieerd met een verhoogd risico op zowel een hartinfarct (5 maal zo hoog risico als vrouwen zonder lupus anticoagulans) als een herseninfarct (43 maal zo hoog risico als vrouwen zonder lupus anticoagulans). Dit kon echter niet bevestigd worden in de studie onder mannen (hoofdstuk 5). Bij mannen was de aanwezigheid van lupus anticoagulans niet geassocieerd met een verhoogd risico op een hartinfarct. Verder bleek uit de studie onder jonge vrouwen dat het risico op een herseninfarct of een hartinfarct nog verder toenam in vrouwen met lupus anticoagulans die roken of de anticonceptiepil gebruiken. Bij deze vrouwen was het risico op een hartinfarct 31 maal verhoogd als deze vrouwen rookten en 20 maal verhoogd als de anticonceptiepil gebruikt werd. Het risico op een herseninfarct was zelfs 87 maal zo groot in rokende vrouwen met lupus anticoagulans en 201 maal zo groot in vrouwen met lupus anticoagulans die de anticonceptiepil gebruiken.

Hartinfarcten en herseninfarcten zijn zeldzaam op jonge leeftijd, maar de gevolgen van deze aandoeningen zijn ernstig. Hoewel ook het lupus anticoagulans zeldzaam is (in de studie beschreven in hoofdstuk 4 komt het slechts bij 0.6% van de vrouwen voor), kan het ongeveer 20% van alle herseninfarcten bij vrouwen onder de 50 jaar verklaren. Toch wordt er bij vrouwen met lupus anticoagulans niet preventief behandeld om een toekomstig infarct te voorkomen, omdat het nut van deze behandeling niet bewezen is. Wel zou men kunnen stellen dat gezonde vrouwen waarbij het lupus anticoagulans is aangetoond sterk ontraden zouden moeten worden te roken of de anticonceptiepil te gebruiken.

Een mogelijk mechanisme achter bloedplaatjesactivatie in het anti-fosfolipiden syndroom

Onderzoek voorafgaand aan dit proefschrift heeft aangetoond dat anti-fosfolipiden antistoffen bloedplaatjes zodanig kunnen activeren dat de initiële plaatjeshechting aan de beschadigde vaatwand excessief toeneemt. Deze bloedplaatjesactivering wordt veroorzaakt door de interactie van anti-β_2-glycoproteïne I antistoffen met twee eiwitten (ook wel receptoren genoemd) op het oppervlak van het bloedplaatje: ApoER2 en glycoproteïne Ibα. In dit proefschrift wordt beschreven dat de activering van bloedplaatjes afhankelijk is van de interactie van anti-fosfolipiden antistoffen met beide receptoren

tegelijk (hoofdstuk 6). Beide receptoren genereren een intracellulair signaal op het moment dat de antistoffen eraan binden. Onder laboratoriumcondities normaliseert de bloedplaatjeshechting aan de vaatwand op het moment dat de interactie van de antistoffen met één van beide receptoren wordt geremd, ondanks de onverminderde intracellulaire signalen die worden afgegeven door de andere receptor. Dit wijst erop dat beide receptoren even belangrijk zijn bij de activering van bloedplaatjes door antifosfolipiden antistoffen.

De voorafgaande gegevens zijn verkregen door het lichaam na te bootsen in het laboratorium. De belangrijke rol van ApoER2 is verder uitgediept in een diermodel (hoofdstuk 7) om aan te tonen dat anti-fosfolipiden antistoffen hun protrombotische invloed daadwerkelijk via deze receptor uitoefenen. Hierbij is er gekeken naar de protrombotische veranderingen in muizen die werden ingespoten met anti-fosfolipiden antistoffen. Er werden zowel gewone muizen, als muizen die de ApoER2-receptor missen gebruikt. Uit deze experimenten bleek dat ApoER2 inderdaad verantwoordelijk is voor de trombotische manifestaties van het anti-fosfolipid syndroom, aangezien de protrombotische veranderingen die optraden in normale muizen na inspuiting van anti-fosfolipiden antistoffen uitbleven in muizen zonder ApoER2.

Een paradoxale waarneming

Hoewel lupus anticoagulans het meest geassocieerd is met een verhoogd risico op trombose in slagaders, is de exacte werking van deze antistoffen tot op heden onduidelijk. Eerder onderzoek en het onderzoek beschreven in dit proefschrift laat zien dat lupus anticoagulans endotheel en bloedplaatjes kan activeren, en dat lupus anticoagulans-antistoffen in een diermodel trombose veroorzaken. Desalniettemin zijn er paradoxale waarnemingen gedaan in patiënten met lupus anticoagulans (hoofdstuk 3). Deze patiënten hebben naast een verlengde stoltijd, ook een afwijking in een diagnostische test van bloedplaatjesfunctie; ze hebben een verlengde bloedingstijd. In een groep patiënten met lupus anticoagulans is er gezocht naar een oorzaak voor de gevonden toename in bloedingstijd. Er konden geen afwijkingen in bloedplaatjesfunctie gevonden worden en er konden dan ook geen uitspraken gedaan worden over de mogelijke oorzaak.

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Dankwoord

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Tsja, als je zo lang op een afdeling zit is het logisch dat je de meesten weer ziet vertrekken. Sommigen kunnen echter iets moeilijker afscheid nemen. Suus, de onbetwiste bloedplaatjessignaleringsexpert van ons lab, we hebben samen veel gelachen, maar ook goede gesprekken gehad. Bedankt voor de vriendschap. Leuk dat je nog iedere week komt buurten. Lisman, je weet het gesprek aan tafel altijd richting de belangrijke dingen des levens te sturen, waarvoor dank :-). Voor goed advies en een goed gesprek kan ik

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Toen ik begon moest iedere AIO minstens drie jaar wachten op een plek in de schrijfkamer. Inmiddels is de wachttijd verkort tot maximaal een half jaar. Een ruimte van 20 m^2 waarin zich 7 AIO-schrijfplekken en 4 computerflexplekken bevinden, dat is nog het best te vergelijken met een kippenhok. Vooral omdat iedereen er altijd komt buurten als er een bakkie gedronken dient te worden en het er daarom nooit stil is als je geconcentreerd probeert te werken. Toch heb ik me daar prima vermaakt en mis ik de aanspraak. Soms. Een beetje. Anja, op het lab zit je achter me, hoewel we daar beiden niet vaak meer te vinden zijn. Jammer, misschien moeten we weer eens meezingen met Queens of the Stone Age. Succes met je laatste jaar! Cees, “de toekomst”, de man van de voetbalpool, de F1-manager, het onder werktijd zelf gebrouwen bier en “het Ceesje” als je de avond ervoor weer eens te veel hebt ingenomen. Succes met de afronding! Coen, officieuze ADHD-er, Boschenaar in hart en nieren, man met een innemend karakter. Volgens mij gaan we heel wat leuke proeven bedenken op onze nieuwe postdockamer. Dianne, de opvolgster van de illustere Bahram. Hoewel je in bijna niets met hem te vergelijken bent, verzamel ook jij gigantische stapels papier om je heen. Zal wel bij je project horen ;-). Van jou heb ik geleerd hoe je worst te drogen moet hangen. Dank voor je onderkoelde humor! Dokter Erik, je hebt als arts (een klein beetje) je best moeten doen om door ons, “hardcore biochemici”, geaccepteerd te worden als serieuze wetenschapper, maar je hoort er nu helemaal bij. Gelukkig zijn er nog artsen te vinden met interesse in de meer basale wetenschap. Nu nog leren luisteren naar “alles moet dood”-muziek. Succes met je promotie. Evelyn, inmiddels lijk je de laatste VWF-AIO van onze afdeling te zijn. Veel succes met de laatste loodjes. Vivian, I’m amazed at how well you fit in. Now that you’ve gained a writing space in the “AIO-kamer”, I hope you don’t find the others too distracting. Good luck! Mirjam, de Leidse tak van ons Utregse lab. Je zit dan wel niet op de AIO kamer, maar ik noem je naam hier toch maar even. Op de een of andere manier werk je altijd in Utrecht op het moment dat er een borrel is georganiseerd. Toeval?

Jan-Willem, dank voor je interesse in mijn werk en je nuttige commentaar tijdens werkbesprekingen. Harry, jouw vaardigheden met de microscoop zijn ongeëvenaard op onze afdeling. Tuna, jouw kritische blik houdt iedereen scherp. Peter en Martijn, ik heb veel van jullie geleerd. Joukje, waar zou ik toch zijn zonder jou? Bedankt voor al het werk dat je voor me hebt gedaan en nog immer doet.

Nog altijd is mijn werkplek op lab 2, al ben ik als enige overgebleven van de oorspronkelijke (lees: die van 6,5 jaar geleden) bezetting. Wessie, koning van de Biabore, je bent inmiddels al weer bijna gepromoveerd Down Under. Jelk Ademier, de menselijke pipetteerrobot, met Ton mee vertrokken naar Groningen. Het was een mooie tijd op het lab. Altijd tijd voor practical jokes. Lucy, jammer dat je slechts zo kort bent gebleven. De bakkies in de ochtend waren altijd gezellig. Remo, vrolijke sloddervos, altijd al geweten

dat je eigenlijk een onzettende computernerd bent. Succes in de game-industrie. Ome Cor, als enige van CB blijven plakken. Eszter, crazy Hungarian woman, who would have thought you'd stay in the thrombosis and haemostasis field? Ronan, the French-connection. Even though you ended your Dutch career in lab 1, I still think of you as belonging in lab 2. The day you came to our lab, you could barely speak English. The day you left, you forgot how to speak French, could not speak Dutch and spoke English the Dutch way. Boy, do you know how to party (I know only one who's better at it, and he's Irish). Glad to be working in France again?

Tinus, heer en meester van de perfusiehoek, manusje van alles, regelneef en toeziend analist van onze afdeling. Bedankt voor alles dat je voor me gedaan hebt. Chantal, Janine en Carina, het was altijd gezellig in de theehoek op lab 1. Vivianne en Silvie, gelukkig hebben jullie de HUVEC kweek overgenomen. Miranda, veel succes als klinisch chemicus.

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List of publications
&
Curriculum Vitae

List of publications

Urbanus R, Derksen R, de Groot P. Platelets and the antiphospholipid syndrome. Lupus 2008;17(10):888-94.

White TC, Berny MA, Tucker EI, Urbanus RT, de Groot PG, Fernandez JA, et al. Protein C supports platelet binding and activation under flow: role of glycoprotein Ib and apolipoprotein E receptor 2. J Thromb Haemost 2008 Jun;6(6):995-1002.

Urbanus RT, Pennings MT, Derksen RH, de Groot PG. Platelet activation by dimeric beta2-glycoprotein I requires signaling via both glycoprotein Ibalpha and apolipoprotein E receptor 2'. J Thromb Haemost 2008 Aug;6(8):1405-12.

Urbanus RT, Derksen RH, de Groot PG. Current insight into diagnostics and pathophysiology of the antiphospolipid syndrome. Blood Rev 2008 Mar;22(2):93-105.

Pennings MT, Derksen RH, Urbanus RT, Tekelenburg WL, Hemrika W, de Groot PG. Platelets express three different splice variants of ApoER2 that are all involved in signaling. J Thromb Haemost 2007 Jul;5(7):1538-44.

Pennings MT, Derksen RH, van Lummel M, Adelmeijer J, VanHoorelbeke K, Urbanus RT, et al. Platelet adhesion to dimeric beta-glycoprotein I under conditions of flow is mediated by at least two receptors: glycoprotein Ibalpha and apolipoprotein E receptor 2'. J Thromb Haemost 2007 Feb;5(2):369-77.

Pennings MT, van Lummel M, Derksen RH, Urbanus RT, Romijn RA, Lenting PJ, et al. Interaction of beta2-glycoprotein I with members of the low density lipoprotein receptor family. J Thromb Haemost 2006 Aug;4(8):1680-90.

van Lummel M, Pennings MT, Derksen RH, Urbanus RT, Lutters BC, Kaldenhoven N, et al. The binding site in {beta}2-glycoprotein I for ApoER2' on platelets is located in domain V. J Biol Chem 2005 Nov 4;280(44):36729-36.

Ferreira IA, Mocking AI, Urbanus RT, Varlack S, Wnuk M, Akkerman JW. Glucose uptake via glucose transporter 3 by human platelets is regulated by protein kinase B. J Biol Chem 2005 Sep 23;280(38):32625-33.

Urbanus RT, de Laat HB, de Groot PG, Derksen RH. Prolonged bleeding time and lupus anticoagulant: a second paradox in the antiphospholipid syndrome. Arthritis Rheum 2004 Nov;50(11):3605-9.

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de Laat B, Derksen RH, Urbanus RT, de Groot PG. IgG antibodies that recognize epitope Gly40-Arg43 in domain I of beta 2-glycoprotein I cause LAC, and their presence correlates strongly with thrombosis. Blood 2005 Feb 15;105(4):1540-5.

de Groot PG, van Lummel M, Pennings M, Urbanus R, de Laat B, Lenting PJ, et al. Beta2-glycoprotein I and LDL-receptor family members. Thromb Res 2004;114(5-6):455-9.

de Laat HB, Derksen RH, Urbanus RT, Roest M, de Groot PG. beta2-glycoprotein I-dependent lupus anticoagulant highly correlates with thrombosis in the antiphospholipid syndrome. Blood 2004 Dec 1;104(12):3598-602.

Fijnheer R, Urbanus RT, Nieuwenhuis HK. [Withdrawing the use of acetylsalicyclic acid prior to an operation usually not necessary]. Ned Tijdschr Geneeskd 2003 Jan 4;147(1):21-5.

Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 16 november 1978 te Amersfoort. Na het afronden van het VWO aan het Meridiaan College, vestiging het Nieuwe Eemland College, te Amersfoort, is hij in 1997 begonnen met de studie Biologie aan de Universiteit Utrecht. Na een jaar verwisselde hij de studie Biologie voor de studie Geneeskunde aan dezelfde universiteit. In 2002 liep hij zijn wetenschappelijke stage bij de afdeling Haematologie in het Universitair Medisch Centrum Utrecht onder begeleiding van prof.dr. Ph.G. de Groot en dr. R.H.W.M. Derksen. In hetzelfde jaar behaalde hij zijn doctoraaldiploma geneeskunde, waarna hij op deze afdeling bleef werken als research-analist. In februari 2004 begon hij aldaar aan het onderzoek dat heeft geleid tot dit proefschrift, wederom onder begeleiding van prof.dr. Ph.G. de Groot en dr. R.H.W.M. Derksen. Sinds juli 2008 is hij werkzaam als Postdoc bij de afdeling Klinische Chemie en Haematologie van het Universitair Medisch Centrum Utrecht.