Evaluation and optimization of laboratory criteria for Antiphospholipid Syndrome Diagnosis

Evaluation and Optimization of Laboratory Criteria for Antiphospholipid Syndrome Diagnosis

Dongmei Yin

The research in this thesis was supported by unrestricted financial support from Synapse Research Institute and by grants from the China Scholarship Council (CSC).

Financial support for printing this thesis was kindly provided by Synapse Research Institute. It is gratefully acknowledged.

Cover design: Dongmei Yin

Layout: Dongmei Yin

Printing: ProefschriftMaken-www.proefschriftmaken.nl

ISBN: 978-94-6423-455-8

Evaluation and Optimization of Laboratory Criteria for Antiphospholipid Syndrome Diagnosis

Dissertation

To obtain the degree of Doctor at the Maastricht University,

on the authority of the Rector Magnificus, Prof.dr. Rianne M. Letschert

in accordance with the decision of the Board of Deans,

to be defended in public on

Wednesday 27th October 2021, at 10:00 hours

By

Dongmei Yin

Born on December 24th,1982

In Suizhou, China

Promotors:

Prof. dr. H. ten Cate

Prof. dr. Katrien M.J. Devreese (Ghent University Hospital, Ghent University, Ghent, Belgium)

Co-promotor:

Dr. B. de Laat

Assessment Committee:

Prof. Dr. Y.M.C. Henskens (Chair)

Prof. Dr. J.W.M. Heemskerk

Prof. Dr. J.C.M. Meijers (University of Amsterdam)

Dr. C. Winckers

Dr. R. T. Urbanus (University Medical Center Utrecht)

Contents

Chapter 1

General introduction

Antiphospholipid syndrome

The antiphospholipid syndrome (APS) is a rare systemic autoimmune disease, that affects approximately 0.05% of the general population [1]. APS contributes to 6.1% of all pregnancy failures, and less than 1% of thrombosis cases [1]. However, APS is the most common cause of acquired hypercoagulability for people under the age of 50 years, accounting for 20% of all thrombotic complications [2]. APS is diagnosed when a patient suffers from thrombosis or pregnancy morbidity and has persistent circulating antiphospholipid antibodies (aPLs) (**Table 1**) [3]. Vascular thrombosis and pregnancy-related morbidity frequently occur in the general population irrespective of APS. As patients with APS often need a more intense anticoagulant therapy, a heavy burden rests on the assays used to detect the presence of circulating aPLs.

The current revised laboratory criteria for APS classification requires a combination of different laboratory tests to detect aPLs. These laboratory criteria include one functional coagulation assay known as lupus anticoagulant (LAC), and two immunological assays measuring immunoglobulin (Ig) G and/or IgM with either cardiolipin (antiCL) or beta2-glycoprotein I as antigen (antiβ2GPI). To avoid false-positive tests related to infections, positive tests should be repeated with an interval of at least 12 weeks [3].

Table 1. Revised classification criteria for the antiphospholipid syndrome. Classification of APS needs to meet at least of one of the clinical criteria and one of the laboratory criteria. Modified from Miyakis S 2006 [3].

Clinical criteria
1. Vascular thrombosis:
One or more clinical episodes of arterial, venous, or small vessel thrombosis, in any tissue or organ.
2. Pregnancy morbidity:
a. One or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation, with normal fetal morphology, or
b. One or more premature births of a morphologically normal neonate before the 34th week of gestation because of: (i) eclampsia or severe preeclampsia 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 (LAC) 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 (ISTH-SSC) on LAs/phospholipid-dependent antibodies
2. Anticardiolipin antibody (antiCL) 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
3. Anti-β2glycoprotein-I antibody (antiβ2GPI) 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.

Antiphospholipid antibodies

Although the exact pathogenesis of APS is unknown, the relevant aPLs antibodies are not directed against phospholipids, but against phospholipid-binding proteins, e.g. β2GPI, which have an affinity for anionic phospholipids [4]. Autoantibodies against many different plasma proteins have been described [4]. β2GPI has proven to be a major antigen target for aPLs [5-7].

Antiβ2GPI antibodies

β2GPI consists of four similar complement control protein (CCP)-like domains (DI, DII, DIII, DIV) and one domain (DV) with extensions of a CCP like domain. DI-DIV have evolutionary conserved sequences and DV harbors a large positively charged lysine patch that has affinity for anionic phospholipids. The phospholipid-binding site is located at the bottom of DV and consists of a hydrophobic loop. β2GPI has two different conformation: a native closed (circular) conformation and an open (fish hook shaped) conformation [8]. It has been hypothesized that both the N-terminal domain I (DI) and the C-terminal domain V (DV) are partly hidden in the native circular β2GPI. When the lysine loop on DV interacts with anionic phospholipid or other negatively charged molecules on cell surfaces [9-11], β2GPI undergoes a conformational change. Consequently, the DI-IV stretches resulting in a more open fish hook shape, thereby exposing a dominant epitope in the glycine40-arginine43 (G40-R43) region of DI and allowing the binding of antiDI of β2GPI antibodies [12-17] (**illustrated in Figure 1**).

It is not immediately clear how antibodies against β2GPI could induce the clinical manifestations of APS. **Figure 2** illustrates the possible mechanisms behind the clinical manifestations of APS by autoantibodies against β2GP. In plasma, β2GP is present as a circular protein in which DI interacts with DV. After a small injury, cells express phospholipids on their surface (the so-called second hit theory). Closed β2GPI will bind to the anionic membrane. On binding to anionic surfaces, the protein patches and changes into a stretched structure, thereby exposing a cryptic epitope on DI for the autoantibodies. The antibodies will bind to and stabilize β2GPI in its stretched conformation. Binding of aPLs to this epitope on β2GPI may subsequently target different cell types (endothelial cells, monocytes, platelets) by several surface receptors thereby activating these cells. Tissue factor expression will be one of the consequences of these activations, and complement activation is also involved in the induction of the clinical manifestation of APS [18,19].

In vivo models demonstrated the pathogenicity of antibodies directed against DI of β2GPI [20-23]. The subpopulation of aPLs that recognizes an epitope on DI of β2GPI comprising at least G40-R43 have been proved to be pathogenic and displays a significant correlation with clinical manifestations [16,24,25]. Antibodies against other domains of β2GPI appear to be unrelated to the clinical symptoms of APS [26-29].

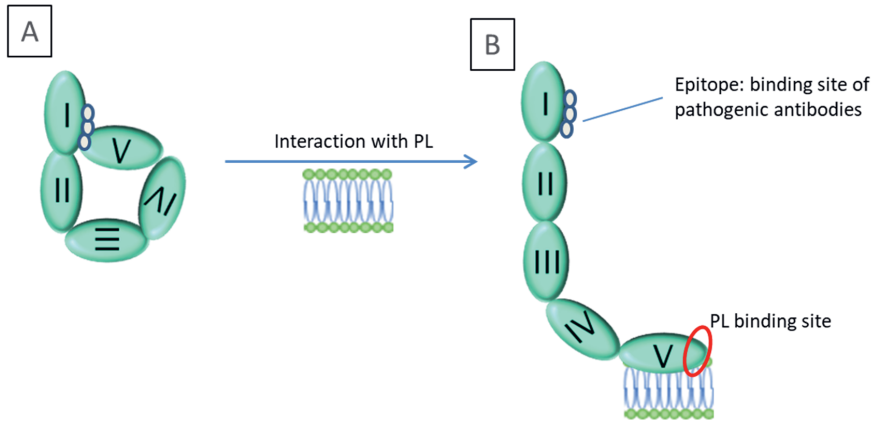

Figure 1. Open structure and closed structure of β2GPI. Domain I-V represent the five domains of β2GPI. (A) β2GPI circulates in the closed circular structure. DI and DV are partly hidden in the native circular β2GPI. (B) The open fish hook form of β2GPI. In this conformation, a cryptic epitope on DI is exposed and allows binding of antiDI of β2GPI antibodies. It is proposed that β2GPI binding to anionic phospholipid, through its phospholipid binding site on DV, results in conformational changes.

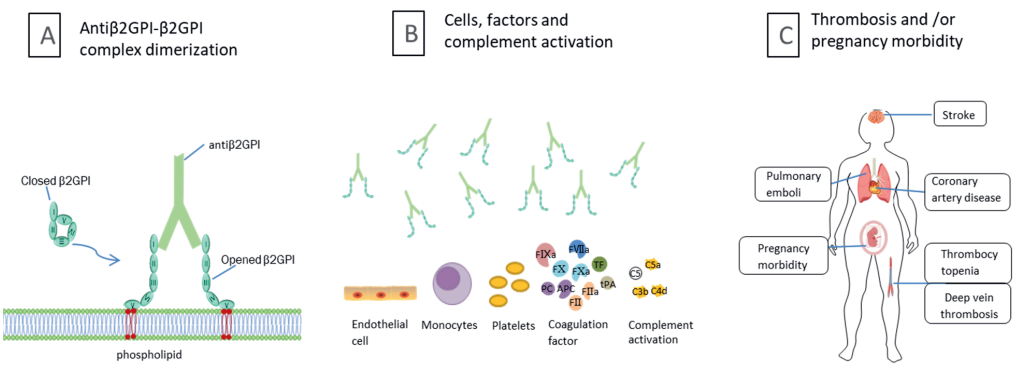

Figure 2. Schematic diagram of the pathogenic mechanisms of autoantibodies against β2GP. (A) Antiβ2GPI-β2GPI complex dimerization. By binding to anionic phospholipid membrane (marked as red color), closed β2GPI will change into a stretched structure thereby exposing a cryptic epitope on domain I for autoantibodies to bind. One antibody can bind to two molecules and stabilizes β2GPI to form a dimer complex. (B) Cells, factors and complement activation. The antiβ2GPI-β2GPI dimer complex may subsequently target surface receptors of different cell types (endothelial cells, monocytes, platelets) resulting in activating of these cells. Activation via soluble coagulation-related factors and complement factors activation will be also involved in these mechanisms. (C) The clinical manifestations of APS. Antibodies initiate clinical events via interactions of these pathways. The clinical manifestations of APS include thrombosis which can occur in any vein, artery, or microcirculation and/or well-defined obstetrical manifestations.

Other autoantibodies than antiβ2GPI

Prothrombin is one of the major coagulation factors in the circulation, and it can be converted into thrombin during coagulation. Antibodies directly against prothrombin (PT) enhance clearance of prothrombin from the circulation, can decrease levels of prothrombin and thereby cause bleeding. Strangely enough, anti-prothrombin antibodies (antiPT) have been described to correlate with thrombosis [30]. Autoantibodies against a phosphatidylserine/prothrombin (PS/PT) complex differ from autoantibodies against prothrombin. Autoantibodies against PS/PT recognize prothrombin only when it is bound to anionic phospholipid, suggesting that prothrombin undergoes a conformational change when it is bound to PS, exposing a cryptic epitope. Antibodies directed against this cryptic epitope correlate better with thrombosis than antibodies against the rest of the prothrombin molecule. Autoantibodies against PS/PT showed a significant correlation with thrombosis in APS in a systematic review [31].

In addition, other than β2GPI and prothrombin, autoantibodies to a number of other proteins, such as protein S, protein C, tissue factor pathway inhibitor, factor X, XI and XII, Factor H, Annexin A5, Annexin A2, or antibodies to phospholipid antigens, including antibodies directed against phosphatidic acid (PA), phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidyl ethanolamine (PE), cardiolipin (CL) are found in a small subgroup of patients with APS [32]. Some correlate with clinical manifestations (level of evidence is even low), and mechanisms regarding how these autoantibodies might induce a prothrombotic state have been proposed, however, most of the studies do not support an association between these antibodies and thrombosis or pregnancy morbidity. There is no convincing evidence that they play a role in thrombosis or pregnancy complications in APS.

Detection of antiphospholipid antibodies

Lupus anticoagulant (LAC)

LAC can be caused by a heterogeneous subset of inhibitors. The antibody is in most cases directed against β2GPI and prothrombin, but a positive LAC can also be found in the absence of these antibodies. Although the current guidelines for LAC detection have proved to be very helpful for a better standardization of this assay, LAC detection still has a number of unresolved issues. The heterogeneity of LAC antibodies leads to differences in detection performance. The high inter-laboratory variability also results from multiple handling procedures between collection of the blood and the actual LAC testing [33] and a wide variation in phospholipid concentration and composition in the commercial clotting regents [34]. Residual platelet material (platelet-derived phospholipids) can shorten the clotting times thereby generating false-negative results [35]. Carrying out LAC tests during anticoagulation therapy increases the risk of false positive or negative results [36]. Moreover, there are still a number of uncertainties in the interpretation of a weak LAC [37].

LAC is a clotting time-based assay used to detect aPLs antibodies. When testing for LAC, it is important to realize that coagulation factor deficiencies should be excluded and that a lupus anticoagulant should be phospholipid dependent. Lupus anticoagulant should be tested applying

three steps: screen, mix and confirm [38,39]. In the screening step, the presence of aPLs is demonstrated by the use of sensitive reagents containing a low concentration of phospholipids. In the mixing step, the presence of an inhibitor (antibodies) is demonstrated and a deficiency of the coagulation factors can be excluded by mixing the patient plasma with normal pooled plasma in a 1:1 proportion. In the confirmation step, the phospholipid dependent character of the inhibitor/antibodies is illustrated by the attenuation of the prolonged clotting time due to increasing the concentration of phospholipid used.

Several coagulation assays have been described to measure LAC. **Figure 3** shows a simplified schematic diagram of the coagulation pathways evaluated by each coagulation test used to detect LAC activity. The activated partial-thromboplastin time (APTT), colloidal-silica clotting time (CSCT) and kaolin clotting time (KCT) assays detect coagulation defects in the intrinsic coagulation pathway via contact activation on glass, silica or kaolin, in the presence of high-molecular-weight kininogen and prekallikrein. The dilute prothrombin time (dPT) assay detects coagulation through the extrinsic coagulation pathway by adding tissue factor (TF) and subsequently the formation of a complex between TF and factor (f) VIIa. Both the intrinsic and extrinsic pathways result in the conversion of FX (i.e. FXa). Finally, both intrinsic and extrinsic pathways converge in a final common pathway, the activation of prothrombin to thrombin followed by the conversion of fibrinogen to fibrin. The dilute Russell's viper venom time (dRVVT) assay starts coagulation by directly activating FX to FXa. The Taipan, Textarin, and Ecarin snake-venoms directly activate prothrombin. The activation of prothrombin to thrombin and several other reactions in the coagulation cascade require the presence of phospholipids and calcium. It is generally believed that the prolongation of phospholipid-dependent coagulation assays is due to competition between coagulation factors and aPLs with lupus anticoagulant activity for negatively charged phospholipids, although recently new data has been published that other mechanisms might also play a role [40].

To measure LAC, all three steps should be performed in two distinct assays with different test principles. To reach more harmonization between the laboratories, the updated International Society on Thrombosis and Haemostasis (ISTH)- Scientific and Standardization Committee (SSC) guidelines recommend performing only aPTT and dRVVT based assays [38]. The Clinical and laboratory Standards Institute (CLSI) guidelines are a bit less outspoken and do not exclude other assays [41]. The conclusion of positivity for LAC can be drawn when all three steps of at least one assay are positive.

Anticardiolipin assay and antiβ2GPI assay

According to the Sydney criteria, the presence of antiCL or antiβ2GPI antibodies are a laboratory criterion for APS in addition to LAC [3]. AntiCL and antiβ2GPI antibodies are measured by solid phase assays according to the ISTH-SSC guideline [42]. In contrast to LAC detecting all functional aPL, the quantification of one group of aPL is detected by solid phase assays. AntiCL assays detect different types of antibodies depending on the coating of the solid phase assays: antibodies directed against cardiolipin itself, directed against the complex of cardiolipin with β2GPI and directed against other cardiolipin-binding protein [43]. The Antiβ2GPI assays detect antibodies directed to the β2GPI protein (**Figure 4**).

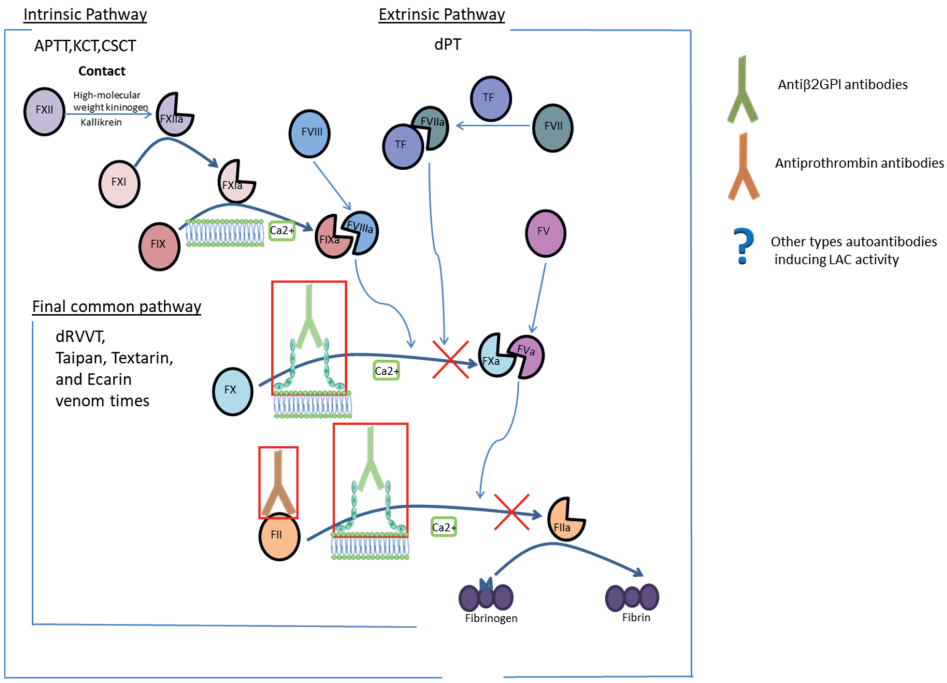

Figure 3. Lupus anticoagulant assays to detect antiphospholipid antibodies. A simplified schematic diagram of the coagulation pathway. Activated partial-thromboplastin time (APTT), colloidal-silica clotting time (CSCT) and kaolin clotting time (KCT), dilute prothrombin time (dPT) or dilute Russell's viper venom time (dRVVT), Taipan, Textarin and Ecarin snake-venom time are shown. It is generally believed that the prolongation of a phospholipid-dependent coagulation time is due to competition between coagulation factors and aPLs with lupus anticoagulant activity for negatively charged phospholipids.

Traditionally, antiCL and antiβ2GPI antibodies were measured by ELISA [3]. Nowadays, automated systems have been introduced into the market using a variety of solid phase surfaces (magnetic beads, microbeads, membranes and coated polystyrene cups) and various detection techniques of chemiluminescence (CLIA), flowcytometry, and multiplex systems[42,44,45]. However, despite the current guidelines and the introduction of internal calibrators to improve solid phase assay comparability, detection of antiCL and antiβ2GPI IgG/M show large inter-platform and inter-laboratory variation [46,47]. The heterogeneity of aPL and the lack of standardization of the assays make the laboratory diagnosis of APS full of challenging.

In theory, the efficacy of the antiCL assay depends on the coating efficiency of cardiolipin and the presence of a cofactor β2GPI. AntiCL antibodies directed against cardiolipin itself are thought to be infection-related and transient. The specificity of antiCL assays can be improved by performing them in a β2GPI-dependent manner [48]. Therefore, Antiβ2GPI assays using immobilized β2GPI antigen to detect antibodies directed towards β2GPI should have a better correlation with clinical manifestations of APS. As there is only a single β2GPI antigen, the antiβ2GPI assay is easier to standardize than antiCL assay. However, it still showed a high variability between antiβ2GPI

assays [49,50]. Futhermore, antiβ2GPI antibodies are frequently encountered in infectious diseases as well [51]. A lack of correlation of antiβ2GPI with thrombosis and pregnancy morbidity was shown in a meta-analysis [52]. This can be explained by differences in the conformation of β2GPI, which will affect the exposure of cryptic epitopes[53].

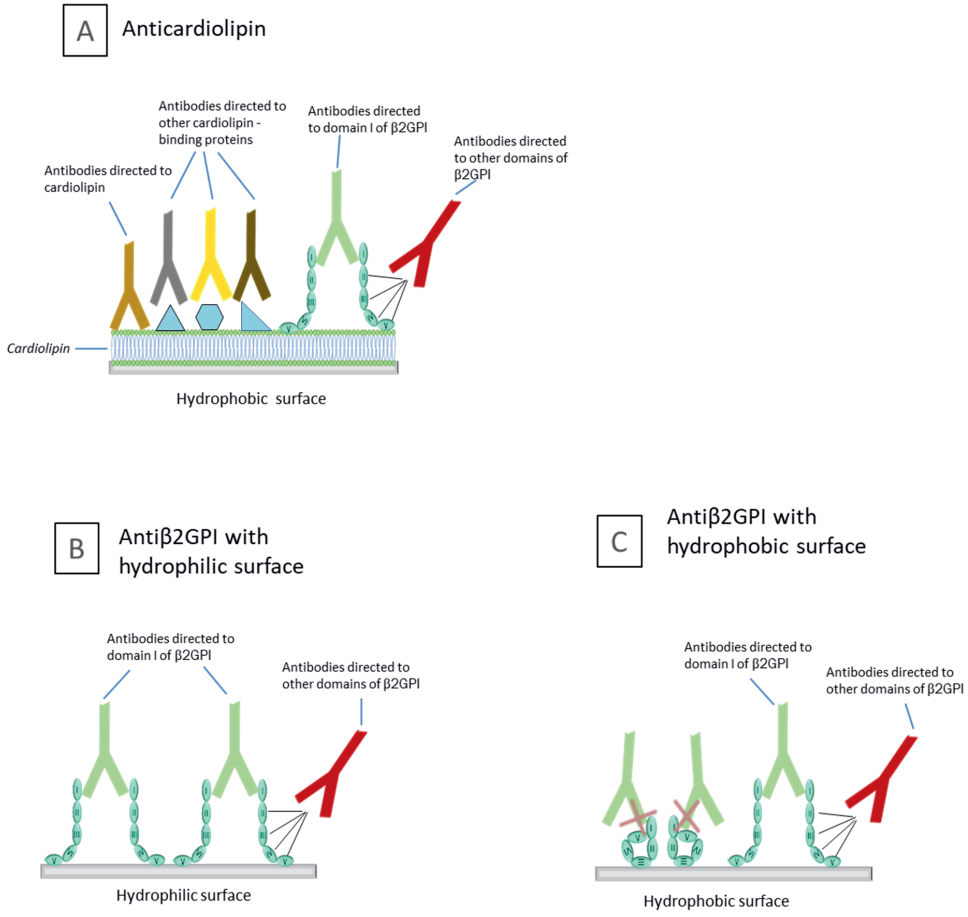

Figure 4. Anticardiolipin and antiβ2GPI assays for detecting antiphospholipid antibodies. (A) The antiCL assay detects a heterogeneous antibody population, including antibodies directly against cardiolipin itself and antibodies against cardiolipin-binding proteins. These cardiolipin-binding proteins can be β2GPI-independent antiCL antibodies, but also β2GPI-dependent antiCL antibodies, as well as antibodies against DI or other domains of β2GPI. (B-C) The antiβ2GPI assay detects a group of antibodies that bind directly to β2GPI, including antibodies against DI of β2GPI and antibodies against other domains of β2GPI. (B) When β2GPI is coated on hydrophilic surface, β2GPI undergoes a conformational change, exposing the cryptic epitope on DI allowing antiDI antibodies to bind. (C) When β2GPI is coated on a hydrophobic surface, the exposure of the epitope on DI of β2GPI is reduced and a part of the antiDI antibodies are not able to recognize β2GPI.

The cryptic epitope theory is that this epitope on DI is only exposed after the conformational change of β2GPI. In the antiCL assay, cardiolipin is first immobilized to a solid surface, β2GPI acts as a cofactor and binds to cardiolipin (which is negatively charged), which results in the conformational change thereby exposing the cryptic epitope on DI. In the antiβ2GPI assay, the nature of solid surface needs to be considered. Whenβ2GPI is bound onto a negatively charged surface (hydrophilic surface), the conformation of β2GPI changes thereby exposing the cryptic epitope on DI, allowing antibodies that recognizes the epitope to bind. When β2GPI is coated on a neutral-charged surface (hydrophobic surface), the exposure of the epitope on DI of β2GPI is reduced resulting in loss of binding by a sub-population of antiβ2GPI antibodies (**Figure 4**).

APLs that recognize the epitope on DI of β2GPI have been shown to be pathogenic [16,20-25], antibodies against other domains of β2GPI appear to be unrelated to the clinical symptoms of APS [26-29]. Based on these findings, the antiDI assays with directly coated DI of β2GPI as antigen were developed and were expected to have a very powerful role in the identification of APS patients. Several in-house antiDI and one commercial antiDI assay have been reported in the literature. Most of the reported assays are solely detecting IgG isotype antiDI antibodies.

Other aPLs assay

In addition to β2GPI, detecting various non-criteria aPLs against other plasma proteins, such as anti- phosphatidic acid (PA), anti-phosphatidylcholine, anti-phosphatidylethanolamine, anti-phosphatidylglycerol, anti-phosphatidylinositol, anti-phosphatidylserine, anti-Protein S, anti-Protein C, anti-annexin A2 anti-annexin5, antiPT and antiPS/PT have been published. However, the clinical significance of most of these antibodies is unclear [37,54-57]. Recent literature showed that antiPS/PT antibodies are associated with thrombosis and pregnancy morbidity [58-60] and the presence of antiPS/PT antibodies increases the risk of thrombosis recurrence [61]. Moreover, antiPS/PT antibodies are frequently present in APS patients and are strongly associated with LAC [62,63]. However, the evidence for a predictive value of antiPS/PT was considered insufficient. Until now, antiPS/PT testing is not included in the official laboratory criteria for diagnosing APS [3].

Isotype of antiCL and antiβ2GPI antibodies

After the revised criteria were published in 2006 [3], the discussion about the relevance of antiCL and antiβ2GPI isotypes was resumed [37]. IgG and IgM have been included in the criteria [3]. A meta-analysis literature review showed that IgG antibodies correlate significantly more with thrombosis than IgM antibodies. There was also a significant association for IgM, but it remains a question whether IgM is an independent risk factor[64]. So far, IgA has not been included in laboratory criteria of APS. In a mouse model, IgA antibodies isolated from APS patients, reactive against DI and DIV/DV, demonstrated the pathogenic potential by increased thrombus area compared to control IgA [5]. Moreover, multiple studies have shown that IgA antiCL and antiβ2GPI antibodies are associated with thrombosis and pregnancy morbidity [65-81]. Furthermore, some studies concluded that the presence of IgA antiCL (and/or antiβ2GPI) is an independent risk factor for thrombosis [73,77]. However, positivity for IgA antiCL and IgA antiβ2GPI is usually associated with IgG and/or IgM positivity, which makes it difficult to understand the role of isolated IgA. A recent systematic review indicated that several studies failed to demonstrate utility of adding IgA antiCL and antiβ2GPI testing, either because of low prevalence of these antibodies, their

association with other aPL, or the lack of improved diagnostic accuracy when routine evaluation of IgA antibodies [82]. Other studies found that isolated IgA antiCL and/or antiβ2GPI positivity was linked to non-criteria clinical manifestations of APS and was not associated with thrombosis or pregnancy morbidity [72,76,77,82-85]. Therefore, the importance of a non-criteria (isolated) IgA isotype of antiCL antibodies and antiβ2GPI antibodies need to be further investigated.

Antiphospholipid profile

The current laboratory criteria include detection of LAC, antiCL IgG and IgM and/or antiβ2GPI IgG and IgM. For the diagnosis of APS, it is sufficient to have one positive test when the patient also has one of the clinical criteria [3]. However, not every test has the same predictive value and positivity in more than one test has a higher predictive value for developing a thrombotic event and/or pregnancy morbidity. Therefore, it has been advised to categorize patients according to the number of positive tests, as well as to investigate the antiphospholipid antibody profile for risk stratification [86]. The concept of antibody profile for risk stratification is shown in **Table 2**. A combination of tests is used to stratify patients at risk for thrombosis and/or pregnancy complications. Taking into account the type and the number of positive tests, triple positivity is defined as combined positivity for LAC, antiCL and antiβ2GPI antibodies. Triple positivity has been shown to be associated with the highest risk of both a first thrombotic event and recurrence [87,88]. Triple positivity is also an independent risk factor for pregnancy failure [89]. Interestingly, patients with triple-positive profile are always still positive when the tests are repeated after 12 weeks [90]. Patients with double positivity for both antiCL and antiβ2GPI positivity, but LAC negative, are at lower risk for clinical events of APS compared to triple-positive patients. The double positive combination of LAC and antiβ2GPI or antiCL correlates much better with thrombosis than does an isolated positive test for LAC or antiβ2GPI or antiCL [86,91,92]. Single positives are positive for only one aPL positive [86]. Single antiCL or antiβ2GPI positivity did not mark an increased risk [93]. Moreover, single positive patients are less likely to develop APS related clinical symptoms [43,89]. Isolated positivity for antiCL, with antibodies directed against cardiolipin itself, has a questionable clinical relevance [94]. Equally, isolated antiβ2GPI positivity does not show any association with thrombosis [43].The precise clinical relevance of isolated LAC positivity is still unknown. As LAC measures the functional effect of a heterogeneous group of aPL antibodies, LAC could be mediated by either antiβ2GPI antibodies [25] or anti-PS/PT antibodies [95], or possibly other inhibitors [55,96] (**Figure 3).** LAC is a much better predictor of clinical events than the antiCL and antiβ2GPI [97-99]. When LAC is positive together with antiCL and antiβ2GPI antibodies, it carries a significant hazard for thrombotic as well as obstetric complications[87,88,100]. In the absence of antiCL and antiβ2GPI IgG and IgM antibodies, the clinical relevance of an isolated LAC has been widely debated. Although the clinical relevance of an isolated LAC has been suggested in both retrospective and prospective studies [98,99,101-103], however, not everybody is convinced by these results [104].

Table 2. Antiphospholipid antibodies profile.

	Lupus anticoagulant	**AntiCardiolipin IgG/IgM**	**Antiβ2GPI IgG/IgM**	**Categorization of patients**
Triple positive	Positive	Positive	Positive	Strongest association with risk events
Double positive	Negative	Positive	Positive	Lower association with thrombotic risk than triple positive Pregnancy complications unknown
Double positive	Positive	Negative	Positive	Better associated with clinical risk compared to isolated positive
Double positive	Positive	Positive	Negative	Better associated with clinical risk compared to isolated positive
Single positive	Positive	Negative	Negative	Most of studies show associated with thrombosis and pregnancy morbidity, although Pengo's study showed poor predictive value for thrombosis event [104]
Single positive	Negative	Positive	Negative	No association with risk events
Single positive	Negative	Negative	Positive	No association with risk events

Outline of my thesis

Laboratory assays used to detect antiphospholipid antibodies are a major criterium for the classification of APS and tailored treatment for patients with APS. Standardization of the assays used to detect antiphospholipid antibodies remains difficult, which makes the laboratory diagnosis of APS challenging. The currently criteria rely on the detection of a heterogeneous antibody population. Which proteins and factors contribute to the production of antiphospholipid antibodies, and how these antibodies induce clinical events (thrombosis and pregnancy morbidity) related to APS, are particularly complicated issues, for which there still are no good explanations. Therefore, non-criteria aPLs assay might be potentially useful in the diagnosis and management of APS. Therefore, in this thesis, data of a multicenter study are used in order to investigate whether applying a new assay (in-house antiDI assay), non-criteria aPL (IgA antiCL and IgA antiβ2GPI) or re-evaluation of the current aPL assays (isolated LAC) contribute to a better laboratory diagnosis of APS.

The following topics were addressed:

1. Is there added value of detecting anti-domain I of β2GPI IgG on top of the current APS laboratory criteria? (**Chapter 2-4**)
2. Is there added value of detecting IgA isotype of antiCL antibodies and antiβ2GPI antibodies in APS? (**Chapter 6**)
3. Does isolated LAC in the absence of antiCL and antiβ2GPI antibodies have a clinical relevance? (**Chapter 7**)

References

1. Duarte-García A, Pham MM, Crowson CS, et al. The epidemiology of antiphospholipid syndrome: a population-based study. *Arthritis & Rheumatology* 2019; **71**(9): 1545-52.
2. 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 & Rheumatism* 2002; **46**(4): 1019-27.
3. Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *Journal of thrombosis and haemostasis* 2006; **4**(2): 295-306.
4. de Groot PG, de Laat B, Rand J. et al. Natural proteins involved in antiphospholipid syndrome. *In: Erkan D, Lockshin M. eds.* Antiphospholipid Syndrome. Cham: Springer; 2017: 15-17.
5. Galli M, Barbui T, Comfurius P, et al. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. *The Lancet* 1990; **335**(8705): 1544-7.
6. Matsuura E. Anticardiolipin cofactor (s) and differential diagnosis of autoimmune disease. *Lancet* 1990; **336**: 177-8.
7. 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). *Proceedings of the National Academy of Sciences of the USA* 1990; **87**(11): 4120-4.
8. Ağar Ç, van Os G, Mörgelin M, et al. β2-Glycoprotein I can exist in 2 conformations: implications for our understanding of the antiphospholipid syndrome. *Blood* 2010; **116**(8): 1336-43.
9. van Lummel M, Pennings MT, Derksen RH, et al. The binding site in β2-glycoprotein I for ApoER2' on platelets is located in domain V. *Journal of Biological Chemistry* 2005; **280**(44): 36729-36.
10. Frank M, Sodin-Šemrl S, Irman Š, Božič B, Rozman B. β2-glycoprotein I and annexin A5 phospholipid interactions: Artificial and cell membranes. *Autoimmunity reviews* 2009; **9**(1): 5-10.
11. Kolyada A, Barrios DA, Beglova N. Dimerized domain V of beta2-glycoprotein I is sufficient to upregulate procoagulant activity in PMA-treated U937 monocytes and require intact residues in two phospholipid-binding loops. *Antibodies* 2017; **6**(2): 8.
12. Ioannou Y, Pericleous C, Giles I, Latchman DS, Isenberg DA, Rahman A. Binding of antiphospholipid antibodies to discontinuous epitopes on domain I of human β2-glycoprotein I: Mutation studies including residues R39 to R43. *Arthritis & Rheumatism* 2007; **56**(1): 280-90.
13. Iverson GM, Victoria EJ, Marquis DM. Anti-β2 glycoprotein I (β2GPI) autoantibodies recognize an epitope on the first domain of β2GPI. *Proceedings of the National Academy of Sciences of the USA* 1998; **95**(26): 15542-6.
14. Iverson GM, Reddel S, Victoria EJ, et al. Use of single point mutations in domain I of β2-glycoprotein I to determine fine antigenic specificity of antiphospholipid autoantibodies. *The Journal of Immunology* 2002; **169**(12): 7097-103.

15. Reddel SW, Wang YX, Sheng YH, Krilis SA. Epitope studies with anti-β2-glycoprotein I antibodies from autoantibody and immunized sources. *Journal of autoimmunity* 2000; **15**(2): 91-6.
16. de Laat B, Derksen RH, van Lummel M, Pennings MT, de Groot PG. Pathogenic anti-β2-glycoprotein I antibodies recognize domain I of β2-glycoprotein I only after a conformational change. *Blood* 2006; **107**(5): 1916-24.
17. de Laat B, van Berkel M, Urbanus RT, et al. Immune responses against domain I of β2-glycoprotein I are driven by conformational changes: Domain I of β2-glycoprotein I harbors a cryptic immunogenic epitope. *Arthritis & Rheumatism* 2011; **63**(12): 3960-8.
18. Gropp K, Weber N, Reuter M, et al. β2-glycoprotein I, the major target in antiphospholipid syndrome, is a special human complement regulator. *Blood* 2011; **118**(10): 2774-83.
19. van den Hoogen LL, van Roon JA, Radstake TR, Fritsch-Stork RD, Derksen RH. Delineating the deranged immune system in the antiphospholipid syndrome. *Autoimmunity reviews* 2016; **15**(1): 50-60.
20. Ioannou Y, Romay-Penabad Z, Pericleous C, et al. In vivo inhibition of antiphospholipid antibody-induced pathogenicity utilizing the antigenic target peptide domain I of β2-glycoprotein I: proof of concept. *Journal of Thrombosis and Haemostasis* 2009; **7**(5): 833-42.
21. McDonnell T, Pericleous C, Laurine E, et al. Development of a high yield expression and purification system for Domain I of Beta-2-glycoprotein I for the treatment of APS. *BMC biotechnology* 2015; **15**(1): 104.
22. Agostinis C, Durigutto P, Sblattero D, et al. A non-complement-fixing antibody to β2 glycoprotein I as a novel therapy for antiphospholipid syndrome. *Blood, The Journal of the American Society of Hematology* 2014; **123**(22): 3478-87.
23. Pericleous C, Ruiz-Limón P, Romay-Penabad Z, et al. Proof-of-concept study demonstrating the pathogenicity of affinity-purified IgG antibodies directed to domain I of β 2-glycoprotein I in a mouse model of anti-phospholipid antibody-induced thrombosis. *Rheumatology* 2015; **54**(4): 722-7.
24. De Laat B, Pengo V, Pabinger I, et al. The association between circulating antibodies against domain I of beta2-glycoprotein I and thrombosis: an international multicenter study. *Journal of Thrombosis and Haemostasis* 2009; **7**(11): 1767-73.
25. De Laat B, Derksen RH, Urbanus RT, De Groot PG. IgG antibodies that recognize epitope Gly40-Arg43 in domain I of β2-glycoprotein I cause LAC, and their presence correlates strongly with thrombosis. *Blood* 2005; **105**(4): 1540-5.
26. Pelkmans Ld, De Laat B. Antibodies against domain I of β2-glycoprotein I: the one and only? *Lupus* 2012; **21**(7): 769-72.
27. Pengo V, Ruffatti A, Tonello M, et al. Antibodies to Domain 4/5 (Dm4/5) of β2-Glycoprotein 1 (β2GP1) in different antiphospholipid (aPL) antibody profiles. *Thrombosis research* 2015; **136**(1): 161-3.
28. Andreoli L, Nalli C, Motta M, et al. Anti-β2-glycoprotein I IgG antibodies from 1-year-old healthy children born to mothers with systemic autoimmune diseases preferentially target domain 4/5: might it be the reason for their 'innocent'profile? *Annals of the rheumatic diseases* 2011; **70**(2): 380-3.

29. Artenjak A, Locatelli I, Brelih H, et al. Immunoreactivity and avidity of IgG anti-β2-glycoprotein I antibodies from patients with autoimmune diseases to different peptide clusters of β2-glycoprotein I. *Immunologic research* 2015; **61**(1-2): 35-44.
30. S P Bajaj, S I Rapaport, D S Fierer, K D Herbst, Schwartz DB. A mechanism for the hypoprothrombinemia of the acquired hypoprothrombinemia-lupus anticoagulant syndrome. *Blood* 1983; **61**(4): 684-92.
31. Sciascia S, Sanna G, Murru V, Roccatello D, Khamashta M, Bertolaccini M. Anti-prothrombin (aPT) and anti-phosphatidylserine/prothrombin (aPS/PT) antibodies and the risk of thrombosis in the antiphospholipid syndrome. A systematic review. *Thrombosis and Haemostasis* 2014; **111**(2): 354-64.
32. Misasi R, Capozzi A, Longo A, et al. "New" antigenic targets and methodological approaches for refining laboratory diagnosis of antiphospholipid syndrome. *Journal of immunology research* 2015; **2015**: 858542.
33. Urbanus RT, de Groot PG. Antiphospholipid antibodies—we are not quite there yet. *Blood reviews* 2011; **25**(2): 97-106.
34. Tripodi A, Chantarangkul V, Cini M, et al. Variability of cut-off values for the detection of lupus anticoagulants: results of an international multicenter multiplatform study. *Journal of Thrombosis and Haemostasis* 2017; **15**(6): 1180-90.
35. Moore G. Commonalities and contrasts in recent guidelines for lupus anticoagulant detection. *International journal of laboratory hematology* 2014; **36**(3): 364-73.
36. Hoxha A, Banzato A, Ruffatti A, Pengo V. Detection of lupus anticoagulant in the era of direct oral anticoagulants. *Autoimmunity reviews* 2017; **16**(2): 173-8.
37. Bertolaccini ML, Amengual O, Andreoli L, et al. 14th International Congress on Antiphospholipid Antibodies Task Force. Report on antiphospholipid syndrome laboratory diagnostics and trends. *Autoimmunity reviews* 2014; **13**(9): 917-30.
38. Pengo V, Tripodi A, Reber G, et al. Update of the guidelines for lupus anticoagulant detection. *Journal of Thrombosis and Haemostasis* 2009; **7**(10): 1737-40.
39. Katrien M.J.Devreese, Philip G. de Groot, Bas de Laat, et al. Guidance from the Scientific and Standardization Committee for lupus anticoagulant/antiphospholipid antibodies of the International Society on Thrombosis and Haemostasis: : Update of the guidelines for lupus anticoagulant detection and interpretation. *Journal of Thrombosis and Haemostasis* 2020; **18**: 2828-39.
40. Noordermeer T, Molhoek JE, Schutgens RE, et al. Anti-β2-glycoprotein I and anti-prothrombin antibodies cause lupus anticoagulant through different mechanisms of action. *Journal of thrombosis and haemostasis* 2021; **19**(4): 1018-28.
41. Wayne P. Clinical and laboratory Standards Institute (CLSI): laboratory testing for the lupus anticoagulant; Approved guideline. *CLSI document H60-A CLSI* 2014.
42. Devreese KM, Pierangeli SS, de Laat B, et al. Testing for antiphospholipid antibodies with solid phase assays: guidance from the SSC of the ISTH. *Journal of thrombosis and haemostasis* 2014; **12**(5): 792-5.
43. Pengo V, Bison E, Denas G, Jose SP, Zoppellaro G, Banzato A. Laboratory Diagnostics of Antiphospholipid Syndrome. *Seminars in thrombosis and hemostasis* 2018; **44**(5): 439-44.

44. Van Hoecke F, Persijn L, Decavele AS, Devreese K. Performance of two new, automated chemiluminescence assay panels for anticardiolipin and anti-beta2-glycoprotein I antibodies in the laboratory diagnosis of the antiphospholipid syndrome. *International journal of laboratory hematology* 2012; **34**(6): 630-40.
45. Devreese K, Poncet A, Lindhoff-Last E, Musial J, De Moerloose P, Fontana P. A multicenter study to assess the reproducibility of antiphospholipid antibody results produced by an automated system. *Journal of Thrombosis and Haemostasis* 2017; **15**(1): 91-5.
46. Favaloro EJ, Wheatland L, Jovanovich S, Roberts-Thomson P, Wong RC. Internal quality control and external quality assurance in testing for antiphospholipid antibodies: Part I—Anticardiolipin and anti-β2-glycoprotein I antibodies. *Seminars in thrombosis and hemostasis* 2012; 38(04): 390-403.
47. Pelkmans L, Kelchtermans H, de Groot PG, et al. Variability in exposure of epitope G40-R43 of domain i in commercial anti-beta2-glycoprotein I IgG ELISAs. *PloS one* 2013; **8**(8): e71402.
48. Levy RA, de Meis E, Pierangeli S. An adapted ELISA method for differentiating pathogenic from nonpathogenic aPL by a beta 2 glycoprotein I dependency anticardiolipin assay. *Thrombosis research* 2004; **114**(5-6): 573-7.
49. Reber G, Schousboe I, Tincani A, et al. Inter-laboratory variability of anti-β2-glycoprotein I measurement. *Thrombosis and Haemostasis* 2002; **88**(07): 66-73.
50. Reber G, Tincani A, Sanmarco M, de Moerloose P, Boffa M-C. Variability of anti-β2 glycoprotein I antibodies measurement by commercial assays. *Thrombosis and Haemostasis* 2005; **94**(09): 665-72.
51. Santiago M, Martinelli R, Ko A, et al. Anti-beta2 glycoprotein I and anticardiolipin antibodies in leptospirosis, syphilis and Kala-azar. *Clinical and Experimental Rheumatology* 2001; **19**(4): 425-30.
52. Galli M, Luciani D, Bertolini G, Barbui T. Anti-β2-glycoprotein I, antiprothrombin antibodies, and the risk of thrombosis in the antiphospholipid syndrome. *Blood* 2003; **102**(8): 2717-23.
53. Brighton TA, Dai YP, Hogg PJ, CN. C. Microheterogeneity of beta-2 glycoprotein I: implications for binding to anionic phospholipids. *The biochemistry journal* 1999; **340**(Pt 1): 59-67.
54. Rodriguez-Garcia V, Ioannou Y, Fernandez-Nebro A, Isenberg DA, Giles IP. Examining the prevalence of non-criteria anti-phospholipid antibodies in patients with anti-phospholipid syndrome: a systematic review. *Rheumatology (Oxford)* 2015; **54**(11): 2042-50.
55. Oosting JD, Derksen R, Bobbink I, Hackeng T, Bouma B, de Groot P. Antiphospholipid antibodies directed against a combination of phospholipids with prothrombin, protein C, or protein S: an explanation for their pathogenic mechanism? *Blood* 1993; **81**(10): 2618-25.
56. Pengo V, Biasiolo A, Brocco T, Tonetto S, Ruffatti A. Autoantibodies to phospholipid-binding plasma proteins in patients with thrombosis and phospholipid-reactive antibodies. *Thrombosis and Haemostasis* 1996; **76**(05): 721-4.
57. de Laat B, Wu XX, van Lummel M, Derksen R, de Groot PG, Rand JH. Correlation between antiphospholipid antibodies that recognize domain I of beta 2-glycoprotein I and a reduction in the anticoagulant activity of annexin A5. *Blood* 2007; **109**(4): 1490-4.

58. Sciascia S, Sanna G, Murru V, Roccatello D, Khamashta M, Bertolaccini M. Anti-prothrombin (aPT) and anti-phosphatidylserine/prothrombin (aPS/PT) antibodies and the risk of thrombosis in the antiphospholipid syndrome. A systematic review. *Thrombosis and Haemostasis* 2014; 111(02): 354-64.

59. Nakamura H, Oku K, Olga Amengual, et al. First-line, Non-criterial Antiphospholipid Antibody Testing for the Diagnosis of Antiphospholipid Syndrome in Clinical Practice: a Combination of Anti-beta2-glycoprotein I Domain I and Phosphatidylserine-dependent Antiprothrombin Antibodies. *Arthritis care & research* 2017: 1-46.

60. Lee JS, Gu J, Park HS, Yoo HJ, Kim HK. Coexistence of anti-beta2-glycoprotein I domain I and anti-phosphatidylserine/prothrombin antibodies suggests strong thrombotic risk. *Clinical Chemistry and Laboratory Medicine* 2017; **55**(6): 882-9.

61. Bizzaro N, Ghirardello A, Zampieri S, et al. Anti-prothrombin antibodies predict thrombosis in patients with systemic lupus erythematosus: a 15-year longitudinal study. *Journal of Thrombosis and Haemostasis* 2007; **5**(6): 1158-64.

62. Pengo V, Del Ross T, Ruffatti A, et al. Lupus anticoagulant identifies two distinct groups of patients with different antibody patterns. *Thrombosis Research* 2018; **172**: 172-8.

63. Radin M, Foddai SG, Cecchi I, et al. Antiphosphatidylserine/Prothrombin Antibodies: An Update on Their Association with Clinical Manifestations of Antiphospholipid Syndrome. *Thrombosis and Haemostasis* 2020; **120**(4): 592-8.

64. Kelchtermans H, Pelkmans L, De Laat B, Devreese K. IgG/IgM antiphospholipid antibodies present in the classification criteria for the antiphospholipid syndrome: a critical review of their association with thrombosis. *Journal of Thrombosis and Haemostasis* 2016; **14**(8): 1530-48.

65. Lakos G, Favaloro EJ, Harris EN, et al. International consensus guidelines on anticardiolipin and anti-beta2-glycoprotein I testing: report from the 13th International Congress on Antiphospholipid Antibodies. *Arthritis & Rheumatism* 2012; **64**(1): 1-10.

66. Shen Y-M, Dyszkiewicz-Korpanty A, Lee R, Balani J, Frenkel E, Sarode R. The Prevalence and Clinical Significance of IgA Antiphospholipid Antibodies (aPL). *Blood, The Journal of the American Society of Hematology* 2005; 106 (11): 2647.

67. Andreoli L, Fredi M, Nalli C, et al. Clinical Significance of IgA Anti-Cardiolipin and IgA Anti-β 2 Glycoprotein I Antibodies. *Current rheumatology reports* 2013; **15**(7): 343.

68. Murthy V, Willis R, Romay-Penabad Z, et al. Value of Isolated IgA Anti-β2-Glycoprotein I Positivity in the Diagnosis of the Antiphospholipid Syndrome. *Arthritis & Rheumatology* 2013; **65**(12): 3186-93.

69. Martínez-Flores JA, Serrano M, Pérez D, et al. Circulating immune complexes of IgA bound to beta 2 glycoprotein are strongly associated with the occurrence of acute thrombotic events. *Journal of atherosclerosis and thrombosis* 2016: 34488.

70. Serrano M, Martínez-Flores JA, Pérez D, et al. β2-glycoprotein I/IgA immune complexes: a marker to predict thrombosis after renal transplantation in patients with antiphospholipid antibodies. *Circulation* 2017; **135**(20): 1922-34.

71. Despierres L, Beziane A, Kaplanski G, et al. Contribution of anti-β2glycoprotein I IgA antibodies to the diagnosis of anti-phospholipid syndrome: potential interest of target domains to discriminate thrombotic and non-thrombotic patients. *Rheumatology* 2014; **53**(7): 1215-8.

72. Carmo-Pereira S, Bertolaccini M, Escudero-Contreras A, Khamashta M, Hughes G. Value of IgA anticardiolipin and anti-β2-glycoprotein I antibody testing in patients with pregnancy morbidity. *Annals of the Rheumatic Diseases* 2003; **62**(6): 540-3.
73. Lakos G, Kiss E, Regeczy N, et al. Isotype distribution and clinical relevance of anti-β2-glycoprotein I (β2-GPI) antibodies: importance of IgA isotype. *Clinical and experimental immunology* 1999; **117**(3): 574-9.
74. Mattia E, Ruffatti A, Tonello M, et al. IgA anticardiolipin and IgA anti-β2 glycoprotein I antibody positivity determined by fluorescence enzyme immunoassay in primary antiphospholipid syndrome. *Clinical Chemistry and Laboratory Medicine* 2014; **52**(9): 1329-33.
75. Mehrani T, Petri M. Association of IgA anti-ß2 glycoprotein i with clinical and laboratory manifestations of systemic lupus erythematosus. *The Journal of rheumatology* 2011; **38**(1): 64-8.
76. Ruiz-García R, Serrano M, Ángel Martínez-Flores J, et al. Isolated IgA anti-β2 glycoprotein I antibodies in patients with clinical criteria for antiphospholipid syndrome. *Journal of immunology research* 2014; **2014**: 704395.
77. Samarkos M, Davies K, Gordon C, Loizou S. Clinical significance of IgA anticardiolipin and anti-β 2-GP1 antibodies in patients with systemic lupus erythematosus and primary antiphospholipid syndrome. *Clinical Rheumatology* 2006; **25**(2): 199-204.
78. Shen YM, Lee R, Frenkel E, Sarode R. IgA antiphospholipid antibodies are an independent risk factor for thromboses. *Lupus* 2008; **17**(11): 996-1003.
79. Sweiss NJ, Bo R, Kapadia R, et al. IgA anti-β2-glycoprotein I autoantibodies are associated with an increased risk of thromboembolic events in patients with systemic lupus erythematosus. *PloS one* 2010; **5**(8): e12280.
80. Tortosa C, Cabrera-Marante O, Serrano M, et al. Incidence of thromboembolic events in asymptomatic carriers of IgA anti ss2 glycoprotein-I antibodies. *PloS one* 2017; **12**(7): e0178889.
81. Murthy V, Willis R, Romay-Penabad Z, et al. Value of isolated IgA anti-beta2 -glycoprotein I positivity in the diagnosis of the antiphospholipid syndrome. *Arthritis & Rheumatology* 2013; **65**(12): 3186-93.
82. Meijide H, Sciascia S, Sanna G, Khamashta MA, Bertolaccini ML. The clinical relevance of IgA anticardiolipin and IgA anti-beta2 glycoprotein I antiphospholipid antibodies: a systematic review. *Autoimmunity reviews* 2013; **12**(3): 421-5.
83. Selva-O'Callaghan A, Ordi-Ros J, Monegal-Ferran F, Martinez N, Cortes-Hernandez F, Vilardell-Tarres M. IgA anticardiolipin antibodies-relation with other antiphospholipid antibodies and clinical significance. *Thrombosis and Haemostasis* 1998; **79**(02): 282-5.
84. Vlagea A, Pascual-Salcedo D, Alvarez Doforno R, et al. IgA anti-beta2 glycoprotein I antibodies: Experience from a large center. *Thrombosis Research* 2018; **162**: 38-43.
85. Bertolaccini ML, Atsumi T, Contreras AE, Khamashta MA, Hughes G. The value of IgA antiphospholipid testing for diagnosis of antiphospholipid (Hughes) syndrome in systemic lupus erythematosus. *The Journal of rheumatology* 2001; **28**(12): 2637-43.
86. Pengo V, Biasiolo A, Pegoraro C, Cucchini U, Noventa F, Iliceto S. Antibody profiles for the diagnosis of antiphospholipid syndrome. *Thrombosis and Haemostasis* 2005; **93**(06): 1147-52.
87. Pengo V, Ruffatti A, Legnani C, et al. Incidence of a first thromboembolic event in asymptomatic carriers of high-risk antiphospholipid antibody profile: a multicenter prospective study. *Blood, The Journal of the American Society of Hematology* 2011; **118**(17): 4714-8.

88. Pengo V, Ruffatti A, Legnani C, et al. Clinical course of high-risk patients diagnosed with antiphospholipid syndrome. *Journal of Thrombosis and Haemostasis* 2010; **8**(2): 237-42.

89. Ruffatti A, Tonello M, Visentin MS, et al. Risk factors for pregnancy failure in patients with anti-phospholipid syndrome treated with conventional therapies: a multicentre, case-control study. *Rheumatology (Oxford)* 2011; **50**(9): 1684-9.

90. Pengo V, Ruffatti A, Del Ross T, et al. Confirmation of initial antiphospholipid antibody positivity depends on the antiphospholipid antibody profile. *Journal of thrombosis and haemostasis* 2013; **11**(8): 1527-31.

91. Zoghlami-Rintelen C, Vormittag R, Sailer T, et al. The presence of IgG antibodies against β2-glycoprotein I predicts the risk of thrombosis in patients with the lupus anticoagulant. *Journal of Thrombosis and Haemostasis* 2005; **3**(6): 1160-5.

92. Ruffatti A, Tonello M, Del Ross T, et al. Antibody profile and clinical course in primary antiphospholipid syndrome with pregnancy morbidity. *Thrombosis and Haemostasis* 2006; **96**(09): 337-41.

93. Mustonen P, Lehtonen K, Javela K, Puurunen M. Persistent antiphospholipid antibody (aPL) in asymptomatic carriers as a risk factor for future thrombotic events: a nationwide prospective study. *Lupus* 2014; **23**(14): 1468-76.

94. Devreese K, Ortel T, Pengo V, de Laat B. Laboratory criteria for antiphospholipid syndrome: reply. *Journal of thrombosis and haemostasis* 2018; **16**: 2117-9.

95. Bevers E, Galli M, Barbui T, Comfurius P, Zwaal R. Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin. *Thrombosis and Haemostasis* 1991; **66**(06): 629-32.

96. de Laat B, Wu X-X, van Lummel M, Derksen RH, de Groot PG, Rand JH. Correlation between antiphospholipid antibodies that recognize domain I of β2-glycoprotein I and a reduction in the anticoagulant activity of annexin A5. *Blood* 2007; **109**(4): 1490-4.

97. Reynaud Q, Lega J-C, Mismetti P, et al. Risk of venous and arterial thrombosis according to type of antiphospholipid antibodies in adults without systemic lupus erythematosus: a systematic review and meta-analysis. *Autoimmunity reviews* 2014; **13**(6): 595-608.

98. Urbanus RT, Siegerink B, Roest M, Rosendaal FR, de Groot PG, Algra A. Antiphospholipid antibodies and risk of myocardial infarction and ischaemic stroke in young women in the RATIO study: a case-control study. *The Lancet Neurology* 2009; **8**(11): 998-1005.

99. Gebhart J, Posch F, Koder S, et al. Increased mortality in patients with the lupus anticoagulant: the Vienna Lupus Anticoagulant and Thrombosis Study (LATS). *Blood, The Journal of the American Society of Hematology* 2015; **125**(22): 3477-83.

100. Ruffatti A, Tonello M, Visentin MS, et al. Risk factors for pregnancy failure in patients with anti-phospholipid syndrome treated with conventional therapies: a multicentre, case-control study. *Rheumatology* 2011; **50**(9): 1684-9.

101. de Mast Q, Molhoek JE, van der Ven AJ, et al. Antiphospholipid Antibodies and the Risk of Stroke in Urban and Rural Tanzania: A Community-Based Case-Control Study. *Stroke* 2016; **47**(10): 2589-95.

102. Lockshin MD, Kim M, Laskin CA, et al. Prediction of adverse pregnancy outcome by the presence of lupus anticoagulant, but not anticardiolipin antibody, in patients with antiphospholipid antibodies. *Arthritis & Rheumatism* 2012; **64**(7): 2311-8.

103. Ruffatti A, Del Ross T, Ciprian M, et al. Risk factors for a first thrombotic event in antiphospholipid antibody carriers: a prospective multicentre follow-up study. *Annals of the Rheumatic Diseases* 2011; **70**(6): 1083-6.
104. Pengo V, Testa S, Martinelli I, et al. Incidence of a first thromboembolic event in carriers of isolated lupus anticoagulant. *Thrombosis Research* 2015; **135**(1): 46-9.

Chapter 2

The clinical value of assays detecting antibodies against domain I of β2-glycoprotein I in the antiphospholipid syndrome

Dongmei Yin, Bas de laat, Katrien M. J. Devreese, Hilde Kelchtermans

Autoimmunity reviews 17 (2018) 1210-1218.

Abstract

As the clinical symptoms of the antiphospholipid syndrome (APS) frequently occur irrespective of the syndrome, diagnosis predominantly depends on the laboratory assays measuring the level or function of antiphospholipid antibodies (aPLs). β2-glycoprotein I (β2GPI) is increasingly accepted as the most important target of aPLs. Antiβ2GPI antibodies constitute a heterogeneous population, but current in vivo and in vitro evidence show that especially the first domain (DI) of β2GPI contains an important pathogenic epitope. This epitope containing Glycine40-Arginine43 (G40-R43) has proven to be cryptic and only exposed when β2GPI is in its open conformation. A previous study demonstrated a highly variable exposure of the cryptic epitope in commercial antiβ2GPI assays, with implications on correct patient classification. Unexpectedly, recent unpublished data revealed impaired exposure of the pathogenic epitope in the commercially available antiDI chemiluminescence immunoassay (CLIA) assay detecting specific antibodies directed to DI.

In this review we summarize the laboratory and clinical performance characteristics of the different antiDI assays in published data and conclude with inconsistent results for both the correlation of antiDI antibodies with clinical symptoms and the added value of antiDI antibodies in the classification criteria of APS. Additionally, we hypothesize on possible explanations for the observed discrepancies. Finally, we highly advise manufacturers to use normal pooled plasma spiked with the monoclonal antiDI antibodies to verify correct exposure of the cryptic epitope.

1. Introduction

APS is a systemic autoimmune disease characterized by thrombotic complications, either venous, arterial, or small-vessel thrombosis, or pregnancy-related morbidity. The latter includes fetal death, premature births attributed to placental insufficiency, eclampsia or severe pre-eclampsia and spontaneous abortions [1]. Given the high frequency of these clinical symptoms irrespective of the syndrome, apart from a clinical characteristic an APS patient needs to fulfil at least one of the laboratory criteria [2]. Current revised laboratory criteria for APS classification detect the presence of aPLs through a combination of laboratory assays, including one functional coagulation assay lupus anticoagulant (LAC) and two immunological assays measuring immunoglobulin (Ig) G and/or IgM anti-cardiolipin antibodies (antiCL) and IgG and/or IgM antiβ2GPI antibodies (antiβ2GPI). To avoid false positive tests due to infections, positive tests should be repeated with an interval of at least 12 weeks [3]. Of note, the presence of these aPLs has also been demonstrated to associate with other clinical symptoms that are not included in the APS criteria, such as epilepsy and migraine [4,5].

Probably due to the fact that LAC measures a functional effect of the antibodies, LAC is a better predictor of thrombosis than the quantitative solid phase immunoassays. However, currently it is not advised to carry out LAC tests during treatment with direct oral anticoagulants because of the risk of false-positive results [6]. The exact pathogenesis of APS is unknown, but aPLs have been described to activate monocytes, neutrophils, dendritic cells and the placental tissue (summarized in [7]). Despite the fact that many different proteins have been identified as being involved in the pathogenesis of APS, accumulating evidence from in vitro experiments as well as animal studies has revealed that β2GPI is the main target for aPLs [8-10]. Based on this evidence, one would expect that testing for antibodies with reactivity towards β2GPI has a good correlation with clinical manifestations. Although numerous studies have demonstrated a significant correlation [11-13], a meta-analysis performed by Galli et al. failed to show a significant correlation between single antiβ2GPI positivity with a history of thrombosis or fetal loss [14]. The lack of correlation with thrombotic complications as well as pregnancy morbidity was also shown in more recent studies [15-17].

The reason for this variation may at least in part result from the availability of numerous commercial and home-made antiβ2GPI assays and the lack of standardization [18,19]. The available assays differ from each other in assay design [method/principle: enzyme-linked immunosorbent assay (ELISA) versus chemiluminescence immunoassays (CLIA)], the source of β2GPI and coating principles, the calculation of cut-off values, the reference material and the units used to express positivity [20,21]. Additionally, various subsets of antiβ2GPI antibodies targeting different domains of the protein have been described with clear differences in clinical potential [22-25]. β2GPI consists of five homologous domains (DI-DV) arranged differently in its native versus open conformation [26]. Importantly, in the native circular or S-shaped β2GPI the critical DI epitope is not exposed. Upon binding to an anionic (phospholipid) surface through the positively charged patch on DV, β2GPI undergoes a conformational change. Consequently, the DI-IV spreads out resulting in a more open J shape, exposing a cryptic epitope G40-R43 in DI and allowing antiDI of β2GPI

autoantibodies to bind [27-29]. This subpopulation of aPLs that recognize an epitope on DI of β2GPI comprising at least G40-R43 proved to be pathogenic [27,30-37] and displayed a significant correlation with clinical manifestations [16,38]. The detection of antibodies to DI versus DIV/V has therefore been suggested to help in the diagnosis of APS [39,40].

As to the in vivo evidence for the pathogenicity of antibodies directed to DI, administration of recombinant human DI was found to inhibit the induction of thrombosis by polyclonal human IgG from patients with APS in a mouse model [41,42]. In addition, a recombinant antibody recognizing DI of β2GPI induced thrombosis and fetal loss in animal models following priming with lipopolysaccharide (LPS) , while a CH2-deleted version of this antibody prevented the procoagulant and abortion-inducing effect of aPLs from APS patients [43]. In a proof-of-concept study, using polyclonal IgG from patients with APS, antiDI-rich IgG significantly induced larger thrombi and enhanced the procoagulant activity in vivo compared with antiDI-poor IgG [44].

Interestingly, a study in our laboratory demonstrated that the exposure of this pathogenic epitope G40-R43 on domain I was highly variable in commercial full-length antiβ2GPI assays. This reduced exposure of G40-R43 may account for the variable results obtained concerning the clinical correlation of the assays as reduced exposure was found to result in false negative classification of APS patients [45]. More recently, various assays specifically measuring antiDI antibodies have been developed. As these assays are measuring a specific pathogenic population, one would expect antiDI assays to highly correlate with clinical symptoms. Nonetheless, as for the full length antiβ2GPI assays, no consensus has been reached for the antiDI antibodies concerning the correlation with clinical symptoms. Whereas some studies have demonstrated a higher correlation with thrombosis compared to the full-length assays, other studies failed to show an added value of antiDI assays. In this review, we summarize the laboratory and clinical performance characteristics of the various antiDI assays and elaborate on the possible reasons for the observed discrepancies.

2. Available antiDI assays

So far five major assays have been described in literature to specifically measure antibodies directed to DI of β2GPI (summarized in **Table 1**). These five assays differ in the source of DI, the coating and measuring principle and the interpretation of results. As these factors may influence the clinical performance of the test, we aim to summarize and compare the sensitivity and specificity, the odds ratio (OR) of antiDI for clinical manifestations, as well as the added value of antiDI to the existing laboratory criteria when measured with the different available assays in various patient populations (summarized in **Table 2-4**).

Table 1. Overview of assays used for the detection of anti-domain I antibodies (antiDI)

Assay	Principle of the method	Source of DI	Solid phase	Surface capacity	Exposure of the epitope G40-R43	Expression of result	Inter-assay CV	References
Two-step ELISA	Antibodies are detected against DI coated on a hydrophilic versus hydrophobic plate by an in-house ELISA	Baculovirus expression system	Hydrophobic and hydrophilic ELISA plate	Normal	Yes	Dichotomous values (the ratio of OD on hydrophobic plate/ hydrophilic plate> 2 is positive)	3.7～14.8%	16,38,46
Direct ELISA	Antibodies are detected against DI coated on a nickel plate by an in-house ELISA	Bacteria expression system	Nickel chelate ELISA plate	Normal	NS	Continuous values (measured in arbitrary units)	<10.0 %	35,47-49
Competitive inhibition ELISA	The percentage inhibition is calculated when IC50 of DI in solution is used to inhibit antibodies binding to β2GPI coated on a flexible plate by an in-house ELISA	Chemical synthesis	ELISA plate	Normal	NS	The median percentage inhibition of antibodies are calculated	10.8%	50,51
Commercial developed ELISA	Antibodies are detected against DI coated on ELISA plates by a commercially developed ELISA	Baculovirus expression system	ELISA plate	Normal	NS	Continuous values (measured in arbitrary units)	15.0%	24,40,52
CLIA for antiDI (HemosIL AcuStar®and QUANTA Flash®, INOVA)	Antibodies are detected against DI coated on paramagnetic beads by a CLIA	Baculovirus expression system	Paramagnetic beads	Higher	NS	Continuous values (measured in arbitrary units)	<10.0 %	15,17,53-67

β2GPI: β2-glycoprotein I; CLIA: chemiluminescence immunoassay; CV: coefficient of variation; DI: domain I of β2GPI; ELISA: enzyme-linked immunosorbent assay; IC50: the concentration of DI able to inhibit 50% of patient IgG antibodies binding to β2GPI; NS: not specified; OD: optical density.

2.1 In-house developed two-step antiDI ELISA assay

In 2005, the first antiDI assay was developed by de Laat et al [38]. The DI used for coating was produced by a baculovirus expression system as described before [34]. In this assay, DI is coated on a hydrophobic as well as a hydrophilic plate. The arbitrary binding of DI on a hydrophobic plate will ensure satisfactory exposure of the G40-R43 epitope. On the contrary, on a hydrophilic plate the positive epitope G40-R43 will be strongly directed downwards making the epitope not available for binding. The result of the assay is expressed as a ratio between the optical densities (OD) measured with the hydrophobic plate versus the hydrophilic plate. A ratio higher than two indicates DI reactivity of the sample.

This two-step antiDI ELISA has been utilized in three studies [16,38,46]. In a single-center study including 198 patients with various systemic autoimmune diseases both IgG antiDI and IgG antiβ2GPI antibodies measured with an in-house ELISA correlated strongly with a history of thrombosis [OR 18.9, 95% confidence interval (95% CI) 6.8-52.3; OR 6.7, 95% CI 3.4-13.5, respectively] [38]. In contrast, those with affinity for other domains of β2GPI were not significantly correlated with thrombosis (OR 1.1, 95% CI 0.4-2.8). Interestingly, only 58% of IgG antiβ2GPI positive patients were IgG antiDI positive, and 93% of the antiDI positive samples proved to be LAC positive [38]. These findings were confirmed in the consecutive multicenter study conducted on 442 patients positive for IgG/IgM antiβ2GPI [16]. The prevalence of IgG antiDI antibodies positivity was 57% and IgG antiDI was significantly associated with thrombosis (OR 3.5, 95% CI 2.3-5.4) and with obstetric complications (OR 2.4, 95% CI 1.4-4.3). On the other hand, no significant positive correlation was found for IgG antiβ2GPI with obstetric complications (OR 1.5, 95% CI 0.6-3.7), nor for IgG reactive against other domains of β2GPI with thrombosis (OR 0.4, 95% CI 0.3-0.6) [16].

In a separate study, 25% of 183 children with systemic lupus erythematosus (SLE) were found to be antiDI positive, whereas all pediatric controls were negative [46]. A multivariate analysis showed that both the presence of antiDI antibodies and LAC are independently associated with reduced annexin A5 anticoagulant activity [46]. Due to the relatively low rate for histories of thrombosis (3.8%) in the population, the study was not sufficiently powered to determine the association of antiDI antibodies with thrombosis.

2.2 In-house developed direct antiDI ELISA

In addition to the baculovirus system for recombinant DI, a bacterial expression system to produce human DI [47] was used to develop a simple direct ELISA that does not require the usage of hydrophobic versus hydrophilic plates. In a population consisting of 22 APS patients, 20 SLE patients and 10 healthy controls, significantly higher binding was observed for polyclonal purified IgG from APS patients compared to that from the other groups [35]. Interestingly, the creation of multiple mutants of DI using the same bacterial system identified that aside from G40-R43 also R39, the domain I-II interlinker, and possibly D8 and D9 play a major role in the binding to antibodies [35].

This assay has been used for detection of IgG antiDI in two studies [48,49]. In a population of 40 seropositive APS and 40 seronegative APS patients (i.e. individuals with typical clinical features

highly suggestive for APS, but persistently negative for laboratory criteria), the prevalence of antiDI positivity was 27.5% in seropositive APS and 7.5% in seronegative APS [48]. Additionally in a population of 111 APS patients, 119 SLE patients and 200 healthy controls, a lower sensitivity of the IgG antiDI compared to IgG antiβ2GPI measured by an in-house ELISA for APS (40.5% versus 64.8%) was demonstrated [49]. Single positivity for IgG antiDI (hazard ratio (HR) 6.6, 95% CI 3.8-11.4) was strongly associated with APS, but to a lesser extent than single IgG antiβ2GPI (HR 33.4, 95% CI 13.0-86.1). However, in the same study, of 136 patients positive for IgG antiCL or antiβ2GPI , 52 were also IgG antiDI positive, and the presence of IgG antiDI positivity raised the HR for APS approximately 3-fold [(HR 36.9, 95% CI 17.7-76.9) versus (HR 11.5, 95% CI 6.3-21.0)]. In addition, positivity for IgG antiDI increased the strength of association between antiCL/antiβ2GPI positivity measured by an in-house ELISA and thrombotic manifestations in the group of 111 patients with APS, suggesting antiDI positivity can be used for thrombotic risk stratification. None of the tested profiles was significantly associated with pregnancy morbidity [49].

2.3 In-house developed competitive inhibition ELISA

A competitive inhibition ELISA assay was developed in which a chemically synthesized DI [50] was used to inhibit binding of antibodies in APS patients plasma to whole β2GP1 immobilized on a 96 wells plate [51]. In this assay, at first the DI concentration able to inhibit 50% of patient IgG binding to β2GPI (IC50) was calculated. Consecutively, this concentration of DI was used to calculate the percentage inhibition obtained in different patient categories. The level of inhibition proved to be higher in samples from triple positive APS patients (positive for LAC, IgG antiCL and IgG antiβ2GPI) compared to double (positive for IgG antiCL and IgG antiβ2GPI) or single positive (positive only for IgG antiβ2GPI) APS patients and healthy controls [51]. Since triple positive APS patients are at high risk of developing future thromboembolic events [52], this result supports the idea that IgG antiDI antibodies play an important pathogenic role [51]. Interestingly, in preliminary experiments, antiDI antibodies were not detected by direct coating of DI onto ELISA plates. More specifically, when plasma from APS patients and healthy controls was tested for IgG binding to the same chemically synthesized DI coated on either a hydrophobic or a hydrophilic plate, the IgG antiDI levels did not differ between APS patients and healthy controls [51].

2.4 Commercially developed antiDI ELISA

The first commercially developed assay to measure antiDI antibodies was from INOVA Diagnostics. Recombinant DI of β2GP1 was expressed and purified from the baculovirus expression system [34]. This assay has been used for detection of IgG antiDI in three patient studies [24,40,53].

Using this ELISA, IgG antiDI antibodies were found to be the most prevalent antibodies (75%) in 64 patients with APS [24]. A low prevalence of IgG antiDI was reported in 57 healthy children born to mothers with various systemic autoimmune diseases (AID) and 33 children with atopic dermatitis (16% and 27%, respectively). On the other hand, IgG antibodies recognizing DIV/DV of β2GPI were preferentially detected in children (37% and 33%, respectively), whereas isolated IgG antiDIV/DV was rare (5%) in APS and not associated with thrombosis. This study speculated that antibodies targeting DI are pathogenic, whereas those reactive with DIV/DV are probably

'innocent' [24]. In another study including 159 antiβ2GPI positive patients (measured by an in-house ELISA) [40], 70% of the 87 patients with primary APS (PAPS) were positive for antiDI reactivity. 30 asymptomatic aPLs-carriers displayed significantly lower levels of antiDI IgG and higher levels of antiDIV/DV compared to the PAPS group and the rheumatic disease (RD) group. Interestingly, no association was found between IgG antiDI and APS classification, thrombosis and obstetric complications in a multivariate logistic regression model, although compared to patients with a single event, those with recurrent thrombosis displayed significantly higher titers of IgG antiDI [40]. Likewise, IgG antiDIV/DV positivity was not associated with APS clinical manifestations thrombosis and pregnancy morbidity [40,54]. However, positive antiDI reactivity was associated with triple positivity, suggesting it may be used as a risk stratification tool in APS patients. Additionally, the ratio of antiDI to antiDIV/DV antibodies in this study emerged as an informative tool to identify those subjects carrying "nonpathogenic" or "less-pathogenic" antiβ2GPI antibodies [40]. Another study including 326 SLE patients showed that IgG antiβ2GPI (measured by Quanta Lite ELISA,INOVA) but not IgG antiDI reactivity was significantly associated with thrombosis (OR 3.3, 95% CI 1.2-8.9; OR 1.1, 95% CI 0.4-2.9, respectively) [53].

2.5 CLIA for antiDI

Using the same recombinant DI of β2GP1 from the baculovirus expression system [34] coupled to paramagnetic beads, more recently a CLIA assay [55] has been developed for the measurement of antiDI antibodies. After incubation of the paramagnetic beads with serum samples, isoluminol-labeled anti-human IgG antibodies are incubated with the aPLs previously captured by the paramagnetic particles. Finally, an agent is added to induce chemiluminescence. Currently, two systems are available that measure antiDI antibodies using this technology, including the BIO-FLASH CLIA from INOVA Diagnostics, Werfen, Austria and the HemosIL Acustar CLIA from Instrumentation Laboratory, Bedford, MA, USA. The BIO-FLASH CLIA and the HemosIL Acustar CLIA are identical assays using the same analytic method and reagent kits. Since 2014, this commercially available CLIA antiDI assay has been evaluated in 17 published studies [15,17,54,56-69].

For the classification of APS, the CLIA IgG antiDI assay has a higher specificity (from 82.1% to 100.0%) and a lower sensitivity (from 35.9% to 62.5%) compared to IgG antiβ2GPI test (from 71.7% to 99.1 % and from 46.2% to 82.3% for specificity and sensitivity, respectively) among seven studies (summarized in **Table 2**). For the association with clinical manifestations of APS, the ORs of IgG antiDI assays varied in eleven studies, ranging from 3.3 to 31.5 for thrombosis and from 1.5 to 14.6 for obstetric symptoms, probably originating from different patient and control cohorts and different cut-off values (summarized in **Table 3**). Few studies failed to demonstrate a significant association of antiDI reactivity with thrombosis [63,68]. In one study on thrombotic APS patients, 54% of the patients displayed antiβ2GPI antibodies, versus only 25% for antiDI [63]. IgG antiDI proved not to be associated with the site of the first event of thrombosis (OR 0.6, 95% CI 0.2-1.9), thrombosis recurrence (OR 1.0, 95% CI 0.4-2.7) nor pregnancy morbidity (OR 1.5, 95% CI 0.3-7.3) [63]. Another study in 178 SLE patients indicated that both the IgG antiDI titer and IgG antiβ2GPI titer were not associated with venous events (n = 22), arterial events (n = 20), composite venous events or arterial events (n = 37), respectively (P = 0.90, 0.76 and 0.89 for IgG antiDI titer and P = 0.86, 0.84 and 0.86 for IgG antiβ2GPI titer, respectively) [68]. Looking at a total population of 426 APS and control patients or at a subpopulation of 74 IgM/IgG antiβ2GPI

positive patients, IgG antiDI positivity was significantly associated with thrombosis (OR 14.4, 95% CI 6.0-34.8 and OR 31.5, 95% CI 5.4-182.1, respectively) [60]. However, in a subgroup of 60 antiβ2GPI IgG positive patients from the same study no significant association of IgG antiDI positivity with thrombosis was observed (OR 10.3, 95% CI 0.6-166.7) [60]. On the other hand, three out of five studies indicated IgG antiDI was associated with pregnancy morbidity [54,59,62]. In a cross-sectional study with 65 positive antiβ2GPI IgG patients, pregnancy loss was present in 16 out of 39 women (41%) positive for antiDI reactivity and in three out of 19 women (16%) with negative values. The association of IgG antiDI with obstetrical APS nearly reached statistical significance (P = 0.07) [59]. In a case-control study including 195 control women, 199 non-severe pre-eclampsia patients and 143 severe pre-eclampsia patients, antiDI IgG reactivity was associated with severe pre-eclampsia patients in the univariate analysis. However, in the final multivariate analysis, positive anti-β2GP1 IgG but not positive IgG antiDI was identified as a risk factor for severe pre-eclampsia [62]. A recently published study including 135 well-characterized female patients with persistent medium-high titre of antiβ2GPI antibodies and at least one pregnancy showed that reactivity against DI is a predictor for pregnancy morbidity (OR 2.4, 95% CI 1.2-5.0) [54]. More specifically, IgG antiDI significantly predicted late pregnancy morbidity (OR 7.3, 95% CI 2.1-25.5) [54]. In addition to the clinical symptoms of APS included in the criteria, one study in 32 APS patients indicated that the non-criteria manifestations livedo reticularis (n = 8) and heart value disease (n = 9) were associated with higher levels of IgG antiDI (p = 0.005 and P = 0.01, respectively) [56].

Furthermore, several studies assessed whether testing for antiDI adds value to current criteria laboratory tests (summarized in **Table 3**). Upon comparison of IgG antiDI assays and IgG antiβ2GPI assays, in four of seven studies the ORs of IgG antiDI exceeded those of IgG antiβ2GPI for clinical manifestations (three for thrombosis, one for thrombosis and pregnancy morbidity) [15,17,57,61]. In the remaining three studies, despite significant correlation of IgG antiDI with the clinical manifestations of APS, ORs of IgG antiDI positivity proved to be lower than IgG antiβ2GPI positivity [60,62,65]. In severe pre-eclampsia patients, a significant association was shown for IgG antiβ2GPI but not IgG antiDI in the final multivariate analysis [62]. Of note, one study also investigated the clinical significance of testing for IgG targeting other domains of β2GPI: neither thrombosis nor pregnancy morbidity was significantly correlated with IgG targeting other domains of β2GPI [17].

Different score systems have been formulated to quantify the risk of thrombosis/obstetric events in APS, including triple positivity (i.e. positive for LAC, IgG or IgM antiCL, IgG or IgM antiβ2GPI) [70] and antiphospholipid score (aPL-S) [71]. Eleven studies showed that triple positive patients tend to have a significantly higher prevalence and higher levels of IgG antiDI than those with double-positive or single-positive profile (summarized in **Table 4)**. In 180 patients with hypercoagulability, the thrombotic risk of the newly defined triple positive group (positive for LAC, IgG antiCL, IgG antiDI) was more than twice than that of the triple positive group (positive for LAC, IgG antiCL, IgG antiβ2GPI) [15]. Similarly, in 138 SLE patients, the test results of IgG antiDI raised the accuracy of predicting thrombosis compared to the test results of antiCL/ antiβ2GPI -ELISA, resulting in an increased area under the receiver operating characteristic (ROC) curve (AUC) (0.84 versus 0.80, respectively) [67]. However, in a retrospective study including 202 AID patients adding positivity for antiDI to the triple positivity profile did not increase the predicting capacity for APS thrombotic

complications [65]. Similar results were obtained in a cohort study in which no added value was demonstrated for antiDI to the criteria panel [60]. Also in this study, patients with a high aPL-S were shown to display higher titers of antiDI IgG [60]. In another study, an adjusted aPL-S was determined, measuring reactivity against DI instead of the whole molecule β2GPI (aPL-S-DI) [57]. When comparing the aPL-S-DI with the traditional aPL-S reaching the same specificity of 95%, the aPL-S-DI resulted in slightly lower OR for clinical symptoms of APS. However, when the optimal cut-off for each aPL-S was calculated, the aPL-S -DI resulted in the highest relative risk of having clinical manifestations of APS [57]. In a separate study, testing IgG antiDI and IgG/M anti-phosphatidylserine/prothrombin (PS/PT) showed a high positive predictive value for the diagnosis of APS and a strong correlation with the aPL-S was obtained [66]. Similarly, the thrombotic risk associated with the combination of IgG anti-PS/PT and IgG antiDI was elevated 4.5 times compared to double positivity for IgG anti-PS/PT and IgG antiβ2GPI [15].

3.Combined results on the clinical value of antiDI antibodies

3.1 Inconsistency on clinical value of antiDI antibodies

Most studies have shown that antiDI positivity significantly correlates with clinical manifestations of APS (**Table 2, 3** and **4)**. In terms of clinical performance, compared with the antiβ2GPI assays the antiDI assays in general seem to be less sensitive, but (slightly) more specific for the diagnosis of APS (**Table 2**). As the antiDI assays detect a pathogenic subpopulation of antibodies, the ORs for manifestations of APS were expected to be higher than the ORs of full length antiβ2GPI assays. However, results were inconsistent and dependent on the assays used to detect both antiβ2GPI and antiDI reactivity (**Table 3**). Nonetheless, significantly higher titers and prevalence of antiDI antibodies were found in high risk patients with triple positivity compared with double and single positive patients (**Table 4**). Moreover, adding of IgG antiDI or IgG antiDI instead of IgG antiβ2GPI in combined positive profile markedly raised the correlation with the risk of thrombosis in APS in several studies [15,49,66,67]. Furthermore, as with triple positivity, positive and negative values of IgG antiDI in initial test were consistently confirmed after 12 weeks, illustrating that IgG antiDI positivity is a robust and reproducible marker [59,72].

For different assays detecting IgG antiDI, the two-step antiDI ELISA in particular showed that the ORs of IgG antiDI are markedly higher than the OR of IgG antibodies targeting the whole length β2GPI or other domains of the protein [16,38]. Moreover, this assay emphasized the exposure of epitope G40-R43 domain I is important for antibody binding. CLIA is currently the most widely used method to detect IgG antiDI antibodies. IgG antiDI measured by CLIA seem to represent a strong indicator for clinical manifestations of APS. However, the results of the added clinical value of IgG antiDI are not consistent. Some studies showed that antiDI display no added clinical value to the classical aPLs panel [60,62,65]. None of the remaining three assays, the direct antiDI ELISA, the competitive inhibition ELISA and the commercial developed INOVA antiDI ELISA showed an added value of IgG antiDI compared to IgG antiβ2GPI. Taken together, the observed inconsistency probably explains why antiDI antibodies have not yet been included in the laboratory criteria [19].

Table 2. Clinical performance characteristics of different IgG antiDI assays: the sensitivity and specificity for APS

Publication		Study population					IgG antiDI				IgG antiβ2GPI			
Year	First author	Design	Patients	N	Control	N	Assay	Cut-off	Sensitivity	Specificity	Assay	Cut-off	Sensitivity	Specificity
2009	de Laat B [16]	R,multi-center	APS	364	SLE+LLD	78	Two-step ELISA	Mean ± 3SD	59.9%	67.9%	In-house ELISA	Mean ± 10SD	NA	NA
2014	Mondejar R [57]	R	APS	39	HC+RD	77	CLIA	20.0 AU (99^{th}p.)	35.9%	97.4%	CLIA	20.0 AU (99^{th}p.)	46.2%	90.9%
2015	Meneghel L [58]	R	PAPS	88	SN-APS + HC+RD	229	CLIA	7.1 CU (99^{th}p.)	54.5%	97.6%	CLIA	34.9 U/ml (99^{th}p.)	56.8%	96.4%
2016	De Craemer AS [60]	R	APS	101	AID+ DC+ HC	325	CLIA	20.0 CU (Manuf.)	53.5%	97.8%	CLIA	60.0 IU/ml (99^{th}p.)	56.4%	99.1%
2016	Oku K [64]	R	APS	61	SLE+ non-SLE CTD + DC+ID+HC	150	CLIA	20.0 CU/ml (99^{th}p.)	52.5%	100.0%	CLIA	20.0 CU/ml (99^{th}p.)	75.0%	90.2%
2016	Pericleous C [49]	R	APS	111	SLE+HC	319	Direct ELISA	10.0 GDIU (99^{th}p.)	40.5%	95.9%	In-house ELISA	8.0 GBU (99^{th}p.)	64.8%	95.6%
2016	Zhang S [17]	R	APS	86	DC+SLE+ HC	143	CLIA	20.0 CU (Manuf.)	46.5%	97.9%	CLIA	NS	66.3%*	92.3%*
2017	Iwaniec T [65]	R,CS	APS	103	SLE	99	CLIA	13.8 CU (99^{th}p.)	62.5%**	82.1%**	CLIA	NS	82.3%**	71.7%**
2017	Nakamura H [66]	CS	APS	51	AID	106	CLIA	20.0 CU (99^{th}p.)	60.8%	100.0%	In-house ELISA	2.2 U/ml (99^{th}p.)	62.8%	96.2%

APS: antiphospholipid syndrome; AID: non- APS autoimmune diseases; AU: arbitrary unit; β2GPI: β2-glycoprotein I; CLIA: chemiluminescence immunoassay; CS: cross-sectional study; CU: chemiluminescence units; CTD: connective tissue diseases; DC: disease control i.e. non-APS with clinical symptoms of APS (thrombosis, pregnancy complication); DI: domain I of β2GPI; ELISA: enzyme-linked immunosorbent assay; GDIU: IgG antiDI units; GBU: IgG anti- β2GPI units; HC: healthy control; ID: infectious disease; IU: international units; LLD: lupus like disease; Mean ± 3 or 10SD: mean optical density (OD) plus 3 or 10 times standard deviation (SD); Manuf.: manufacturer; N: number of population; NA: not application; NS: not specified; SLE: systemic lupus erythematosus; non-SLE CTD: non-SLE connective tissue diseases; PAPS: primary antiphospholipid syndrome; R: retrospective study; RD: rheumatic disease; SN-APS: seronegative APS; U: units; 99^{th}p.: 99^{th} percentile. *IgG and/or IgM positive, ** for APS thrombotic complications.

Table 3. Clinical performance characteristics of different IgG antiDI assays: the correlation of IgG antiDI with clinical manifestations of APS by odd radios (ORs)

Publication		Study			IgG antiDI					IgG antiβ2GPI				
Year	First author	Design	Population	N	Assay	Cut-off	OR (95%CI) for T	OR (95%CI) for P	OR (95%CI) for T/P	Assay	Cut-off	OR (95%CI) for T	OR (95%CI) for P	OR (95%CI) for T/P
2005	de Laat B [38]	R	176 SLE+16 LLD+6 PAPS	198	Two-step ELISA	Mean ± 3SD	**18.9 (6.8-53.2)**	NS	NS	In-house ELISA	Mean ± 3SD	**6.7 (3.4-13.5)**	NS	NS
			IgG antiβ2GPI positive	52	Two-step ELISA	Mean ± 3SD	**10.7**	NA	NA	In-house ELISA	Mean ± 3SD	NA	NA	NA
2009	de laat B [16]	R, multicenter	IgG/ IgM antiβ2GPI positive (364 APS+93 SLE+ 35 LLD)	442	Two-step ELISA	Mean ± 3SD	**3.5 (2.3-5.4)**	**2.4 (1.4-4.3)** [#]	NS	In-house ELISA	Mean ± 10SD	NA	1.5 (0.6-3.7) [#]	NA
			IgG antiβ2GPI positive	420	Two-step ELISA	Mean ± 3SD	**3.3 (2.1-5.2)**	NS	NS	In-house ELISA	Mean ± 10SD	NA	NA	NA
2013	Akhter E [53]	CS	SLE	326	Commercial ELISA	25.0 U (97thp.)	1.1 (0.4-2.9)	NS	NS	Commercial ELISA	NS	**3.3 (1.2-8.9)**	NS	NS
2014	Mondejar R [57]	R	39 APS+47 RD+30 HC	116	CLIA	20.0 AU (99thp.)	NS	NS	**21.0 (4.5-98.9)**	CLIA	20.0 AU (99thp.)	NS	NS	**8.6 (3.2-23.3)**
2015	Andreoli L [40]	R	IgG antiβ2GPI positive (87 PAPS+42 RD+ 30 aPLs-carriers)	159	Commercial ELISA	15.0AU (95thp.)	1.7	2.4[#]	NS	Commercial ELISA	NS	NA	NA	NA
2015	Pengo V [59]	CS	IgG antiβ2GPI positive	65	CLIA	14.2 CU (99thp.)	**7.3**	**3.7**[$]	NS	CLIA	19.4 CU (99thp.)	NA	NA	NA
2016	De Craemer AS [60]	R	101 APS+123 AID+ 82 DC+120 HC	426	CLIA	20.0 CU (Manuf.)	**14.4 (6.0-34.8)**	NS	17.0 (7.1-40.5)	CLIA	60.0 IU/ml (99thp.)	**29.2 (8.8-95.9)**	NS	**36.2 (11.1-117.9)**
			IgG/ IgM antiβ2GPI positive	74	CLIA	20.0 CU (Manuf.)	**31.5 (5.4-182.1)**	NS	**27.0 (5.0-145.9)**	CLIA	60.0 IU/ml (99thp.)	NS	NS	NS
			IgG antiβ2GPI positive	60	CLIA	20.0 CU (Manuf.)	10.3 (0.6-166.7)	NS	6.6 (0.5-89.8)	CLIA	60.0 IU/ml (99thp.)	NA	NA	NA
2016	Mahler M [61]	CS	APS	106	CLIA	20.0 CU (99.5thp.)	**4.0**	NS	NS	CLIA	20.0 CU (99.5thp.)	**2.3**	NS	NS
					CLIA	190.2 CU (optimized)	**8.7**	NS	NS	CLIA	164.6 CU (optimize)	**4.1**	NS	NS
2016	Marchetti T [62]	CC	143 S-PEecl+199 NS-PEecl+195 HC	537	CLIA	14.4 AU(99thp.)	NA	**14.6 (1.8-115.6)** *[#]	NS	CLIA	17.0 IU/ml (99thp.)	NS	**16.9 (3.7-77.1)** **[#]	NA
2016	Zhang S [17]	R	86 APS+62 DC+42 SLE+39 HC	229	CLIA	20.0 CU (Manuf.)	**3.3 (1.6-6.7)**	1.6 (0.6-3.7) [§]	NS	CLIA	NS	**2.8 (1.5-5.1)**	1.3 (0.6-2.7) [§]	NS
2017	Iwaniec T [65]	R, CS	103 APS+99 SLE	202	CLIA	13.8 CU (99thp.)	**7.6 (4.0-14.5)**	NS	NS	CLIA	NS	**11.8 (6.0-23.0)**	NS	NS
2017	Lee JS [15]	R	hypercoagulability	180	CLIA	40.0 CU (99th p.)	**15.4 (4.3-54.9)**	NS	NS	CLIA	20.0 CU (99th p.)	2.5 (0.98-6.5)	NS	NS
2017	Nojima J [67]		SLE	138	CLIA	NS	**9.2 (2.5-34.2)***	NS	NS	ELISA	NS	NS	NS	NS
2018	Chighizola CB [54]	CC	women IgG antiβ2GPI positive	130	CLIA	20,0 CU (NS)	**5.4 (2.4-12.0)**	**2.4 (1.2-5.0)**	NS	CLIA	20.0 CU (NS)	NA	NA	NA

APS: antiphospholipid syndrome; AID: non- APS autoimmune diseases; aPLs-carriers: asymptomatic antiphospholipid antibodies carriers; AU: arbitrary units; β2GPI: β2 glycoprotein I; CC: case-control; CLIA: chemiluminescence immunoassay; CS: cross-sectional study; CU: chemiluminescence units; DC: disease control, i.e. non-APS with clinical symptoms of APS (thrombosis, pregnancy complication); DI: domain I of β2-glycoprotein I (β2GPI); ELISA: enzyme-linked immunosorbent assay; HC: healthy control; IU: international units; LLD: lupus like disease; Mean ± 3 or 10SD: mean optical density (OD) plus 3 or 10 times standard deviation (SD); Manuf.: manufacturer; N: number of population; NA: not application; NS: not specified; NS-PEecl: non-severe pre-eclampsia; OR (95% CI): odds ratio (95% confidence interval); P: pregnancy morbidity; PAPS: primary antiphospholipid syndrome; R: retrospective study; RD: rheumatic disease; SLE: systemic lupus erythematosus; S-PEecl: serve pre-eclampsia; T: thrombosis; U: units; 95th/97th/99th/99.5thp.: 95th/97th/99th/99.5th percentile. *by univariate analysis; **by multivariate analysis. [®]OR (95% CI) for venous thrombosis; [#]OR (95% CI) for P: only women with at least 1 previous pregnancy were included; [$]OR (95% CI) for P: only women were included; [§]OR (95% CI) for P: only married women of reproductive age were included. Bold: significant association of assay with clinical symptoms.

Table 4. The prevalence of IgG antiDI positive in triple, double and single positive patients

Publication		Study			IgG antiDI positive			
Year	First author	Design	Population	N	Assay	Cut-off	Prevalence	P value
2011	Banzato A [51]	R	Triple positive [A]	22	Competitive inhibition ELISA	NA	25.5%	S*
			Double positive	15			5.0%	
			Single positive	9			2.0%	
			Control	20			0.0%	
2015	Andreoli L [40]	R	Triple positive [B]	87	Commercial ELISA	15.0 AU (95thp.)	80.4%	S*
			Double/single positive	72			48.6%	
2015	Meneghel L [58]	R	Triple positive [B]	NS	CLIA	7.1 CU (99thp.)	94.1%	S*
			Double positive	NS			45.5%	
			Single positive	NS			4.8%	
			SN-APS/HC	NS			1.6%/0.6%	
2015	Pengo V [59]	CS	Triple positive [A]	32	CLIA	14.2 CU (99thp.)	97.0%	S*
			Double positive	23			43.0%	
			Single positive	10			10.0%	
2016	De Craemer AS [60]	R	Triple positive [B]	62	CLIA	20.0 CU (Manuf.)	83.9%	S*
			Double positive	4			50.0%	
			Single positive	4			0.0%	
			Triple negative	222			0.9%	
2016	Montalvão S [63]	R	Triple positive [B]	13	CLIA	20.0 CU (Manuf.)	46.0%	S*
			Double/single positive	28			17.0%	
2016	Zhang S [17]	R	Triple/Double/single positive [B]	229	CLIA	20.0 CU (Manuf.)	NS	S*
2017	Iwaniec T [65]	R,CS	Triple positive [B]	79	CLIA	13.8 CU (99thp.)	308.2 [&]	S*
			Double positive	10			6.2 [&]	
			Single positive	14			2.0[&]	
2017	Lee JS [15]	R	Triple positive [A]	17	CLIA	40.0 CU (99thp.)	58.8%	S*
			Double positive	27			7.4%	
			Single positive	93			0.0%	
			Triple negative	43			0.0%	
2018	Chighizola CB [54]	CC	Triple positive [B]	82	CLIA	20.0 CU (NS)	195.0 ± 628.3[§]	S*
			Double/single positive	53			4.0 ± 11.0[§]	
2018	Marchetti T [68]	Longitudinal study	Triple positive [B]	22	CLIA	14.4 AU (99 thp.)	365.4 ± 596.1[§]	S*
			Double positive	13			137.9 ± 434.1[§]	
			Single positive	40			3.8 ± 8.7[§]	

AU: arbitrary units; CLIA: chemiluminescence immunoassay; CS: cross-sectional study; CU: chemiluminescence unit; DI: domain I β2-glycoprotein I (β2GPI); ELISA: enzyme-linked immunosorbent assay; HC: healthy control; Manuf.: manufacturer; N: number of population; NA: not applicable; NS: not specified; R: retrospective study; SN-APS: seronegative APS; 95th/99thp.: 95th/99th percentile; S*: significantly higher prevalence or levels of IgG antiDI positive were found in patients with triple positivity, compared with patients with double and single aPL positivity; &: median value of IgG antiDI; §: mean ± standard deviation (SD) value of IgG antiDI; A: IgG triple positive = positive lupus anticoagulant (LAC) , IgG antiCL, IgG antiβ2GPI ; B: Triple positive = positive LAC, IgG or IgM antiCL, and IgG or IgM antiβ2GPI .

3.2 Towards an explanation for the observed discrepancies

The studies included in this review differ in study design, study population and the methodology to measure the antiDI antibodies. These factors, together with the absence of standardization or calibration, make it very difficult to compare results of clinical studies and accurately assess the clinical value of measuring antiDI. Even using the same assay for detection of antiDI has led to discrepant results in determination of the added value of antiDI antibodies. Differences in methods of calculating cut-off values, statistical analysis method, laboratory-specific handlings or protocols and different study populations may affect the interpretation of the results and lead to the observed discrepancies. Two studies independently showed the effect of different cut-off values: increasing cut-off values resulted in significantly higher ORs [61,73]. One study including patients with APS, AID, disease controls and healthy controls showed different results for correlation of IgG antiDI positivity with thrombosis in the total population of 426 patients versus in 60 IgG antiβ2GPI positive patients [60].

A similar situation is observed for the antiβ2GPI assays, in which external quality assessment program reports show a wide variability in results over different centers [74-77]. Previous research has shown the importance of a certain antigen density to enable divalent binding of antibodies [78,79], as well as a hydrophilic solid phase surface to coat β2GPI thereby inducing a conformational change resulting in the exposure of a cryptic pathogenic epitope in DI. Antibodies directed to this cryptic epitope G40-R43 are a major pathological subset of antibodies [27-29,35-38,41]. Hence, results obtained by antiβ2GPI assays depend on the density and the conformation of the coated β2GPI (i.e. the exposure of the epitope G40-R43), which are affected by the type of solid phase surface used to immobilize β2GPI and source of protein. As in antiDI assays DI is coated instead of the full protein, similar problems were not expected. However, taken into account the positive charge of epitope G40-R43, the charge of the coating surface possibly influences the availability of the G40-R43 epitope. A neutral coating surface is hypothesized to result in an arbitrary orientation of the DI, resulting in exposure of epitope G40-R43. On the other hand, a negative surface binds the positive epitope preventing its availability for antibodies. Based on this hypothesis and the evidence provided with the two-step ELISA, we assume that the differences in the type of solid phase surface used to immobilize DI and hence the exposure of the pathogenic DI epitope in the available antiDI assays that add up to the variation in results. Looking at the other antiDI assays, a possible charge of the beads cannot be excluded, rendering the exposure of epitope G40-R43 on DI uncertain. Exactly the same question can be made for the other available assays, as except for the two- step antiDI ELISA, the charge of the solid phase surfaces used to immobilize DI are unknown. This may provide an explanation why both studies using the commercially developed antiDI ELISA from INOVA Diagnostics to measure antiDI showed that there is no significant association with thrombosis [40,53]. A clinical study using an in-house developed direct antiDI ELISA showed significantly but less clinical value of antiDI compared to antiβ2GPI [49]. Similarly, although with a competitive inhibition antiDI ELISA a significant difference was found in patients with triple positivity compared with patients with double or single positivity and healthy controls, a direct antiDI ELISA with the same DI did not find differences in IgG antiDI between APS patients and controls, independent of using hydrophobic or hydrophilic plates, different coating/washing/blocking buffers and concentrations of DI [51]. In addition, although CLIA

can provide a greater surface for antibodies binding to DI, three studies showed significantly but less clinical value of IgG antiDI compared to IgG antiβ2GPI [60,62,65]. This problem seems to be avoided in the in-house developed two-step ELISA, where the ratio between the OD measured with a hydrophobic plate versus hydrophilic plate is used to determine positivity.

We aimed to verify our hypothesis by determining the exposure of the pathogenic G40-R43 epitope in the commercially available aDI CLIA method. Therefore, normal pooled plasma was spiked with two human-derived monoclonal antibodies P1-117 and P2-6. Antibody P1-117, recognizing epitope G40-R43 only available when β2GPI is in its open conformation, and P2-6, recognizing domain I independently of its conformation [80]. Our unpublished results show that P2-6 can be detected while no signal is obtained for P1-117 (data not shown). Contrary to the expectation, this antiDI assay thereby does not expose the epitope G40-R43 on the surface of the beads, therefore patient samples with antibodies recognizing the epitope G40-R43 will be missed. Our results are consistent with previous results obtained with the CLIA antiβ2GPI assay (HemosIL Acustar) in which P1-117 showed lower reactivity compared to P2-6, demonstrating a reduced G40-R43 availability [81]. In addition, a high agreement (69% ~ 92%) was observed between antiβ2GPI and antiDI in the same CLIA device [17,59,60,65], suggesting that both assays measure almost the same antibody population directed against domain I but not to the G40-R43 epitope. On the other hand, two studies detecting IgG antiDI using the two-step ELISA found that approximately half (58% and 57%, respectively) of IgG antiβ2GPI bound DI [16,38]. Taken together, these results suggest that the two-step ELISA measures a more specific antiDI antibody population directed against G40-R43, compared to the commercially available antiDI CLIA assay detecting all antibodies to domain I, as well as to other domains. As a result, the two-step ELISA did show an added clinical value of the antiDI positivity compared to the full-length antiβ2GPI , demonstrated by the higher OR for thrombosis and pregnancy morbidity [16,38]. Our data highlight the importance of not only measuring G40-R43 exposure in the full length antiβ2GPI assays, but also in the antiDI assays to be certain that at least this pathogenic antibody population is not missed.

Apart from the coating principle, also the density of the coated DI and the source of DI may influence the results. Regarding the density, especially the CLIA with paramagnetic beads provide a three-dimensional platform and larger surface for antibodies binding to DI [82]. Three different sources of DI are used in the available assays: DI obtained through a baculovirus expression system, an Escherichia Coli expression system or by a chemical synthesis. So far, the effect of production on the conformation of DI and hence the availability of the pathogenic G40-R43 epitope is not known.

4. Conclusion

In summary, given all the in vitro and in vivo evidence, antiDI assays were expected to be very powerful in the classification of APS patients. The majority of clinical studies did find a significant association of antiDI antibodies with clinical symptoms of APS and a higher specificity was demonstrated compared with antiβ2GPI antibodies. As specifically antibodies to DI are detected, a more uniform result was expected, resulting in an improved risk stratification and tailored

treatment for APS patients. However, multiple antiDI assays are available and suffer from the same problems as the full-length antiβ2GPI assays. To assess the added value of antiDI assays, several important issues urgently need to be addressed. As for antiβ2GPI assays, standardization of antiDI assays is of utmost importance. This standardization in our view also includes the confirmation of a satisfactory exposure of the epitope G40-R43 to ascertain that at least one specific pathogenic antibody population is measured. Additionally, so far only retrospective studies were performed to determine the added value of DI antibodies. Prospective, well designed multicenter studies are urgently required to clarify the clinical utility of the antiDI assays.

References

1. Ruiz-Irastorza G, Crowther M, Branch W, Khamashta MA. Antiphospholipid syndrome. *Lancet* 2010; **376**: 1498-509.
2. Ortel TL. Antiphospholipid syndrome: laboratory testing and diagnostic strategies. *American journal of hematology* 2012; **87 Suppl 1**: S75-81.
3. Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *Journal of thrombosis and haemostasis* 2006; **4**(2): 295-306.
4. Asiful Islam M, Alam F, Cavestro C, et al. Antiphospholipid antibodies in epilepsy: A systematic review and meta-analysis. *Autoimmunity reviews* 2018: doi: 10.1016/j.autrev.2018.01.025. [Epub ahead of print].
5. Islam MA, Alam F, Wong KK. Comorbid association of antiphospholipid antibodies and migraine: A systematic review and meta-analysis. *Autoimmunity reviews* 2017; **16**(5): 512-22.
6. Hoxha A, Banzato A, Ruffatti A, Pengo V. Detection of lupus anticoagulant in the era of direct oral anticoagulants. *Autoimmunity reviews* 2017; **16**(2): 173-8.
7. van den Hoogen LL, van Roon JA, Radstake TR, Fritsch-Stork RD, Derksen RH. Delineating the deranged immune system in the antiphospholipid syndrome. *Autoimmunity reviews* 2016; **15**(1): 50-60.
8. 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**(7): 2340-6.
9. Arad A, Proulle V, Furie RA, Furie BC, Furie B. beta(2)-Glycoprotein-1 autoantibodies from patients with antiphospholipid syndrome are sufficient to potentiate arterial thrombus formation in a mouse model. *Blood* 2011; **117**(12): 3453-9.
10. Jankowski M, Vreys I, Wittevrongel C, et al. Thrombogenicity of β2-glycoprotein I-dependent antiphospholipid antibodies in a photochemically induced thrombosis model in the hamster. *Blood* 2003; **101**(1): 157-62.
11. Kelchtermans H, Pelkmans L, de Laat B, Devreese KM. IgG/IgM antiphospholipid antibodies present in the classification criteria for the antiphospholipid syndrome: a critical review of their association with thrombosis. *Journal of thrombosis and haemostasis* 2016; **14**(8): 1530-48.
12. Abou-Nassar K, Carrier M, Ramsay T, Rodger MA. The association between antiphospholipid antibodies and placenta mediated complications: A systematic review and meta-analysis. *Thrombosis Research* 2011; **128**(1): 77-85.
13. Gris JC, Bouvier S, Nouvellon E, et al. Antiphospholid antibodies and the risk of pregnancy complications. *Thrombosis Research* 2017; **151 Suppl 1**: S34-S7.
14. 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**(8): 2717-23.
15. Lee JS, Gu J, Park HS, Yoo HJ, Kim HK. Coexistence of anti-beta2-glycoprotein I domain I and anti-phosphatidylserine/prothrombin antibodies suggests strong thrombotic risk. *Clinical Chemistry and Laboratory Medicine* 2017; **55**(6): 882-9.

16. de Laat B, Pengo V, Pabinger I, et al. The association between circulating antibodies against domain I of beta2-glycoprotein I and thrombosis: an international multicenter study. *Journal of thrombosis and haemostasis* 2009; **7**(11): 1767-73.
17. Zhang S, Wu Z, Chen S, et al. Evaluation of the diagnostic potential of antibodies to beta2-glycoprotein 1 domain 1 in Chinese patients with antiphospholipid syndrome. *Scientific reports* 2016; **6**: 23839.
18. Devreese K. Standardization of antiphospholipid antibody assays.Where do we stand? *Lupus* 2012; **21**: 718-21.
19. Devreese KM, Ortel TL, Pengo V, de Laat B. Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibodies. Laboratory criteria for antiphospholipid syndrome: communication from the SSC of the ISTH. *Journal of thrombosis and haemostasis* 2018; **16**(4): 809-13.
20. Bertolaccini ML, Amengual O, Andreoli L, et al. 14th International Congress on Antiphospholipid Antibodies Task Force. Report on antiphospholipid syndrome laboratory diagnostics and trends. *Autoimmunity reviews* 2014; **13**(9): 917-30.
21. Devreese KM, Pierangeli SS, de Laat B, et al. Testing for antiphospholipid antibodies with solid phase assays: guidance from the SSC of the ISTH. *Journal of thrombosis and haemostasis* 2014; **12**(5): 792-5.
22. Pelkmans L, de Laat B. Antibodies against domain I of beta2-glycoprotein I: the one and only? *Lupus* 2012; **21**(7): 769-72.
23. Pengo V, Ruffatti A, Tonello M, et al. Antibodies to Domain 4/5 (Dm4/5) of beta2-Glycoprotein 1 (beta2GP1) in different antiphospholipid (aPL) antibody profiles. *Thrombosis Research* 2015; **136**(1): 161-3.
24. Andreoli L, Nalli C, Motta M, et al. Anti-beta(2)-glycoprotein I IgG antibodies from 1-year-old healthy children born to mothers with systemic autoimmune diseases preferentially target domain 4/5: might it be the reason for their 'innocent' profile? *Annals of the Rheumatic Diseases* 2011; **70**(2): 380-3.
25. Artenjak A, Locatelli I, Brelih H, et al. Immunoreactivity and avidity of IgG anti-beta2-glycoprotein I antibodies from patients with autoimmune diseases to different peptide clusters of beta2-glycoprotein I. *Immunologic research* 2015; **61**(1-2): 35-44.
26. de Groot PG, Meijers JC. beta(2) -Glycoprotein I: evolution, structure and function. *Journal of thrombosis and haemostasis* 2011; **9**(7): 1275-84.
27. 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**(5): 1916-24.
28. Agar C, van Os GM, Morgelin M, et al. Beta2-glycoprotein I can exist in 2 conformations: implications for our understanding of the antiphospholipid syndrome. *Blood* 2010; **116**(8): 1336-43.
29. de Laat B, van Berkel M, Urbanus RT, et al. Immune responses against domain I of beta(2)-glycoprotein I are driven by conformational changes: domain I of beta(2)-glycoprotein I harbors a cryptic immunogenic epitope. *Arthritis & Rheumatology* 2011; **63**(12): 3960-8.

30. By Makoto Igarashi EM, Yoshiko Igarashi, Hisato Nagae, Kenji Ichikawa, Douglas A. Triplett,Takao Koike. Human β2-Glycoprotein I as an Anticardiolipin Cofactor Determined Using Deleted Mutants Expressed by a Baculovirus System. *Blood* 1996; **87**(8): 3262-327.

31. Iverson GM, Victoria EJ, Marquis DM. Anti-β2 glycoprotein I (β2GPI) autoantibodies recognize an epitope on the first domain of β2GPI. *Proceedings of the National Academy of Sciences of the U S A* 1998; **95**(26): 15542-6.

32. Reddel SW, Wang YX, Sheng YH, Krilis SA. Epitope studies with anti-beta 2-glycoprotein I antibodies from autoantibody and immunized sources. *Journal of Autoimmunity* 2000; **15**(2): 91-6.

33. Patricia A. McNeeley JSD, Richard A. Furie, Richard M. Jack,Thomas L. Ortel, Douglas A.Triplett, Edward J. Victoria,Matthew D. Linnik. beta2-glycoprotein I-dependent Anticardiolipin Antibodies Preferentially Bind the Amino Terminal Domain of beta2-glycoprotein I. *Thrombosis and Haemostasis* 2001; **86**: 590-5.

34. Iverson GM, Reddel S, Victoria EJ, et al. Use of Single Point Mutations in Domain I of 2-Glycoprotein I to Determine Fine Antigenic Specificity of Antiphospholipid Autoantibodies. *Journal of immunology* 2002; **169**(12): 7097-103.

35. Ioannou Y, Pericleous C, Giles I, Latchman DS, Isenberg DA, Rahman A. Binding of antiphospholipid antibodies to discontinuous epitopes on domain I of human beta(2)-glycoprotein I: mutation studies including residues R39 to R43. *Arthritis and rheumatism* 2007; **56**(1): 280-90.

36. Giannakopoulos B, Krilis SA. The pathogenesis of the antiphospholipid syndrome. *The New England journal of medicine* 2013; **368**(11): 1033-44.

37. de Groot PG, de Laat B. Mechanisms of thrombosis in systemic lupus erythematosus and antiphospholipid syndrome. *Best Practice & Research Clinical Rheumatology* 2017; **31**(3): 334-41.

38. 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**(4): 1540-5.

39. Pengo V, Bison E, Zoppellaro G, et al. APS - Diagnostics and challenges for the future. *Autoimmunity reviews* 2016; **15**(11): 1031-3.

40. Andreoli L, Chighizola CB, Nalli C, et al. Clinical characterization of antiphospholipid syndrome by detection of IgG antibodies against beta2 -glycoprotein i domain 1 and domain 4/5: ratio of anti-domain 1 to anti-domain 4/5 as a useful new biomarker for antiphospholipid syndrome. *Arthritis & Rheumatology* 2015; **67**(8): 2196-204.

41. Ioannou Y, Romay-Penabad Z, Pericleous C, et al. In vivo inhibition of antiphospholipid antibody-induced pathogenicity utilizing the antigenic target peptide domain I of beta2-glycoprotein I: proof of concept. *Journal of thrombosis and haemostasis* 2009; **7**(5): 833-42.

42. McDonnell T, Pericleous C, Laurine E, et al. Development of a high yield expression and purification system for Domain I of Beta-2-glycoprotein I for the treatment of APS. *BMC biotechnology* 2015; **15**: 104.

43. Agostinis C, Durigutto P, Sblattero D, et al. A non-complement-fixing antibody to beta2 glycoprotein I as a novel therapy for antiphospholipid syndrome. *Blood* 2014; **123**(22): 3478-87.

44. Pericleous C, Ruiz-Limon P, Romay-Penabad Z, et al. Proof-of-concept study demonstrating the pathogenicity of affinity-purified IgG antibodies directed to domain I of beta2-

glycoprotein I in a mouse model of anti-phospholipid antibody-induced thrombosis. *Rheumatology (Oxford)* 2015; **54**(4): 722-7.

45. Pelkmans L, Kelchtermans H, de Groot PG, et al. Variability in exposure of epitope G40-R43 of domain i in commercial anti-beta2-glycoprotein I IgG ELISAs. *PloS one* 2013; **8**(8): e71402.

46. Wahezi DM, Ilowite NT, Wu XX, et al. Annexin A5 anticoagulant activity in children with systemic lupus erythematosus and the association with antibodies to domain I of beta2-glycoprotein I. *Lupus* 2013; **22**(7): 702-11.

47. Ioannou Y, Giles I, Lambrianides A, et al. A novel expression system of domain I of human beta2 glycoprotein I in Escherichia coli. *BMC biotechnology* 2006; **6**: 8.

48. Cousins L, Pericleous C, Khamashta M, et al. Antibodies to domain I of β-2-glycoprotein I and IgA antiphospholipid antibodies in patients with 'seronegative'antiphospholipid syndrome. *Annals of the rheumatic diseases* 2015; **74**: 317-9.

49. Pericleous C, Ferreira I, Borghi O, et al. Measuring IgA Anti-beta2-Glycoprotein I and IgG/IgA Anti-Domain I Antibodies Adds Value to Current Serological Assays for the Antiphospholipid Syndrome. *PloS one* 2016; **11**(6): e0156407.

50. Pozzi N, Banzato A, Bettin S, Bison E, Pengo V, De Filippis V. Chemical synthesis and characterization of wild-type and biotinylated N-terminal domain 1-64 of beta2-glycoprotein I. *Protein Science* 2010; **19**(5): 1065-78.

51. Banzato A, Pozzi N, Frasson R, et al. Antibodies to Domain I of beta(2)Glycoprotein I are in close relation to patients risk categories in Antiphospholipid Syndrome (APS). *Thrombosis Research* 2011; **128**(6): 583-6.

52. Pengo V, Ruffatti A, Legnani C, et al. Clinical course of high-risk patients diagnosed with antiphospholipid syndrome. *Journal of thrombosis and haemostasis* 2010; **8**(2): 237-42.

53. Akhter E, Shums Z, Norman GL, Binder W, Fang H, Petri M. Utility of antiphosphatidylserine/prothrombin and IgA antiphospholipid assays in systemic lupus erythematosus. *The Journal of Rheumatology*2013; **40**(3): 282-6.

54. Chighizola CB, Pregnolato F, Andreoli L, et al. Beyond thrombosis: Anti-β2GPI domain 1 antibodies identify late pregnancy morbidity in anti-phospholipid syndrome. *Journal of Autoimmunity* 2018; **90**: 76-83.

55. Mahler M, Radice A, Yang W, et al. Development and performance evaluation of novel chemiluminescence assays for detection of anti-PR3 and anti-MPO antibodies. *Clinica chimica acta; international journal of clinical chemistry* 2012; **413**(7-8): 719-26.

56. Cieśla M, Wypasek E, Undas A. IgA antiphospholipid antibodies and anti-domain 1 of beta 2 glycoprotein 1 antibodies are associated with livedo reticularis and heart valve disease in antiphospholipid syndrome. 2014; **23**(5): 729-33.

57. Mondejar R, Gonzalez-Rodriguez C, Toyos-Saenz de Miera FJ, et al. Role of antiphospholipid score and anti-beta2-glycoprotein I Domain I autoantibodies in the diagnosis of antiphospholipid syndrome. *Clinica chimica acta; international journal of clinical chemistry* 2014; **431**: 174-8.

58. Meneghel L, Ruffatti A, Gavasso S, et al. Detection of IgG anti-Domain I beta2 Glycoprotein I antibodies by chemiluminescence immunoassay in primary antiphospholipid syndrome. *Clinica chimica acta; international journal of clinical chemistry* 2015; **446**: 201-5.

59. Pengo V, Ruffatti A, Tonello M, et al. Antiphospholipid syndrome: antibodies to Domain 1 of beta2-glycoprotein 1 correctly classify patients at risk. *Journal of thrombosis and haemostasis* 2015; **13**(5): 782-7.
60. De Craemer AS, Musial J, Devreese KM. Role of anti-domain 1-beta2 glycoprotein I antibodies in the diagnosis and risk stratification of antiphospholipid syndrome. *Journal of thrombosis and haemostasis* 2016; **14**(9): 1779-87.
61. Mahler M, Albesa R, Zohoury N, et al. Autoantibodies to domain 1 of beta 2 glycoprotein I determined using a novel chemiluminescence immunoassay demonstrate association with thrombosis in patients with antiphospholipid syndrome. *Lupus* 2016; **25**(8): 911-6.
62. Marchetti T, de Moerloose P, Gris JC. Antiphospholipid antibodies and the risk of severe and non-severe pre-eclampsia: the NOHA case-control study. *Journal of thrombosis and haemostasis* 2016; **14**(4): 675-84.
63. Montalvão S, Elídio PS, da Silva Saraiva S, et al. Clinical implications of the detection of antibodies directed against domain 1 of beta2-glycoprotein 1 in thrombotic antiphospholipid syndrome. *Thrombosis Research* 2016; **148**: 32-7.
64. Oku K, Amengual O, Kato M, et al. Significance of fully automated tests for the diagnosis of antiphospholipid syndrome. *Thrombosis Research* 2016; **146**: 1-6.
65. Iwaniec T, Kaczor MP, Celinska-Lowenhoff M, Polanski S, Musial J. Clinical significance of anti-domain 1 beta2-glycoprotein I antibodies in antiphospholipid syndrome. *Thrombosis Research* 2017; **153**: 90-4.
66. Nakamura H, Oku K, Olga Amengual, et al. First-line, Non-criterial Antiphospholipid Antibody Testing for the Diagnosis of Antiphospholipid Syndrome in Clinical Practice: a Combination of Anti-beta2-glycoprotein I Domain I and Phosphatidylserine-dependent Antiprothrombin Antibodies. *Arthritis care & research* 2017: 1-46.
67. Nojima J, Motoki Y, Hara K, Sakata T, Tsuneoka H, Ichihara K. Detection of antibodies against domain 1 of β2-glycoprotein I is key in predicting thromboembolic complications in patients with systemic lupus erythematosus. *Thrombosis Research* 2017; **153**: 83-4.
68. Marchetti T, Ribi C, Perneger T, et al. Prevalence, persistence and clinical correlations of classic and novel antiphospholipid antibodies in systemic lupus erythematosus. *Rheumatology (Oxford)* 2018: Epub ahead of print.
69. Zohoury N, Bertolaccini ML, Rodriguez-Garcia JL, et al. Closing the Serological Gap in the Antiphospholipid Syndrome: The Value of "Non-criteria" Antiphospholipid Antibodies. *The Journal of Rheumatology*2017; **44**(11): 1597-602.
70. Sciascia S, Cosseddu D, Montaruli B, Kuzenko A, Bertero MT. Risk Scale for the diagnosis of antiphospholipid syndrome. *Annals of the Rheumatic Diseases* 2011; **70**(8): 1517-8.
71. Otomo K, Atsumi T, Amengual O, et al. Efficacy of the antiphospholipid score for the diagnosis of antiphospholipid syndrome and its predictive value for thrombotic events. *Arthritis & Rheumatology* 2012; **64**(2): 504-12.
72. Pengo V, Ruffatti A, Del Ross T, et al. Confirmation of initial antiphospholipid antibody positivity depends on the antiphospholipid antibody profile. *Journal of thrombosis and haemostasis* 2013; **11**(8): 1527-31.

73. De Craemer AS, Devreese KM. Role of anti-domain 1 ß2glycoprotein I antibodies in the diagnosis and risk stratification of antiphospholipid syndrome: reply. *Journal of thrombosis and haemostasis* 2016; **14**: 2078-80.
74. 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**(2): 181-5.
75. Reber G TA, Sanmarco M, de Moerloose P, Boffa M. C. Proposals for the measurement of anti-b2-glycoprotein I antibodies. Standardization Group of the European Forum on Antiphospholipid Antibodies. *Journal of thrombosis and haemostasis* 2004; **2**: 1860-2.
76. Tincani A, Allegri F, Balestrieri G, et al. Minimal requirements for antiphospholipid antibodies ELISAs proposed by the European Forum on antiphospholipid antibodies. *Thrombosis Research* 2004; **114**(5-6): 553-8.
77. Pengo V, Biasiolo A, Bison E, Chantarangkul V, Tripodi A, Italian Federation of Anticoagulation C. 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. *Thrombosis Research* 2007; **120**(1): 127-33.
78. Urbanus RT, de Groot PG. Antiphospholipid antibodies--we are not quite there yet. *Blood reviews* 2011; **25**(2): 97-106.
79. de Laat B, de Groot PG. Autoantibodies directed against domain I of beta2-glycoprotein I. *Current rheumatology reports* 2011; **13**(1): 70-6.
80. Dienava-Verdoold I, Boon-Spijker MG, de Groot PG, et al. Patient-derived monoclonal antibodies directed towards beta2 glycoprotein-1 display lupus anticoagulant activity. *Journal of thrombosis and haemostasis* 2011; **9**(4): 738-47.
81. Devreese K, Kelchtermans H, de Laat B. Differences in sensitivity of two automated panels for anticardiolipin and anti-beta2glycoprotein I antibodies in the laboratory diagnosis of antiphospholipid syndrome due to the exposure of the domain I epitope of beta2glycoprotein I on the solid phase [abstract]. *Thrombosis and Haemostasis* 2014; **12**(S1): 55.
82. Mahler M, Bentow C, Serra J, Fritzler MJ. Detection of autoantibodies using chemiluminescence technologies. *Immunopharmacology and immunotoxicology* 2016; **38**(1): 14-20.

Chapter 3

Detection of anti-domain I antibodies by chemiluminescence enables the identification of high-risk antiphospholipid syndrome patients: a multicenter multiplatform study

Dongmei Yin, Walid Chayoua , Hilde Kelchtermans, Philip G. de Groot , Gary W. Moore, Jean-Christophe Gris, Stéphane Zuily, Jacek Musial, Bas de Laat, Katrien M. J. Devreese

Journal of Thrombosis and Haemostasis, 18 (2020) 463-478.

Abstract

Background: Classification of the antiphospholipid syndrome (APS) relies predominantly on detecting antiphospholipid antibodies (aPLs). Antibodies against a domain I (DI) epitope of anti-β2 glycoprotein I (β2GPI) proved to be pathogenic, but are not included in the current classification criteria.

Objectives: Investigate the clinical value of detecting antiDI IgG in APS.

Patients/Methods: From eight European centers 1005 patients were enrolled. Anti-cardiolipin (CL) and antiβ2GPI were detected by four commercially available solid phase assays; antiDI IgG by the QUANTA Flash® β2GPI domain I assay.

Results: Odds ratios (ORs) of antiDI IgG for thrombosis and pregnancy morbidity proved to be higher than those of the conventional assays. Upon restriction to patients positive for antiβ2GPI IgG, antiDI IgG positivity still resulted in significant ORs. When antiDI IgG was added to the criteria aPLs or used as a substitute for antiβ2GPI IgG/antiCL IgG, ORs for clinical symptoms hardly improved. Upon removing antiDI positive patients, LAC remained significantly correlated with clinical complications. AntiDI IgG are mainly present in high-risk triple positive patients, showing higher levels. Combined antiDI and triple positivity confers a higher risk for clinical symptoms compared to only triple positivity.

Conclusions: Detection of antiDI IgG resulted in higher ORs for clinical manifestations than the current APS classification criteria. Regardless of the platform used to detect antiβ2GPI/antiCL, addition of antiDI IgG measured by QUANTA Flash® did not improve the clinical associations, possibly due to reduced exposure of the pathogenic epitope of DI. Our results demonstrate that antiDI IgG potentially helps in identifying high risk patients.

Introduction

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by recurrent thrombosis and/or pregnancy morbidity in combination with the persistent presence of antiphospholipid antibodies (aPLs) [1]. Due to the high prevalence of thrombosis and pregnancy morbidity in the general population, classification of APS mainly relies on laboratory results [2]. Five different assays that detect aPLs are included in the current revised laboratory criteria: lupus anticoagulant (LAC), detecting a phospholipid-dependent prolongation of in vitro clotting times and two immunological quantitative assays measuring immunoglobulin (Ig) G and/or IgM anti-cardiolipin antibodies (antiCL) and antiβ2GPI antibodies (antiβ2GPI). Positive tests should be repeated with an interval of at least 12 weeks, to avoid transient positivity due to infections [3].

Although laboratory testing of aPLs is critical to the classification of APS, the application and interpretation of these tests remain challenging [4,5]. The variable clinical performance in conventional commercial and home-made aPL assays not only results from the lack of standardization [6,7], but also from the heterogeneity in aPLs [8].

Accumulating evidence revealed that aPLs are directed against phospholipid-bound plasma proteins, of which β2GPI proved to be the main target [9-11]. β2GPI consists of five homologous domains (Domain (D)I-DV). In the native circular or S-shaped conformation, the critical DI epitope is not exposed. Upon binding to an anionic phospholipid (PL) surface through the positively charged patch on DV, β2GPI undergoes a conformational change [12]. Consequently, the DI-IV spreads out resulting in a more open J shape, exposing a cryptic epitope G40-R43 on DI and allowing a subset of antiDI of β2GPI autoantibodies to bind [13-15]. Various subsets of antiβ2GPI antibodies targeting different domains of the protein have been described with clear differences in clinical potential. The subpopulation of aPLs that recognize this epitope comprising at least G40-R43 on DI proved to be pathogenic in vitro/in vivo, and in clinical studies [16-20], while aPLs that recognize other domains of β2GPI seem to be innocent [21-24] .

The aim of this study is to assess the clinical relevance of antibodies against DI of β2GPI in APS patients in an international multicenter study and evaluate the added value of detecting antiDI IgG compared to the conventional assays, as well as whether the added value of the antiDI IgG assay measured by this QUANTA Flash® depends on the platform used to detect antiβ2GPI and antiCL IgG. The commercially available chemiluminescence (CLIA) assay for antiDI IgG was used in combination with antiCL and antiβ2GPI assays of different manufacturers. Assays were selected based on frequently used assays in the external quality control program of the ECAT (External quality Control of diagnostic Assays and Tests, Voorschoten, The Netherlands) and willingness of manufacturers for collaboration.

Methods

Study population

Patient and control samples were collected from eight medical European centers. The study was approved by the local ethical committees. General characteristics (e.g. age, gender, ethnicity), clinical characteristics (e.g. thrombotic events with specification of the type, pregnancy morbidity, autoimmune disease) and previous laboratory determinations (e.g. LAC tests, IgG/IgM antiCL and antiβ2GPI reactivity) were recorded. In total, the database enrolled 1005 samples and samples were allocated to six different groups according to the information of the centers.

The classification of APS was based on the Sydney criteria [3]. Classification was determined using the local aPL assay panel for LAC and IgG/IgM antiCL or antiβ2GPI antibodies. Control populations consisted of patients with an autoimmune disease other than APS (e.g. systemic lupus erythematosus and systemic sclerosis), meeting the criteria for the classification of autoimmune disease without Sydney criteria thrombotic or pregnancy morbidity complications (AID controls); patients that were referred for aPLs testing for other reasons than the clinical criteria of APS (e.g. subfertility and prolonged activated partial thromboplastin time (aPTT), normal controls); patients with a previous thrombotic event negatively tested for aPLs (non-APS thrombosis); and patients that experienced obstetric complications in the absence of aPLs (non-APS obstetric). In the normal control-female population (n=169) there was no history of pregnancy morbidity. Of the 169 women 119 (70.4%) were characterized by subfertility without previous pregnancy, of the 50 other control females information on whether they were (successfully) pregnant before was not available.

Methodology

AntiCL IgG, antiCL IgM, antiβ2GPI IgG and antiβ2GPI IgM were detected by four solid phase assays: BioPlex®2200 (Bio-Rad, Bio-Rad Laboratories, Hercules, USA), Phadia® (Thermo Fisher Scientific/Phadia, Uppsala, Sweden), HemosIL AcuStar® (Instrumentation Laboratories, Bedford, USA) and QUANTA Lite® ELISA (Inova Diagnostics, San Diego, USA). AntiDI IgG by the CLIA of QUANTA Flash® β2GPI domain I assay (Inova Diagnostics) on the ACL AcuStar® platform. All tests were performed between February 2016 and October 2016 by a single technician in the Ghent University Hospital following the instructions of the manufacturer. Values below the calculated limit of detection (LOD) were replaced by the LOD. The cut-off values from the manufacturers' recommendation were confirmed in 20 healthy individuals according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [25] and guidance from the SSC of the ISTH [5]. Based on the cut-off values (20 arbitrary units i.e. U/mL, GPL, MPL, SGU, SMU on platforms of HemosIL AcuStar®, BioPlex®2200 and QUANTA Lite® ELISA, 10 arbitrary units on Phadia® for antiCL IgG/IgM and antiβ2GPI IgG/IgM, 20 chemiluminescence units (CU) on QUANTA Flash® for antiDI IgG), positive samples were identified. LAC positivity was determined by the individual center, according to the ISTH guidelines [7].

Statistics

Statistical analyses were performed using IBM SPSS® Statistics 24 (IBM SPSS, New York, NY, USA). To avoid a possible bias induced by the classification performed by the eight individual centers, we selected 'clinically affected versus clinically not-affected' as outcome variable instead of 'APS versus non-APS classification'. Relationships between the different laboratory assays and the clinical events (thrombosis or pregnancy morbidity) of the patients were investigated by calculating odds ratios (ORs) and 95% confidence intervals (95% CI) in the respective subpopulations. To determine the association with thrombosis and pregnancy morbidity, analysis was restricted to the thrombosis subpopulation (thrombotic APS, non-APS thrombosis, AID and normal controls (n = 851)) and the pregnancy morbidity subpopulation (obstetric APS, non-APS obstetric, female AID and female normal controls (n = 481)), respectively. Subsequently, the chi-squared test was used for the comparison of dichotomous variables, and antiDI IgG titers were compared between groups by the Kruskal–Wallis H test (more than 2 groups) or Mann–Whitney U test (2 groups). Finally, the kappa agreement of antiDI IgG and conventional aPL tests was studied via the chi-squared test, and the correlation between the titer of antiDI IgG, antiβ2GPI IgG and antiCL IgG was performed by a Spearman rank correlation test. P-values less than 0.05 (two-tailed) were considered as statistically significant.

Results

Characteristics of the study population

The patients' demographic data and clinical characteristics are summarized in Table 1. To evaluate associations with thrombotic events and pregnancy morbidity separately, the characteristics of two subpopulations are shown in **Table 1A and 1B**.

Association between antiDI IgG and clinical symptoms of APS

Independent of the platform, a significant association with clinical events was found for all tested aPL assays, with ORs varying from 2.7 (95%CI 1.9-3.9) to 5.4 (95%CI 3.0-9.6) for thrombosis, 2.3 (95%CI 1.5-3.3) to 4.1 (95%CI 2.5-6.6) for arterial thrombosis, 2.0 (95%CI 1.4-2.8) to 2.8 (95%CI 1.9-4.2) for venous thrombosis and 2.7 (95%CI 1.7-4.3) to 5.6 (95%CI 3.1-9.9) for pregnancy morbidity **(Table 2)**. Compared to the criteria aPL assays, detection of antiDI IgG was less sensitive (21.9% / 26.6% for thrombosis / pregnancy morbidity), but more specific (94.9% / 93.9% for thrombosis / pregnancy morbidity), overall resulting in higher ORs for both clinical criteria of APS compared to LAC, antiβ2GPI or antiCL IgG except for the QUANTA Lite® ELISA platform. Similar ORs of antiDI were found for thrombosis and pregnancy morbidity, and no difference in association was observed between venous versus arterial thrombosis. Interestingly, upon restriction to patients positive for antiβ2GPI IgG, antiDI IgG positivity still resulted in significant - albeit lower - ORs for both clinical criteria of APS except for the QUANTA Lite® ELISA platform **(Table 2).**

Table 1. Demographic and clinical characteristics of the study population.

A. The thrombosis subpopulation (N = 851)				
	Thrombotic APS	**AID**	**Non-APS thrombosis**	**Normal controls**
Patients (N)	258	196	204	193
Gender				
Female [N(%)]	165 (64.0)	158 (80.6)	116 (56.9)	169 (87.6)
Age (Mean ± SD, years)	49.6 ± 14.7	46.4 ± 14.2	46.5 ± 14.1	39.4 ± 11.0
Clinical features [N (%)]				
Thrombosis	258 (100.0)	0	204 (100)	0
AT	54 (20.9)	0	47 (23.0)	0
VT	160 (62.0)	0	149 (73.0)	0
AT + VT	26 (10.1)	0	5 (2.5)	0
Small vessel	4 (1.6)	0	0 (0)	0
Pregnancy morbidity	23 (8.9)	2 (1.0)	0	0
A	6 (2.3)	0	0	0
B	6 (2.3)	0	0	0
C	6 (2.3)	0	0	0
NS/ non-Sydney criteria	5 (1.9)	2 (1.0)	0	0
B. The pregnancy morbidity subpopulation (N = 481)				
	Obstetric APS	**AID-female**	**Non-APS obstetric**	**Normal controls-female**
Patients (N)	121	158	33	169
Age (Mean ± SD, years)	34.3 ± 6.6	45.4 ± 14.5	32.7 ± 5.6	38.4 ± 9.6
Clinical features [N (%)]				
Thrombosis	9 (7.4)	0	0	0
AT	3 (2.5)	0	0	0
VT	5 (4.1)	0	0	0
AT + VT	1 (0.8)	0	0	0
Small vessel	0 (0)	0	0	0
Pregnancy morbidity	121 (100.0)	2 (1.3)	33 (100.0)	0
A	35 (28.9)	0	9 (27.3)	0
B	10 (8.3)	0	4 (12.1)	0
C	67 (55.4)	0	6 (18.2)	0
B+C	2 (1.7)	0	1 (3.0)	0
A+C	3 (2.5)	0	1 (3.0)	0
NS/non-Sydney criteria	4 (3.3)	2 (1.3)	12 (36.4)	0

APS, antiphospholipid syndrome; AID, autoimmune disease other than APS; AT, arterial thrombosis; N, number of patients; SD, standard deviation; VT, venous thrombosis; A, history of fetal death after the 10th weeks of gestation; B, history of premature birth (s) before the 34th weeks due to preeclampsia or placental insufficiency; C, history of three or more consecutive unexplained losses before the 10th weeks of gestation based on the Sydney classification criteria. NS, non-specified pregnancy complications; non-Sydney criteria, not fulfilling Sydney criteria for pregnancy morbidity

Table 2. ORs of aPLs for the indicated clinical manifestation of APS.

In the thrombosis population																
	Manufacturer	Thrombosis*					Arterial thrombosis*$					Venous thrombosis*&				
		N	Sensitivity (%)	Specificity (%)	OR	95%CI	N#	Sensitivity (%)	Specificity (%)	OR	95%CI	N#	Sensitivity (%)	Specificity (%)	OR	95%CI
LAC		851	43.7	81.5	**3.4**	**2.5-4.7**	834	47.0	71.8	**2.3**	**1.5-3.3**	834	43.2	77.1	**2.6**	**1.9-3.5**
AntiCL IgG	HemosIL AcuStar®	851	24.7	92.5	**4.1**	**2.6-6.3**	834	31.8	86.2	**2.9**	**1.9-4.4**	834	24.4	88.7	**2.5**	**1.7-3.7**
	BioPlex® 2200	851	26.4	89.7	**3.1**	**2.1-4.6**	834	34.1	83.9	**2.7**	**1.8-4.1**	834	25.9	85.8	**2.1**	**1.5-3.0**
	Phadia®	851	23.6	93.1	**4.1**	**2.7-6.5**	834	31.1	87.2	**3.1**	**2.0-4.7**	834	22.6	89.1	**2.4**	**1.6-3.5**
	QUANTA Lite ELISA®	851	21.4	94.3	**4.6**	**2.8-7.4**	834	28.8	88.6	**3.1**	**2.0-4.9**	834	20.9	90.5	**2.5**	**1.7-3.7**
Antiβ2GPI IgG	HemosIL AcuStar®	851	30.1	86.4	**2.7**	**1.9-3.9**	834	36.4	80.2	**2.3**	**1.6-3.5**	834	30.0	82.8	**2.1**	**1.5-2.9**
	BioPlex® 2200	851	26.2	88.7	**2.8**	**1.9-4.1**	834	34.1	83.5	**2.6**	**1.7-3.9**	834	25.6	85.0	**2.0**	**1.4-2.8**
	Phadia®	851	22.5	92.3	**3.5**	**2.6-5.4**	834	29.5	87.0	**2.8**	**1.8-4.3**	834	21.8	88.7	**2.2**	**1.5-3.2**
	QUANTA Lite ELISA®	851	16.7	96.4	**5.4**	**3.0-9.6**	834	25.8	92.2	**4.1**	**2.5-6.6**	834	15.3	92.5	**2.2**	**1.4-3.5**
AntiDI IgG	QUANTA Flash®	851	21.9	94.9	**5.2**	**3.1-8.5**	834	27.3	88.5	**2.9**	**1.8-4.5**	834	21.5	91.1	**2.8**	**1.9-4.2**
Triple positivity	HemosIL AcuStar®	851	24.0	94.1	**5.0**	**3.1-8.1**	834	28.8	87.2	**2.7**	**1.8-4.3**	834	24.1	90.7	**3.1**	**2.1-4.6**
	BioPlex® 2200	851	25.5	92.8	**4.4**	**2.9-6.9**	834	31.1	86.2	**2.8**	**1.8-4.3**	834	24.7	89.1	**2.7**	**1.8-3.9**
	Phadia®	851	21.9	93.8	**4.3**	**2.7-6.8**	834	26.5	88.0	**2.7**	**1.7-4.2**	834	21.5	90.7	**2.7**	**1.8-4.0**
	QUANTA Lite ELISA®	851	19.3	95.6	**5.2**	**3.0-8.9**	834	24.2	90.3	**3.0**	**1.9-4.8**	834	18.5	92.5	**2.8**	**1.8-4.3**
AntiDI IgG plus triple positivity	HemosIL AcuStar®	851	18.8	96.7	**6.7**	**3.7-12.2**	834	24.2	90.9	**3.2**	**2.0-5.1**	834	18.2	93.1	**3.0**	**1.9-4.7**
	BioPlex® 2200	851	19.0	96.7	**6.8**	**3.7-12.4**	834	24.2	90.7	**3.1**	**2.0-5.0**	834	18.5	93.1	**3.1**	**2.0-4.8**
	Phadia®	851	16.9	96.9	**6.4**	**3.4-11.9**	834	22.0	91.9	**3.2**	**1.9-5.2**	834	16.2	93.7	**2.9**	**1.8-4.6**
	QUANTA Lite ELISA®	851	15.6	97.4	**7.0**	**3.6-13.8**	834	21.2	92.7	**3.4**	**2.1-5.7**	834	14.7	94.1	**2.8**	**1.7-4.5**

In the pregnancy population						
	Manufacturer	Pregnancy complications**				
		N	Sensitivity (%)	Specificity (%)	OR	95%CI
LAC		481	48.7	81.7	**4.2**	**2.8-6.4**
AntiCL IgG	HemosIL AcuStar®	481	29.2	91.4	**4.4**	**2.6-7.4**
	BioPlex® 2200	481	31.8	88.1	**3.4**	**2.1-5.5**
	Phadia®	481	29.2	92.4	**5.0**	**2.9-8.5**
	QUANTA Lite ELISA®	481	24.7	93.9	**5.0**	**2.8-9.0**
Antiβ2GPI IgG	HemosIL AcuStar®	481	33.1	84.7	**2.7**	**1.7-4.3**
	BioPlex® 2200	481	31.8	87.5	**3.3**	**2.0-5.2**
	Phadia®	481	25.3	91.1	**3.5**	**2.1-5.9**
	QUANTA Lite ELISA®	481	14.3	96.0	**4.0**	**2.0-8.2**
AntiDI IgG	QUANTA Flash®	481	26.6	93.9	**5.6**	**3.1-9.9**
Triple positivity	HemosIL AcuStar®	481	22.1	93.3	**3.9**	**2.2-7.0**
	BioPlex® 2200	481	22.7	91.7	**3.3**	**1.9-5.6**
	Phadia®	481	19.5	93.6	**3.5**	**1.9-6.4**
	QUANTA Lite ELISA®	481	13.0	95.1	**2.9**	**1.5-5.8**
AntiDI IgG plus triple positivity	HemosIL AcuStar®	481	18.8	96.0	**5.6**	**2.8-11.1**
	BioPlex® 2200	481	18.8	96.0	**5.6**	**2.8-11.1**
	Phadia®	481	16.2	96.3	**5.1**	**2.5-10.4**
	QUANTA Lite ELISA®	481	12.3	96.9	**4.5**	**2.0-9.8**

OR of antiDI IgG QUANTA Flash® for the indicated clinical manifestation of APS in antiβ2GPI IgG positive individuals											
	Manufacturer for antiβ2GPI	Thrombosis*					Pregnancy complications**				
		N	Sensitivity (%)	Specificity (%)	OR	95%CI	N	Sensitivity (%)	Specificity (%)	OR	95%CI
antiDI IgG	HemosIL AcuStar®	192	69.8	62.3	**3.8**	**2.0-7.4**	101	78.4	60.0	**5.5**	**2.3-13.1**
	BioPlex® 2200	165	79.3	54.5	**4.6**	**2.2-9.6**	90	81.6	51.2	**4.7**	**1.8-12.0**
	Phadia®	134	82.7	40.0	**3.2**	**1.3-7.8**	68	87.2	37.9	**4.2**	**1.3-13.8**
	QUANTA Lite ELISA®	91	89.6	14.3	1.4	0.3-7.6	35	95.5	7.7	1.8	0.1-30.6

aPLs, antiphospholipid antibodies; APS, antiphospholipid syndrome; β2GPI, β2glycoprotein I; CL, cardiolipin; DI, domain I; IgG, immunoglobulin G; LAC, lupus anticoagulant; N, number of patients; OR, odds ratio; 95% CI, 95% confidence interval;Triple positivity, LAC positive, antiCL IgG/IgM positive and antiβ2GPI IgG/IgM positive.

Significant ORs are shown in bold and calculated according to the outcome variable 'clinically affected versus clinically not-affected patients'.

*: Thrombotic APS + non-APS thrombosis versus non-thrombotic population (AID + normal controls); **: Obstetric APS + non-APS obstetric versus non-obstetric population (AID-female + normal controls-female).

#: In the thrombosis subpopulation, 17 individuals without specified thrombosis type were defined as missing data.

$: Arterial thrombosis versus non-arterial thrombosis; &: Venous thrombosis versus non-venous thrombosis.

Additional clinical value of detecting antiDI IgG on top of the currently used laboratory tests

Table 3 shows the number of patients testing positive or negative for antiDI IgG in relation to positivity for LAC or antiβ2GPI IgG or at least one of the criteria aPL panel measured by HemosIL AcuStar® considering the previously defined subpopulations. The results of the other three solid phase assays can be found in **Supplementary Table 1.**

From **Table 3**, looking at the thrombosis subpopulation, 13 out of the 19 LAC negative antiDI positive patients had thrombosis. Additionally, the four samples testing negative for antiβ2GPI IgG by HemosIL AcuStar® but positive for antiDI IgG, all had thrombosis. Similarly, the two individuals testing negative for the criteria aPL panel by HemosIL AcuStar® and positive for antiDI IgG, both suffered from thrombosis. Concerning the pregnancy morbidity subpopulation, 12 out of the 18 LAC negative antiDI IgG positive patients experienced pregnancy morbidity. One patient testing negative for antiβ2GPI IgG by HemosIL AcuStar® and positive for antiDI IgG, had pregnancy morbidity. Moreover, only one individual negative for the criteria aPL panel by HemosIL AcuStar® tested positive for antiDI IgG and suffered from pregnancy morbidity. Looking at **Supplementary Table 1,** both for the thrombosis subpopulation or the pregnancy morbidity subpopulation, similar results were found when antiβ2GPI or antiCL IgG/IgM were detected by BioPlex®2200. Although more samples tested negative for antiβ2GPI IgG by Phadia® and QUANTA Lite® ELISA but positive for antiDI IgG, and most of them had thrombosis or pregnancy

morbidity. Including LAC, fewer individuals negative for the criteria aPL panel tested positive for antiDI IgG and suffered from clinical events.

To assess the additional clinical value of antiDI IgG on top of the currently used aPL measured by different platforms, antiDI IgG was added to the criteria aPL panel or used as a substitute for antiβ2GPI IgG and antiCL IgG in the criteria aPL panel. For all platforms, the addition of antiDI IgG to the current criteria aPL panel hardly resulted in an increase of the OR for thrombosis or pregnancy morbidity. Replacement of antiβ2GPI IgG and antiCL IgG by antiDI IgG resulted in comparable ORs for both clinical symptoms. For all platforms, a small increase was observed in specificity when replacing antiβ2GPI IgG and antiCL IgG by antiDI IgG, mostly accompanied by a decrease in sensitivity **(Table 4).**

To investigate if antiDI antibodies are the only pathogenic antibodies, we re-evaluated the correlation of LAC, antiCL IgG and antiβ2GPI IgG with clinical manifestations of APS in antiDI IgG negative patients. Interestingly, upon removal of antiDI positive samples, positivity for LAC still resulted in significant ORs for thrombosis (2.5, 95%CI 1.7-3.5) and for pregnancy morbidity (3.9, 95%CI 2.4-6.4) while correlations for antiβ2GPI IgG and antiCL IgG antibodies were no longer statistically significant except for when the antiCL IgG was measured by Phadia® and QUANTA Lite® ELISA (OR for thrombosis of 2.3 (95%CI 1.1-4.8) and 2.4 (95%CI 1.0-5.5), respectively) **(Table 5)**.

Table 3. Number of patients testing positive (+) or negative (-) for antiDI IgG in relation to positivity for LAC or antiβ2GPI IgG or at least one of the criteria aPL panel measured by HemosIL AcuStar®, see supplementary table 1 for the other platforms.

		Thrombosis subpopulation (N = 851)		With T (N = 462)		Without T (N = 389)		Pregnancy morbidity (N = 481)		With P (N = 154)		Without P (N = 327)	
		LAC		LAC		LAC		LAC		LAC		LAC	
		+	-	+	-	+	-	+	-	+	-	+	-
Anti-DI IgG	+	102	19	88	13	14	6	43	18	29	12	14	6
	-	172	558	114	247	58	311	92	328	46	67	46	261
Total		274	577	202	260	72	317	135	346	75	79	60	267
		Anti-CL IgG		Anti-CL IgG		Anti-CL IgG		Anti-CL IgG		Anti-CL IgG		Anti-CL IgG	
		+	-	+	-	+	-	+	-	+	-	+	-
Anti-DI IgG	+	114	7	95	6	19	1	59	2	40	1	19	1
	-	29	701	19	342	10	359	14	406	5	108	9	298
Total		143	708	114	348	29	360	73	408	45	109	28	299
		Anti-β2GPI IgG		Anti-β2GPI IgG		Anti-β2GPI IgG		Anti-β2GPI IgG		Anti-β2GPI IgG		Anti-β2GPI IgG	
		+	-	+	-	+	-	+	-	+	-	+	-
Anti-DI IgG	+	117	4	97	4	20	0	60	1	40	1	20	0
	-	75	655	42	319	33	336	41	379	11	102	30	277
Total		192	659	139	323	53	336	101	380	51	103	50	277
		Criteria aPL panel*		Criteria aPL panel*		Criteria aPL panel*		Criteria aPL panel*		Criteria aPL panel*		Criteria aPL panel*	
		+	-	+	-	+	-	+	-	+	-	+	-
Anti-DI IgG	+	119	2	99	2	20	0	60	1	40	1	20	0
	-	230	500	142	219	88	281	134	286	63	50	71	236
Total		349	502	241	221	108	281	194	287	103	51	91	236

aPL, antiphospholipid antibody; β2GPI, β2glycoprotein I; DI, domain I; IgG (M), immunoglobulin G (M); LAC, lupus anticoagulant; N, number of patients; P, pregnancy morbidity; T, thrombosis.

* Criteria aPL panel positivity: patients testing positive for at least one of the criteria aPL panel (i.e. LAC, antiCL IgG/M and/or antiβ2GPI IgG/M).

Table 4. ORs of the current criteria aPL panel and two newly defined aPL panels including antiDI IgG for clinical manifestations of APS.

Manufacturer		Thrombosis*					Pregnancy morbidity**				
		N	Sensitivity (%)	Specificity (%)	OR	95% CI	N	Sensitivity (%)	Specificity (%)	OR	95% CI
HemosIL AcuStar®	Criteria aPL panel$	851	52.2	72.2	**2.8**	**2.1-3.8**	481	66.9	72.2	**5.2**	**3.5-7.9**
	Criteria aPL panel with addition of antiDI IgG	851	52.6	72.2	**2.9**	**2.2-3.8**	481	67.5	72.2	**5.4**	**3.6-8.2**
	Criteria aPL panel with anti-DI IgG replacing antiβ2GPI IgG and antiCL IgG	851	49.6	73.5	**2.7**	**2.0-3.6**	481	64.3	73.4	**5.0**	**3.3-7.5**
BioPlex® 2200	Criteria aPL panel$	851	48.9	75.1	**2.9**	**2.2-3.9**	481	63.0	74.6	**5.0**	**3.3-7.5**
	Criteria aPL panel with addition of antiDI IgG	851	49.6	75.1	**3.0**	**2.2-4.0**	481	63.0	74.6	**5.0**	**3.3-7.5**
	Criteria aPL panel with anti-DI IgG replacing antiβ2GPI IgG and antiCL IgG	851	48.5	76.6	**3.1**	**2.3-4.1**	481	61.7	76.5	**5.2**	**3.5-7.9**
Phadia®	Criteria aPL panel$	851	53.2	68.1	**2.4**	**1.8-3.2**	481	69.5	68.2	**4.9**	**3.2-7.4**
	Criteria aPL panel with addition of antiDI IgG	851	54.1	68.1	**2.5**	**1.9-3.3**	481	70.1	68.2	**5.0**	**3.3-7.6**
	Criteria aPL panel with anti-DI IgG replacing antiβ2GPI IgG and antiCL IgG	851	51.1	69.9	**2.4**	**1.8-3.2**	481	66.9	70.0	**4.7**	**3.1-7.1**
QUANTA Lite ELISA®	Criteria aPL panel$	851	50.4	73.5	**2.8**	**2.1-3.8**	481	69.5	74.3	**6.6**	**4.3-10.1**
	Criteria aPL panel with addition of antiDI IgG	851	51.7	73.0	**2.9**	**2.2-3.9**	481	70.1	73.7	**6.6**	**4.3-10.1**
	Criteria aPL panel with anti-DI IgG replacing antiβ2GPI IgG and antiCL IgG	851	51.3	73.3	**2.9**	**2.2-3.9**	481	66.9	74.0	**5.8**	**3.8-8.7**

aPL, antiphospholipid antibody; APS, antiphospholipid syndrome; β2GPI, β2glycoprotein I; CL, cardiolipin; DI, domain I; IgG(M), immunoglobulin G(M); LAC, lupus anticoagulant; N, number of patients; OR, odds ratio; 95% CI, 95% confidence interval; $Criteria aPL panel positivity: patients testing positive for at least one of the criteria aPL panel (i.e. LAC, antiCL IgG/M and/or antiβ2GPI IgG/M).

Significant ORs are shown in bold and calculated according to the outcome variable 'clinically affected versus clinically not-affected patients'.

*: Thrombotic APS + non-APS thrombosis versus non-thrombotic population (AID + normal controls); **: Obstetric APS + non-APS obstetric versus non-obstetric population (AID - female + normal controls - female).

Table 5. ORs of LAC, antiCL IgG and antiβ2GPI IgG with clinical manifestations of APS upon the removal of antiDI IgG positive patients.

	Manufacturer	Thrombosis*				Pregnancy morbidity**			
		Population ($N^{\#}$)	Positive ($N^{\$}$)	OR	95% CI	Population ($N^{\#}$)	Positive ($N^{\$}$)	OR	95% CI
LAC		730	172	**2.5**	**1.7-3.5**	420	92	**3.9**	**2.4-6.4**
AntiCL IgG	HemosIL AcuStar®	730	29	2.0	0.9-4.4	420	14	1.5	0.5-4.7
	BioPlex® 2200	730	47	1.3	0.7-2.3	420	29	1.2	0.5-2.8
	Phadia®	730	35	**2.3**	**1.1-4.8**	420	16	2.2	0.8-6.0
	QUANTA Lite ELISA®	730	26	**2.4**	**1.0-5.5**	420	11	2.3	0.7-7.8
Antiβ2GPI IgG	HemosIL AcuStar®	730	75	1.3	0.8-2.2	420	41	1.0	0.5-2.1
	BioPlex® 2200	730	49	1.1	0.6-1.9	420	30	1.2	0.5-2.7
	Phadia®	730	30	1.6	0.7-3.3	420	16	1.2	0.4-3.7
	QUANTA Lite ELISA®	730	10	4.2	0.9-19.7	420	2	2.7	0.2-44.1

APS, antiphospholipid syndrome; β2GPI, β2glycoprotein I; CL, cardiolipin; DI, domain I; IgG, immunoglobulin G; LAC, lupus anticoagulant; N#, number of patients; N$, number of biomarker positive cases; OR, odds ratio; 95% CI, 95% confidence interval.

Significant ORs are shown in bold and calculated according to the outcome variable 'clinically affected versus clinically not-affected patients'.

*: Thrombotic APS + non-APS thrombosis versus non-thrombotic population (AID + normal controls); **: Obstetric APS + non-APS obstetric versus non-obstetric population (AID - female + normal controls - female).

Distribution of antiDI IgG titers according to the patient's antibody profile

For both the thrombosis and pregnancy morbidity subpopulation, all individuals were grouped according to their aPL profile into triple positive (LAC +, antiCL IgG/IgM +, antiβ2GPI IgG/IgM +), double positive (LAC -, antiCL IgG/IgM +, antiβ2GPI IgG/IgM + or LAC +, antiCL IgG/IgM +, antiβ2GPI IgG/IgM - or LAC +, antiCL IgG/IgM -, antiβ2GPI IgG/IgM +), and single positive (isolated positive for LAC, antiCL IgG/IgM or antiβ2GPI IgG/IgM). Results were compared with those of patients without any aPL reactivity (negative control). The different antibody profiles with their characteristics are shown in **Table 6** for antiβ2GPI and antiCL measured by HemosIL AcuStar® and in **Supplementary Table 2-4** for antiβ2GPI and antiCL measured by the three other solid phase assays.

Furthermore, we compared antiDI IgG titers in the triple positive, solid phase double positive (antiCL IgG/IgM positive and antiβ2GPI IgG/IgM positive but LAC negative), combined single positive group (isolated LAC +, and isolated antiCL IgG/IgM +, and isolated antiβ2GPI IgG/IgM +) and the negative control. Comparison of antiDI IgG titers between four aPL profile groups for antiβ2GPI and antiCL measured by four assays is shown in **Figure 1**. Looking at HemosIL AcuStar®, in both the thrombosis subpopulation and pregnancy morbidity subpopulation antiDI titers were significantly higher in triple positive samples compared to the other aPL profile groups, followed by the solid phase double positive samples. No difference was observed between antiDI titers of combined single positive and negative samples. Similar results were found for the three other

assays, except that there was a significant difference between antiDI titers of combined single positive and negative samples for antiβ2GPI and antiCL measured by QUANTA Lite® ELISA (**Figure 1**).

Association of triple positivity with antiDI positivity

The association between DI positivity and triple positivity was studied in detail for antiβ2GPI and antiCL measured by the Acustar® (**Table 6**). As to the thrombosis subpopulation (**Table 6**), positive values of antiDI IgG were found in 100 out of 134 individuals with triple positivity (74.6%), compared with 44.1% (15/34) in the solid phase double positive group (Pearson Chi-Square, P = 0.002). Combined single positive (2/146 = 1.4%) and aPL negative patients (2/502 = 0.4%) hardly showed any antiDI IgG reactivity (Fisher's Exact Test, P < 0.0001). A good agreement was found between triple positivity and antiDI IgG positivity (Kappa value = 0.746, P < 0.0001). In this triple positive group, 87.0% (87/100) of antiDI IgG-positive individuals had a history of thrombosis, compared with 70.6% (24/34) of antiDI IgG negative patients (Pearson Chi-Square, P = 0.028).

Concerning the pregnancy morbidity subpopulation (**Table 6**), positive values of antiDI IgG were found in 42 out of 56 individuals with triple positivity (75.0%), compared with 48.6% (17/35) in the solid phase double positive group (Pearson Chi-Square, P = 0.013). As seen in the thrombosis subpopulation, the combined single positive group (0/81 = 0%) and aPL negative patients (1/287 = 0.3%) hardly showed antiDI IgG reactivity (Fisher's Exact Test, P < 0.0001). Albeit lower than for the thrombosis subpopulation, also in the pregnancy morbidity subpopulation a good agreement was demonstrated between triple positivity and antiDI IgG (Kappa value = 0.679, P < 0.0001). In this triple positive group, 69.0% (29/42) of antiDI IgG positive individuals had a history of pregnancy morbidity, compared with 35.7% (5/14) of antiDI IgG negative patients (Fisher's Exact Test, P = 0.027). In general, similar conclusions can be drawn when antiβ2GPI and antiCL were measured by the other assays (**Supplementary Table 2-5**).

Interestingly, both for thrombosis and pregnancy morbidity, the ORs of antiDI IgG were higher than the ORs of triple positivity except when antiCL and antiβ2GPI were measured by the QUANTA Lite® ELISA platform for thrombosis. To verify if antiDI positivity has an added value in risk stratification compared to triple positivity, ORs of combined triple positivity and DI positivity were calculated for thrombosis and pregnancy morbidity. As to thrombosis, combined positivity resulted in higher OR compared to the separate OR for both triple positivity and DI reactivity. For pregnancy morbidity, ORs of antiDI plus triple positivity were higher compared to ORs of triple positivity, but hardly improved compared to OR of antiDI positivity **(Table 2).**

Furthermore, as to the thrombosis subpopulation, within the triple positive group for antiβ2GPI and antiCL measured by Acustar®, antiDI IgG titers varied greatly but no significant difference was observed between patients with thrombosis (n = 111) and unaffected individuals (n = 23), with a median (quartile(Q)25- Q75) antiDI IgG titer of, respectively, 169.0 CU (25.7-761.3) and 63.7 CU (3.7-625.7) (Mann–Whitney U test, P = 0.084). Similarly, concerning the pregnancy morbidity subpopulation, antiDI IgG titers differed greatly but no significant difference between patients with pregnancy morbidity (n = 34) and unaffected individuals (n = 22) could be demonstrated median (quartile(Q)25- Q75) antiDI IgG titer of 200.2 CU (49.6-462.1) and 65.0 CU

(3.7-654.3), respectively, Mann–Whitney U test, P = 0.179). Similar results were obtained with the three other assays, although for the BioPlex®2200 (thrombosis and pregnancy morbidity) and Phadia® (thrombosis) results reached statistical significance (**Supplementary Table 6**).

Correlation between detection of antiDI IgG and antiβ2GPI IgG and antiCL IgG

Concordance was evaluated by comparing the results of the detection of IgG antiDI antibodies with antiβ2GPI IgG and antiCL IgG measured by four solid phase assays **(Table 7).** A good agreement was established between IgG antiDI antibodies and IgG antiCL as well as with IgG antiβ2GPI antibodies (Kappa value = 0.656 - 0.861) in the thrombosis subpopulation **(Table 7A)** and the pregnancy morbidity subpopulation **(Table 7B)**. The IgG antiDI antibody titers were likewise significantly correlated with IgG antiCL titers and IgG antiβ2GPI titers (Spearman's rho = 0.563 - 0.842) in the thrombosis subpopulation **(Table 7A)** and the pregnancy morbidity subpopulation **(Table 7B)** (all P < 0.0001).

Table 6. Number of cases with distinct antibody profiles (antiCL IgG/IgM and antiβ2GPI IgG/IgM measured by HemosIL AcuStar®), see supplementary table2-3-4 for the other platforms.

In the thrombosis subpopulation		**N (%)#**	**aPL profile**			**AntiDI IgG positive**			**AntiDI IgG negative**		
			LAC	**antiCL IgG/IgM**	**antiβ2GPI IgG/IgM**	**Total N (%)&**	**with T N (%)***	**without T N (%)***	**Total N (%)&**	**with T N (%)***	**without T N (%)***
Goup1	Triple positive	134 (16)	+	+	+	100 (75)	87 (87)	13 (13)	34 (25)	24 (71)	10 (29)
Goup2	Double positive	34 (4)	-	+	+	15 (44)	9 (60)	6 (40)	19 (56)	7 (37)	12 (63)
Goup3	Double positive	14 (2)	+	+	-	0	0	0	14 (100)	7 (50)	7 (50)
Goup4	Double positive	21 (2)	+	-	+	2 (10)	1 (50)	1 (50)	19 (90)	7 (37)	12 (63)
Goup5	Single positive	105 (12)	+	-	-	0	0	0	105 (100)	76 (72)	29 (28)
Goup6	Single positive	26 (3)	-	+	-	1 (4)	1	0	25 (96)	11 (44)	14 (56)
Goup7	Single positive	15 (2)	-	-	+	1 (7)	1 (100)	0	14 (93)	10 (71)	4 (29)
Goup8	Negative control	502 (59)	-	-	-	2 (0.4)	2 (100)	0	500 (99.6)	219 (44)	281 (56)
Total		851 (100)				121 (14)	101 (83)	20 (17)	730 (86)	361 (49)	369 (51)
In the pregnancy morbidity subpopulation		**N (%)#**	**aPL profile**			**AntiDI IgG positive**			**AntiDI IgG negative**		
			LAC	**antiCL IgG/IgM**	**antiβ2GPI IgG/IgM**	**Total N (%)&**	**with P N (%)***	**without P N (%)***	**Total N (%)&**	**with P N (%)***	**without P N (%)***
Goup1	Triple positive	56 (12)	+	+	+	42 (75)	29 (69)	13 (31)	14 (25)	5 (36)	9 (64)
Goup2	Double positive	35 (7)	-	+	+	17 (49)	11 (65)	6 (35)	18 (51)	8 (44)	10 (56)
Goup3	Double positive	9 (2)	+	+	-	0	0	0	9 (100)	2 (22)	7 (78)
Goup4	Double positive	13 (3)	+	-	+	1 (8)	0	1(100)	12 (92)	1 (8)	11 (92)
Goup5	Single positive	57 (12)	+	-	-	0	0	0	57 (100)	38 (67)	19 (33)
Goup6	Single positive	16 (3)	-	+	-	0	0	0	16 (100)	5 (31)	11 (69)
Goup7	Single positive	8 (2)	-	-	+	0	0	0	8 (100)	4 (50)	4 (50)
Goup8	Negative control	287 (60)	-	-	-	1 (0.3)	1 (100)	0	286 (99.7)	50 (17)	236 (83)
Total		481 (100)				61 (13)	41 (67)	20 (33)	420 (87)	113 (27)	307 (73)

aPL, antiphospholipid antibody; β2GPI, β2glycoprotein I; CL, cardiolipin; DI, domain I; IgG (M), immunoglobulin G (M); LAC, lupus anticoagulant; N, number of patients in each group; P, pregnancy morbidity; T, Thrombosis.

#: the percentage of each group antibody profile; &: the percentage of antiDI IgG positive/negative in each group antibody profile; *: the percentage of individuals with/without clinical events.

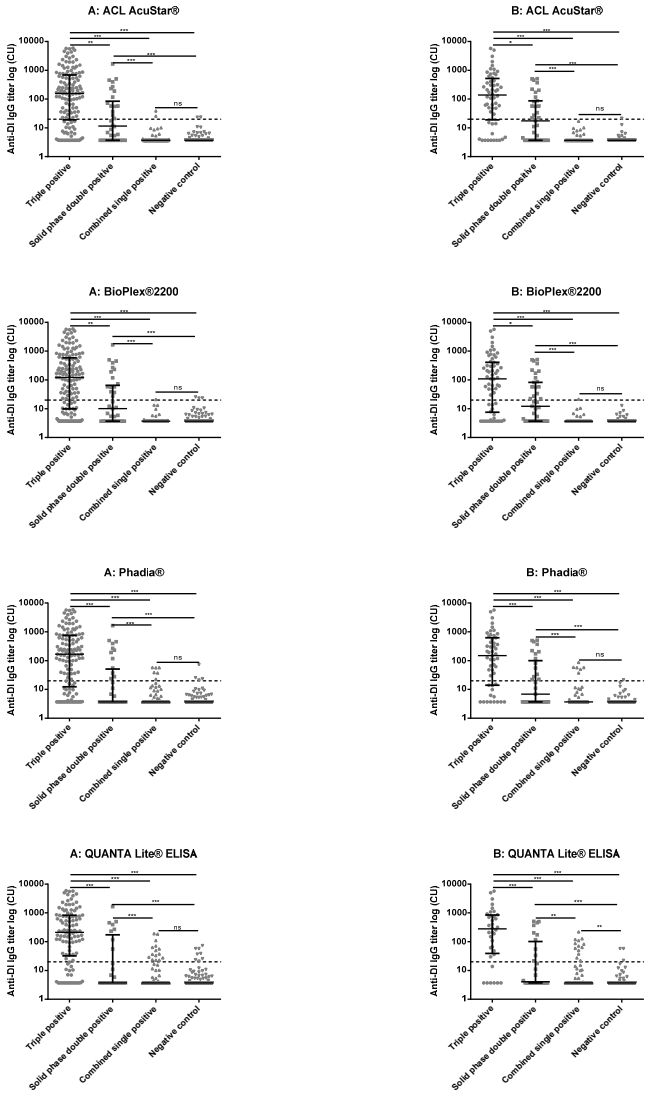

Figure 1. **Distribution of the titer of antiDI IgG according to distinct antibody profiles for antiCL and antiβ2GPI measured by HemosIL AcuStar®, BioPlex®2200, Phadia® and QUANTA Lite® ELISA in the thrombosis subpopulation (A) and pregnancy morbidity subpopulation (B).** Investigated aPL profiles include triple positive (LAC+, antiCL IgG/IgM +, antiβ2GPI IgG/IgM +), solid phase double positive (LAC–, antiCL IgG/IgM +, antiβ2GPI IgG/IgM +) and combined single positive (isolated LAC +, and isolated antiCL IgG/IgM +, and isolated antiβ2GPI IgG/IgM +). Patients without any aPL reactivity were defined as negative controls. Titer of antiDI IgG are expressed as the median with interquartile ranges within each profile; dashed lines indicate the cut-off value of antiDI IgG (20 CU). *P < 0.05, **P < 0.01, ***P < 0.0001, ns: not significant. Abbreviations: aPL, antiphospholipid antibody; DI, domain I; IgG, immunoglobulin G; CU: chemiluminescence units.

Table7. Matrix of correlation between antiDI IgG and antiβ2GPI IgG or antiCL IgG in the indicated subpopulation.

A. In the thrombosis subpopulation					B. In the pregancy morbidity subpopulation				
Agreement of pos/neg (Kappa value)	**HemosIL AcuStar®**	**BioPlex® 2200**	**Phadia®**	**QUANTA Lite® ELISA**	**Agreement of pos/neg (Kappa value)**	**HemosIL AcuStar®**	**BioPlex ®2200**	**Phadia®**	**QUANTA Lite® ELISA**
antiDI IgG vs. antiβ2GPI IgG	0.694***	0.774***	0.783***	0.731***	antiDI IgG vs. antiβ2GPI IgG	0.692***	0.758***	0.776***	0.656***
antiDI IgG vs. antiCL IgG	0.839***	0.776***	0.748***	0.750***	antiDI IgG vs. antiCL IgG	0.861***	0.755***	0.797***	0.760***
Correlation of titers (Spearman's rho)	**HemosIL AcuStar®**	**BioPlex® 2200**	**Phadia®**	**QUANTA Lite® ELISA**	**Correlation of titers(Spearman's rho)**	**HemosIL AcuStar®**	**BioPlex ®2200**	**Phadia®**	**QUANTA Lite® ELISA**
antiDI IgG vs. antiβ2GPI IgG	0.808***	0.842***	0.679***	0.581***	antiDI IgG vs. antiβ2GPI IgG	0.797***	0.838***	0.647***	0.563***
antiDI IgG vs. antiCL IgG	0.756***	0.800***	0.645***	0.734***	antiDI IgG vs. antiCL IgG	0.729***	0,783***	0.662***	0.747***

aPL, antiphospholipid antibody; β2GPI, β2glycoprotein I; CL, cardiolipin; DI, domain I; IgG, immunoglobulin G; LAC, lupus anticoagulant; ***P < 0.0001.

Discussion

Solid phase aPL assays included in the laboratory criteria suffer from a lack of standardization, limiting their utility in clinical practice. Variability results not only from methodological shortcomings but also from the heterogeneity of aPLs [5,7]. More and more literature evidence suggests that detection of a subset of aPLs reactive against DI of β2GPI is a promising classification or risk stratification tool in APS [26,27]. However, a recent review concluded inconsistent results about the added value of the detection of antiDI IgG compared to conventional aPL test, most probably due to differences in study design, study population and the methodology to detect the antiDI antibodies [28].

In this multicenter study, all samples were measured for aPL reactivity by one technician in the same lab to avoid variability coming from differences in working conditions. In most studies evaluating the added value of antiDI IgG, the only commercially available assay (CLIA of QUANTA Flash®) is compared with antiβ2GPI and antiCL of the same manufacturer [29-35]. Importantly, we hypothesized that the added value of the antiDI IgG assay measured by QUANTA Flash® depends on the platform used to detect antiβ2GPI and antiCL IgG. This hypothesis comes from our previously published results demonstrating a variable exposure of the G40-R43 epitope on domain I of β2GPI coated in the different commercially available antiCL and antiβ2GPI assays [8,36,37]. In this multicenter study, we therefore determined the added value of antiDI testing to the current APS classification criteria, when antiCL and antiβ2GPI were measured by four different commercially available assays.

As literature is inconclusive whether the same aPL subset induces thrombosis and pregnancy morbidity, separate analyses were performed in a thrombosis and pregnancy morbidity

subpopulation, including proper control populations. Both for the thrombosis and the pregnancy morbidity subpopulation, we found that the detection of antiDI IgG was less sensitive but more specific compared to the laboratory criteria aPL tests, resulting in a higher OR for thrombosis and pregnancy morbidity compared to LAC, antiβ2GPI or antiCL IgG, except for the QUANTA Lite® ELISA. Interestingly, upon restriction to patients positive for antiβ2GPI IgG, antiDI IgG positivity still resulted in significant ORs for clinical complications, except for the QUANTA Lite® ELISA (**Table 2**), that could be explained by the lower number of antiβ2GPI IgG detected by this assay (**Supplemental Table 1).** These findings are consistent with previous studies [19,20,29,30,32]. Based on these results, apart from those obtained by the QUANTA Lite® ELISA, we expected antiDI IgG to have an additional value on top of the current laboratory criteria, or to be a candidate to replace the antiβ2GPI IgG detection. Contrary to our expectation, the addition of antiDI or replacement of antiβ2GPI IgG by antiDI hardly improved the ORs for thrombosis or pregnancy morbidity. The absence of an added value of antiDI to the current aPL criteria panel was also found in previous studies [34,35].

This at first sight contradiction can be explained by the results presented in **Table 3 and Supplementary Table 1**. Independent of the assay used, only a few individuals (n=11) negative for the criteria aPL panel (with one or more platforms) become positive when antiDI IgG is added to the criteria aPLs panel or used as a substitute for antiβ2GPI IgG and/or antiCL IgG, even though most of them experienced clinical events (n=7 with thrombosis, n=2 with pregnancy morbidity). Of note, despite of antiDI IgG positivity, the titers of antiDI IgG in these individuals proved to be low (from 21.1 to 73.2 CU).

Interestingly, upon removal of antiDI IgG positive patients, antiCL IgG and antiβ2GPI IgG were no longer significantly correlated with thrombosis and pregnancy morbidity, except for antiCL IgG measured by Phadia® and by QUANTA Lite® ELISA (OR for thrombosis of 2.3 (95%CI 1.1-4.8) and 2.4 (95%CI 1.0-5.5), respectively) (**Table 5**). Although the OR for thrombosis of antiCL IgG measured by Phadia® and by QUANTA Lite® ELISA was still significant, the lower limit was adjacent to 1. These findings are in agreement with previous studies demonstrating that especially antiDI antibodies are pathogenic while antibodies targeting other domains such as domain 4/5 are innocent or even protective antibodies [20,23,38,39].

We previously demonstrated that the large variability observed in commercially available antiβ2GPI assays results at least in part from a variable exposure of the pathogenic G40-R43 DI epitope [8]. Importantly, our recent data show that, as for antiβ2GPI IgG assays, the pathogenic DI epitope is not exposed correctly in the commercially available antiDI CLIA assay [28]. Furthermore, both as categorical variables (positive/negative) and as quantitative variables (titer), antiDI IgG strongly correlated with antiCL IgG and antiβ2GPI IgG on the same AcuStar® CLIA. The high correlation between antiDI IgG and antiβ2GPI IgG suggests a high overlap between both assays performed on the automated CLIA platform. Previous studies also observed a high agreement (69% - 92%) between antiDI IgG and antiβ2GPI IgG when measured using the CLIA methodology [29,34,35,40]. These results may explain the absence of an added value of measuring antiDI using this assay. In this study, also for the other platforms, a high agreement was found between antiDI

and the antiCL IgG and antiβ2GPI IgG and no added value of measuring antiDI could be demonstrated (**Table 7**).

Of note, apart from the antiDI CLIA assay used in this study, other methods are available to detect antiDI antibodies [28]. Previously a home-made two-step ELISA strongly indicated that testing for IgG antiDI enables identification of the patients at highest risk for developing thrombosis or pregnancy morbidity [19,20]. In contrast to the results obtained by CLIA assays, only 50% of the antiβ2GPI IgG antibodies testing by this home-made two-step ELISA were demonstrated to be reactive against DI. Importantly, using this two-step ELISA assay, correct exposure of the G40-R43 epitope was already confirmed [20]. The added value of measuring antiDI reactivity using this assay remains to be determined.

Interestingly, LAC remained significantly correlated with thrombosis and pregnancy morbidity upon removal of antiDI IgG positive patients. Further illustrated by the only fair to moderate agreement between IgG antiDI and functional LAC in the thrombosis subpopulation (Kappa value = 0.398) and the pregnancy morbidity subpopulation (Kappa value = 0.320), these data illustrate that the CLIA antiDI IgG assay only detects part of the pathogenic aPLs. The presence of pathogenic anti-phosphatidylserine/prothrombin (anti-PS/PT) antibodies able to induce LAC positivity may explain the remaining correlation between LAC and thrombosis. Indeed, previous studies have demonstrated correlations of anti-PS/PT with clinical symptoms in APS patients and positivity proved to be associated with LAC [41,42] .

Evidence is growing that the determination of combined antibody positivity helps to categorize patients according to their risk profile. Especially triple positive patients (positive for LAC and antiCL and antiβ2GPI IgG/IgM) show a strong association with thrombotic and obstetric manifestations [43,44]. Clinical studies confirm that triple positivity in APS patients and asymptomatic aPL carriers indicates a high risk of recurrence of thrombosis or development of a first thrombotic event, respectively [44,45]. Although identification of triple positives is dependent on the solid phase assay used [46], the percentage of antiDI IgG positives in individuals with distinct antibody profiles were comparable for the four tested solid phase assays. In this study, a very good agreement was found between triple positivity and antiDI IgG positivity, irrespective of the solid phase assay used (**Supplementary Table 5**), which is consistent with previous studies [30-32,35,40]. Moreover, independent of the used platform to measure antiCL and antiβ2GPI IgG/IgM, we observed that antiDI IgG antibodies are mainly present in triple positive individuals, also showing significantly higher titers compared to patients with other aPL profiles. More importantly, within the triple positive group, a higher percentage of clinically affected cases was present in the antiDI IgG positive compared to the antiDI IgG negative group. Furthermore, the higher ORs of combined DI and triple positivity for both thrombosis and pregnancy morbidity compared to only triple positivity, support the idea that detection of antiDI IgG is interesting to identify patients at risk.

We acknowledge that our study has some limitations. First, as in both the thrombotic and obstetric patient groups, a small number of patients (8.9% and 7.4% respectively) also suffers from the other clinical manifestation, the correlation with the specific clinical manifestation may be affected by the comorbidity of the other manifestation. Also, the different groups in our study

population did not match according to age and gender, but were representative to population groups tested in daily practice. Another limitation of our study is the retrospective design. Interestingly, in the AID and normal control group, a number of the triple positives were also positive for antiDI reactivity (depending on the platform 11/22 (50%) - 9/14 (64%) for AID; 1/3 (33%) – 2/4 (50%) for the normal controls). Prospective studies are necessary to verify the risk of patients with these characteristics to develop clinical manifestations of APS, with possible implications for their treatment.

This study showed again that the choice of the commercial assays used to detect the presence of antiphospholipid antibodies influences the classification of APS. It is impossible to determine which commercial assay is the best because we lack a gold standard. However, a good agreement with a domain I specific assay seems to be essential because antibodies against DI have shown to be pathogenic in animal models of APS.

Conclusion

Despite the higher OR of antiDI antibody detection for clinical manifestations of APS, our study was unable to demonstrate an added value of measuring antiDI IgG on top of the laboratory criteria, independent of the platform used to measure antiCL and antiβ2GPI. We put the hypothesis forward that the reduced exposure of the pathogenic DI epitope in this automated assay possibly explains the absence of an added value. Therefore, it may be interesting to re-evaluate the added value of antiDI using the in-house antiDI assay previously developed, for which correct exposure of the pathogenic epitope was already demonstrated. The high correlation between antiDI IgG and triple positivity indicates that antiDI IgG positivity confirms the patients at higher risk for clinical events in APS. Importantly, combined DI and triple positivity confirms a higher risk for both thrombosis and pregnancy morbidity compared to only triple positivity. As LAC positivity remains significantly correlated with thrombosis and pregnancy morbidity upon removal of antiDI positive patients, the antiDI IgG assay only detects part of the pathogenic aPLs.

References

1. Ruiz-Irastorza G, Crowther M, Branch W, Khamashta MA. Antiphospholipid syndrome. *Lancet* 2010; **376**: 1498-509.
2. Ortel TL. Antiphospholipid syndrome: laboratory testing and diagnostic strategies. *American journal of hematology* 2012; **87 Suppl 1**: S75-81.
3. Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *Journal of thrombosis and haemostasis* 2006; **4**(2): 295-306.
4. Bertolaccini ML, Amengual O, Andreoli L, et al. 14th International Congress on Antiphospholipid Antibodies Task Force. Report on antiphospholipid syndrome laboratory diagnostics and trends. *Autoimmunity reviews* 2014; **13**(9): 917-30.
5. Devreese KM, Pierangeli SS, de Laat B, et al. Testing for antiphospholipid antibodies with solid phase assays: guidance from the SSC of the ISTH. *Journal of thrombosis and haemostasis* 2014; **12**(5): 792-5.
6. Devreese K. Standardization of antiphospholipid antibody assays.Where do we stand? *Lupus* 2012; **21**: 718-21.
7. Devreese KM, Ortel TL, Pengo V, de Laat B. Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibodies. Laboratory criteria for antiphospholipid syndrome: communication from the SSC of the ISTH. *Journal of thrombosis and haemostasis* 2018; **16**(4): 809-13.
8. Pelkmans L, Kelchtermans H, de Groot PG, et al. Variability in exposure of epitope G40-R43 of domain i in commercial anti-beta2-glycoprotein I IgG ELISAs. *PloS one* 2013; **8**(8): e71402.
9. 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**(7): 2340-6.
10. Arad A, Proulle V, Furie RA, Furie BC, Furie B. beta(2)-Glycoprotein-1 autoantibodies from patients with antiphospholipid syndrome are sufficient to potentiate arterial thrombus formation in a mouse model. *Blood* 2011; **117**(12): 3453-9.
11. Jankowski M, Vreys I, Wittevrongel C, et al. Thrombogenicity of β2-glycoprotein I-dependent antiphospholipid antibodies in a photochemically induced thrombosis model in the hamster. *Blood* 2003; **101**(1): 157-62.
12. de Groot PG, Meijers JC. beta(2) -Glycoprotein I: evolution, structure and function. *Journal of thrombosis and haemostasis* 2011; **9**(7): 1275-84.
13. 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**(5): 1916-24.
14. Agar C, van Os GM, Morgelin M, et al. Beta2-glycoprotein I can exist in 2 conformations: implications for our understanding of the antiphospholipid syndrome. *Blood* 2010; **116**(8): 1336-43.
15. de Laat B, van Berkel M, Urbanus RT, et al. Immune responses against domain I of beta(2)-glycoprotein I are driven by conformational changes: domain I of beta(2)-glycoprotein I harbors a cryptic immunogenic epitope. *Arthritis & Rheumatology* 2011; **63**(12): 3960-8.

16. Iverson GM, Reddel S, Victoria EJ, et al. Use of Single Point Mutations in Domain I of 2-Glycoprotein I to Determine Fine Antigenic Specificity of Antiphospholipid Autoantibodies. *Journal of immunology* 2002; **169**(12): 7097-103.
17. Pericleous C, Ruiz-Limon P, Romay-Penabad Z, et al. Proof-of-concept study demonstrating the pathogenicity of affinity-purified IgG antibodies directed to domain I of beta2-glycoprotein I in a mouse model of anti-phospholipid antibody-induced thrombosis. *Rheumatology (Oxford)* 2015; **54**(4): 722-7.
18. Ioannou Y, Romay-Penabad Z, Pericleous C, et al. In vivo inhibition of antiphospholipid antibody-induced pathogenicity utilizing the antigenic target peptide domain I of beta2-glycoprotein I: proof of concept. *Journal of thrombosis and haemostasis* 2009; **7**(5): 833-42.
19. 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**(4): 1540-5.
20. de Laat B, Pengo V, Pabinger I, et al. The association between circulating antibodies against domain I of beta2-glycoprotein I and thrombosis: an international multicenter study. *Journal of thrombosis and haemostasis* 2009; **7**(11): 1767-73.
21. Pelkmans L, de Laat B. Antibodies against domain I of beta2-glycoprotein I: the one and only? *Lupus* 2012; **21**(7): 769-72.
22. Pengo V, Ruffatti A, Tonello M, et al. Antibodies to Domain 4/5 (Dm4/5) of beta2-Glycoprotein 1 (beta2GP1) in different antiphospholipid (aPL) antibody profiles. *Thrombosis Research* 2015; **136**(1): 161-3.
23. Andreoli L, Nalli C, Motta M, et al. Anti-beta(2)-glycoprotein I IgG antibodies from 1-year-old healthy children born to mothers with systemic autoimmune diseases preferentially target domain 4/5: might it be the reason for their 'innocent' profile? *Annals of the Rheumatic Diseases* 2011; **70**(2): 380-3.
24. Artenjak A, Locatelli I, Brelih H, et al. Immunoreactivity and avidity of IgG anti-beta2-glycoprotein I antibodies from patients with autoimmune diseases to different peptide clusters of beta2-glycoprotein I. *Immunologic research* 2015; **61**(1-2): 35-44.
25. Wayne P. Defining, establishing, and verifying reference intervals in the clinical laboratory: approved guidelinethird edition. *CLSI documalet C28-A3c 3rd ed Wayne (PA): Clinical and Laboratory Standards Institute* 2008.
26. Arachchillage DRJ, Laffan M. Pathogenesis and management of antiphospholipid syndrome. *British journal of haematology* 2017; **178**(2): 181-95.
27. Kelchtermans H, Chayoua W, Laat B. The Significance of Antibodies against Domain I of Beta-2 Glycoprotein I in Antiphospholipid Syndrome. *Seminars in thrombosis and hemostasis* 2018; **44**(5): 458-65.
28. Yin D, de Laat B, Devreese KMJ, Kelchtermans H. The clinical value of assays detecting antibodies against domain I of beta2-glycoprotein I in the antiphospholipid syndrome. *Autoimmunity reviews* 2018; **17**(12): 1210-8.
29. Zhang S, Wu Z, Chen S, et al. Evaluation of the diagnostic potential of antibodies to beta2-glycoprotein 1 domain 1 in Chinese patients with antiphospholipid syndrome. *Scientific reports* 2016; **6**: 23839.

30. Mondejar R, Gonzalez-Rodriguez C, Toyos-Saenz de Miera FJ, et al. Role of antiphospholipid score and anti-beta2-glycoprotein I Domain I autoantibodies in the diagnosis of antiphospholipid syndrome. *Clinica chimica acta; international journal of clinical chemistry* 2014; **431**: 174-8.
31. Meneghel L, Ruffatti A, Gavasso S, et al. Detection of IgG anti-Domain I beta2 Glycoprotein I antibodies by chemiluminescence immunoassay in primary antiphospholipid syndrome. *Clinica chimica acta; international journal of clinical chemistry* 2015; **446**: 201-5.
32. Mahler M, Albesa R, Zohoury N, et al. Autoantibodies to domain 1 of beta 2 glycoprotein I determined using a novel chemiluminescence immunoassay demonstrate association with thrombosis in patients with antiphospholipid syndrome. *Lupus* 2016; **25**(8): 911-6.
33. Oku K, Amengual O, Kato M, et al. Significance of fully automated tests for the diagnosis of antiphospholipid syndrome. *Thrombosis Research* 2016; **146**: 1-6.
34. Iwaniec T, Kaczor MP, Celinska-Lowenhoff M, Polanski S, Musial J. Clinical significance of anti-domain 1 beta2-glycoprotein I antibodies in antiphospholipid syndrome. *Thrombosis Research* 2017; **153**: 90-4.
35. De Craemer AS, Musial J, Devreese KM. Role of anti-domain 1-beta2 glycoprotein I antibodies in the diagnosis and risk stratification of antiphospholipid syndrome. *Journal of thrombosis and haemostasis* 2016; **14**(9): 1779-87.
36. Devreese K, Kelchtermans H, de Laat B. Differences in sensitivity of two automated panels for anticardiolipin and anti-beta2glycoprotein I antibodies in the laboratory diagnosis of antiphospholipid syndrome due to the exposure of the domain I epitope of beta2glycoprotein I on the solid phase [abstract]. ISTH SSC 2014 Meeting Abstracts. *Journal of thrombosis and haemostasis* 2014; **12**: 55.
37. Chayoua W, Kelchtermans H, Moore GW, et al. Detection of Anti-Cardiolipin and Anti-beta2glycoprotein I Antibodies Differs between Platforms without Influence on Association with Clinical Symptoms. *Thrombosis and Haemostasis* 2019; **119**(05): 797-806.
38. Andreoli L, Chighizola CB, Nalli C, et al. Clinical characterization of antiphospholipid syndrome by detection of IgG antibodies against beta2 -glycoprotein i domain 1 and domain 4/5: ratio of anti-domain 1 to anti-domain 4/5 as a useful new biomarker for antiphospholipid syndrome. *Arthritis & Rheumatology* 2015; **67**(8): 2196-204.
39. Chighizola CB, Pregnolato F, Andreoli L, et al. Beyond thrombosis: Anti-beta2GPI domain 1 antibodies identify late pregnancy morbidity in anti-phospholipid syndrome. *Journal of Autoimmunity* 2018; **90**: 76-83.
40. Pengo V, Ruffatti A, Tonello M, et al. Antiphospholipid syndrome: antibodies to Domain 1 of beta2-glycoprotein 1 correctly classify patients at risk. *Journal of thrombosis and haemostasis* 2015; **13**(5): 782-7.
41. Sciascia S, Sanna G, Murru V, Roccatello D, Khamashta MA, Bertolaccini ML. Anti-prothrombin (aPT) and anti-phosphatidylserine/prothrombin (aPS/PT) antibodies and the risk of thrombosis in the antiphospholipid syndrome. A systematic review. *Thrombosis and Haemostasis* 2014; **111**(2): 354-64.
42. Hoxha A, Mattia E, Tonello M, Grava C, Pengo V, Ruffatti A. Antiphosphatidylserine/prothrombin antibodies as biomarkers to identify severe primary antiphospholipid syndrome. *Clinical Chemistry and Laboratory Medicine* 2017; **55**(6): 890-8.

43. Ruffatti A, Tonello M, Visentin MS, et al. Risk factors for pregnancy failure in patients with anti-phospholipid syndrome treated with conventional therapies: a multicentre, case-control study. *Rheumatology (Oxford)* 2011; **50**(9): 1684-9.
44. Pengo V, Ruffatti A, Legnani C, et al. Clinical course of high-risk patients diagnosed with antiphospholipid syndrome. *Journal of thrombosis and haemostasis* 2010; **8**(2): 237-42.
45. Pengo V, Ruffatti A, Legnani C, et al. Incidence of a first thromboembolic event in asymptomatic carriers of high-risk antiphospholipid antibody profile: a multicenter prospective study. *Blood* 2011; **118**(17): 4714-8.
46. Chayoua W, Kelchtermans H, Moore GW, et al. Identification of high thrombotic risk triple-positive antiphospholipid syndrome patients is dependent on anti-cardiolipin and anti-beta2glycoprotein I antibody detection assays. *Journal of thrombosis and haemostasis* 2018; **16**(10): 2016-23.

Supporting Information

Supplementary Table 1. Number of patients testing positive (+) or negative (-) for antiDI IgG in relation to positivity for LAC or antiβ2GPI IgG or at least one of the criteria aPL panel measured by other three solid phase assays.

		Manufacturer	Thrombosis subpopulation (N = 851)		With T (N = 462)		Without T (N = 389)		Pregnancy morbidity subpopulation (N = 481)		With P (N = 154)		Without P (N = 327)	
		BioPlex®2200	AntiCL IgG		AntiCL IgG		AntiCL IgG		AntiCL IgG		AntiCL IgG		AntiCL IgG	
			+	-	+	-	+	-	+	-	+	-	+	-
AntiDI IgG	+		115	6	96	5	19	1	59	2	40	1	19	1
	-		47	683	26	335	21	348	29	391	9	104	20	287
Total			162	689	122	340	40	349	88	393	49	105	39	288
		Phadia®	AntiCL IgG		AntiCL IgG		AntiCL IgG		AntiCL IgG		AntiCL IgG		AntiCL IgG	
			+	-	+	-	+	-	+	-	+	-	+	-
AntiDI IgG	+		101	20	85	16	16	4	54	7	38	3	16	4
	-		35	695	24	337	11	358	16	404	7	106	9	298
Total			136	715	109	353	27	362	70	411	45	109	25	302
		QUANTA Lite® ELISA	AntiCL IgG		AntiCL IgG		AntiCL IgG		AntiCL IgG		AntiCL IgG		AntiCL IgG	
			+	-	+	-	+	-	+	-	+	-	+	-
AntiDI IgG	+		95	26	81	20	14	6	47	14	33	8	14	6
	-		26	704	18	343	8	361	11	409	5	108	6	301
Total			121	730	99	363	22	367	58	423	38	116	20	307
		BioPlex®2200	Antiβ2GPI IgG		Antiβ2GPI IgG		Antiβ2GPI IgG		Antiβ2GPI IgG		Antiβ2GPI IgG		Antiβ2GPI IgG	
			+	-	+	-	+	-	+	-	+	-	+	-
AntiDI IgG	+		116	5	96	5	20	0	60	1	40	1	20	0
	-		49	681	25	336	24	345	30	390	9	104	21	286
Total			165	686	121	341	44	345	90	391	49	105	41	286
		Phadia®	Antiβ2GPI IgG		Antiβ2GPI IgG		Antiβ2GPI IgG		Antiβ2GPI IgG		Antiβ2GPI IgG		Antiβ2GPI IgG	
			+	-	+	-	+	-	+	-	+	-	+	-
AntiDI IgG	+		104	17	86	15	18	2	52	9	34	7	18	2
	-		30	700	18	343	12	357	16	404	5	108	11	296
Total			134	717	104	358	30	359	68	413	39	115	29	298
		QUANTA Lite® ELISA	Antiβ2GPI IgG		Antiβ2GPI IgG		Antiβ2GPI IgG		Antiβ2GPI IgG		Antiβ2GPI IgG		Antiβ2GPI IgG	
			+	-	+	-	+	-	+	-	+	-	+	-
AntiDI IgG	+		81	40	69	32	12	8	33	28	21	20	12	8
	-		10	720	8	353	2	367	2	418	1	112	1	306
Total			91	760	77	385	14	375	35	446	22	132	13	314
		BioPlex®2200	Criteria aPL panel *		Criteria aPL panel *		Criteria aPL panel *		Criteria aPL panel *		Criteria aPL panel *		Criteria aPL panel *	
			+	-	+	-	+	-	+	-	+	-	+	-
AntiDI IgG	+		118	3	98	3	20	0	61	0	41	0	20	0
	-		205	525	128	233	77	292	119	301	56	57	63	244
Total			323	528	226	236	97	292	180	301	97	57	83	244
		Phadia®	Criteria aPL panel *		Criteria aPL panel *		Criteria aPL panel *		Criteria aPL panel *		Criteria aPL panel *		Criteria aPL panel *	
			+	-	+	-	+	-	+	-	+	-	+	-
AntiDI IgG	+		117	4	97	4	20	0	60	1	40	1	20	0
	-		253	477	149	212	104	265	151	269	67	46	84	223
Total			370	481	246	216	124	265	211	270	107	47	104	223
		QUANTA Lite® ELISA	Criteria aPL panel *		Criteria aPL panel *		Criteria aPL panel *		Criteria aPL panel *		Criteria aPL panel *		Criteria aPL panel *	
			+	-	+	-	+	-	+	-	+	-	+	-
AntiDI IgG	+		113	8	95	6	18	2	58	3	40	1	18	2
	-		223	507	138	223	85	284	133	287	67	46	66	241
Total			336	515	233	229	103	286	191	290	107	47	84	243

aPL, antiphospholipid antibody; β2GPI, β2glycoprotein I; DI, domain I; IgG (M), immunoglobulin G (M); LAC, lupus anticoagulant; N, number of patients; P, pregnancy morbidity; T, thrombosis. * Criteria aPL panel positivity: patients testing positive for at least one of the criteria aPL panel (i.e. LAC, antiCL IgG/M and/or antiβ2GPI IgG/M).

Supplementary Table 2. Number of cases with distinct antibody profiles (antiCL IgG/IgM and antiβ2GPI IgG/IgM measured by BioPlex®2200).

In the thrombosis subpopulation		N (%)[#]	aPL profile			Anti-DI IgG positive			Anti-DI IgG negative		
			LAC	anti-CL IgG/IgM	anti-β2GPI IgG/IgM	Total	with T	without T	Total	with T	without T
						N (%)[&]	N (%)	N (%)	N (%)[&]	N (%)	N (%)
Goup1	Triple positive	146 (17)	+	+	+	101 (69)	88 (87)	13 (13)	45 (31)	30 (67)	15 (33)
Goup2	Double positive	37 (4)	-	+	+	15 (41)	9 (60)	6 (40)	22 (59)	8 (36)	14 (64)
Goup3	Double positive	3 (0.4)	+	+	-	0	0	0	3 (100)	1 (33)	2 (67)
Goup4	Double positive	5 (1)	+	-	+	1 (20)	0	1 (100)	4 (80)	1 (25)	3 (75)
Goup5	Single positive	120 (14)	+	-	-	0	0	0	120 (100)	82 (68)	38 (32)
Goup6	Single positive	6 (1)	-	+	-	1 (17)	1 (100)	0	5 (83)	2 (40)	3 (60)
Goup7	Single positive	6 (1)	-	-	+	0	0	0	6 (100)	4 (67)	2 (33)
Goup8	Negative control	528 (62)	-	-	-	3 (1)	3 (100)	0	525 (99)	233 (44)	292 (56)
Total		851 (100)				121 (14)	101 (83)	20 (17)	730 (86)	361 (49)	369 (51)
In the pregnancy morbidity subpopulation		**N (%)[#]**	**aPL profile**			**Anti-DI IgG positive**			**Anti-DI IgG negative**		
			LAC	anti-CL IgG/IgM	anti-β2GPI IgG/IgM	Total	with P	without P	Total	with P	without P
						N (%)[&]	N (%)	N (%)	N (%)[&]	N (%)	N (%)
Goup1	Triple positive	62 (13)	+	+	+	42 (68)	29 (69)	13 (31)	20 (32)	6 (30)	14 (70)
Goup2	Double positive	39 (8)	-	+	+	17 (44)	11 (65)	6 (35)	22 (56)	9 (41)	13 (59)
Goup3	Double positive	2 (0.4)	+	+	-	0	0	0	2 (100)	1 (50)	1 (50)
Goup4	Double positive	3 (1)	+	-	+	1 (33)	0	1 (100)	2 (67)	0	2 (100)
Goup5	Single positive	68 (14)	+	-	-	0	0	0	68 (100)	39 (57)	29 (43)
Goup6	Single positive	2 (0.4)	-	+	-	0	0	0	2 (100)	0	2 (100)
Goup7	Single positive	4 (1)	-	-	+	1 (25)	1 (100)	0	3 (75)	1 (33)	2 (67)
Goup8	Negative control	301 (63)	-	-	-	0	0	0	301 (100)	57 (19)	244 (81)
Total		481 (100)				61 (13)	41 (67)	20 (33)	420 (87)	113 (27)	307 (73)

aPL, antiphospholipid antibody; β2GPI, β2glycoprotein I; CL, cardiolipin; DI, domain I; IgG (M), immunoglobulin G (M); LAC, lupus anticoagulant; N, number of patients in each group; P, pregnancy morbidity; T, Thrombosis.

[#]: the percentage of each group antibody profile; [&]: the percentage of antiDI IgG positive/negative in each group antibody profile; *: the percentage of individuals with/without clinical events.

Supplementary Table 3. Number of cases with distinct antibody profiles (antiCL IgG/IgM and antiβ2GPI IgG/IgM measured by Phadia®).

In the thrombosis subpopulation		N (%)[#]	aPL profile			Anti-DI IgG positive			Anti-DI IgG negative		
			LAC	anti-CL IgG/IgM	anti-β2GPI IgG/IgM	Total	with T	without T	Total	with T	without T
						N (%)[&]	N (%)	N (%)	N (%)[&]	N (%)	N (%)
Goup1	Triple positive	125 (15)	+	+	+	90 (72)	78 (87)	12 (13)	35 (28)	23 (66)	12 (34)
Goup2	Double positive	36 (4)	-	+	+	12 (33)	8 (67)	4 (33)	24 (67)	12 (50)	12 (50)
Goup3	Double positive	18 (2)	+	+	-	3 (17)	3 (100)	0	15 (83)	12 (80)	3 (20)
Goup4	Double positive	14 (2)	+	-	+	6 (43)	5 (83)	1 (17)	8 (57)	2 (25)	6 (75)
Goup5	Single positive	117 (14)	+	-	-	3 (3)	2 (67)	1 (33)	114 (97)	77 (68)	37 (32)
Goup6	Single positive	52 (6)	-	+	-	2 (4)	1 (50	1 (50)	50 (96)	20 (40)	30 (60)
Goup7	Single positive	8 (1)	-	-	+	1 (13)	0	1 (100)	7 (88)	3 (43)	4 (57)
Goup8	Negative control	481 (57)	-	-	-	4 (1)	4 (100)	0	477 (99)	212 (44)	265 (56)
Total		851 (100)				121 (14)	101 (83)	20 (17)	730 (86)	361 (49)	369 (51)
In the pregnancy morbidity subpopulation		**N (%)[#]**	**aPL profile**			**Anti-DI IgG positive**			**Anti-DI IgG negative**		
			LAC	anti-CL IgG/IgM	anti-β2GPI IgG/IgM	Total	with P	without P	Total	with P	without P
						N (%)[&]	N (%)	N (%)	N (%)[&]	N (%)	N (%)
Goup1	Triple positive	51 (11)	+	+	+	37 (73)	25 (68)	12 (32)	14 (27)	5 (36)	9 (64)
Goup2	Double positive	33 (7)	-	+	+	14 (42)	10 (71)	4 (29)	19 (58)	10 (53)	9 47()
Goup3	Double positive	9 (2)	+	+	-	2 (22)	2 (100)	0	7 (78)	4 (57)	3 (43)
Goup4	Double positive	7 (1)	+	-	+	1 (14)	0	1 (100)	6 (86)	1 (17)	5 (83)
Goup5	Single positive	68 (14)	+	-	-	3 (4)	2 (67)	1 (33)	65 (96)	36 (55)	29 (45)
Goup6	Single positive	34 (7)	-	+	-	2 (6)	1 (50)	1 (50)	32 (94)	7 (22)	25 (78)
Goup7	Single positive	9 (2)	-	-	+	1 (11)	0	1 (100)	8 (89)	4 (50)	4 (50)
Goup8	Negative control	270 (56)	-	-	-	1 (0.4)	1 (100)	0	269 (100)	46 (17)	223 (83)
Total		481 (100)				61 (13)	41 (67)	20 (33)	420 (87)	113 (27)	307 (73)

Abbreviations: aPL, antiphospholipid antibody; β2GPI, β2glycoprotein I; CL, cardiolipin; DI, domain I; IgG (M), immunoglobulin G (M); LAC, lupus anticoagulant; N, number of patients in each group; P, pregnancy morbidity; T, Thrombosis.

[#]: the percentage of each group antibody profile; [&]: the percentage of antiDI IgG positive/negative in each group antibody profile; *: the percentage of individuals with/without clinical events.

Supplementary Table 4. Number of cases with distinct antibody profiles (antiCL IgG/IgM and antiβ2GPI IgG/IgM measured by QUANTA Lite ELISA®).

In the thrombosis subpopulation		N (%)[#]	aPL profile			Anti-DI IgG positive			Anti-DI IgG negative		
			LAC	anti-CL IgG/IgM	anti-β2GPI IgG/IgM	Total	with T	without T	Total	with T	without T
						N (%)[&]	N (%)	N (%)	N (%)[&]	N (%)	N (%)
Goup1	Triple positive	106 (12)	+	+	+	82 (77)	72 (88)	10 (12)	24 (23)	17 (71)	7 (29)
Goup2	Double positive	25 (3)	-	+	+	8 (32)	5 (62)	3 (38)	17 (68)	6 (35)	11 (65)
Goup3	Double positive	22 (3)	+	+	-	6 (27)	5 (83)	1 (17)	16 (73)	9 (56)	7 (44)
Goup4	Double positive	13 (2)	+	-	+	2 (15)	2 (100)	0	11 (85)	8 (73)	3 (27)
Goup5	Single positive	133 (16)	+	-	-	12 (9)	9 (75)	3 (25)	121 (91)	80 (66)	41 (34)
Goup6	Single positive	28 (3)	-	+	-	3 (11)	2 (67)	1 (33)	25 (89)	15 (60)	10 (40)
Goup7	Single positive	9 (1)	-	-	+	0	0	0	9 (100)	3 (33)	6 (67)
Goup8	Negative control	515 (61)	-	-	-	8 (2)	6 (75)	2 (25)	507 (98)	223 (44)	284 (56)
Total		851 (100)				121 (14)	101 (83)	20 (13)	730 (86)	361 (49)	369 (51)
In the pregnancy morbidity subpopulation		**N (%)[#]**	**aPL profile**			**Anti-DI IgG positive**			**Anti-DI IgG negative**		
			LAC	**anti-CL IgG/IgM**	**anti-β2GPI IgG/IgM**	**Total**	**with P**	**without P**	**Total**	**with P**	**without P**
						N (%)[&]	**N (%)**	**N (%)**	**N (%)[&]**	**N (%)**	**N (%)**
Goup1	Triple positive	36 (7)	+	+	+	29 (81)	19 (66)	10 (34)	7 (19)	1 (14)	6 (86)
Goup2	Double positive	28 (6)	-	+	+	10 (36)	7 (70)	3 (30)	18 (64)	9 (50)	9 (50)
Goup3	Double positive	13 (3)	+	+	-	6 (46)	5 (83)	1 (17)	7 (54)	1 (14)	6 (86)
Goup4	Double positive	6 (1)	+	-	+	0	0	0	6 (100)	3 (50)	3 (50)
Goup5	Single positive	80 (17)	+	-	-	8 (10)	5 (62)	3 (37)	72 (90)	41 (57)	31 (43)
Goup6	Single positive	20 (4)	-	+	-	4 (20)	3 (75)	1 (25)	16 (80)	9 (56)	7 (44)
Goup7	Single positive	8 (2)	-	-	+	1 (13)	1 (100)	0	7 (88)	3 (43)	4 (57)
Goup8	Negative control	290 (60)	-	-	-	3 (1)	1 (33)	2 (67)	287 (99)	46 (16)	241 (84)
Total		481 (100)				61 (13)	41 (67)	20 (33)	420 (87)	113 (27)	307 (73)

aPL, antiphospholipid antibody; β2GPI, β2glycoprotein I; CL, cardiolipin; DI, domain I; IgG (M), immunoglobulin G (M); LAC, lupus anticoagulant; N, number of patients in each group; P, pregnancy morbidity; T, Thrombosis.

[#]: the percentage of each group antibody profile; [&]: the percentage of antiDI IgG positive/negative in each group antibody profile; *: the percentage of individuals with/without clinical events.

Supplementary Table 5. Agreement between antiDI IgG positivity and triple positivity for four platforms in the different subpopulations.

Agreement[1]	HemosIL AcuStar®	BioPlex®2200	Phadia®	QUANTA Lite® ELISA
In the thrombosis subpopulation	0.746***	0.712***	0.686***	0.680***
In the pregnancy morbidity subpopulation	0.679***	0.636***	0.616***	0.556***

[1] Kappy values are shown

*** P < 0.0001

Supplementary Table 6. Comparison of antiDI IgG titers between triple positive patients with and without clinical events.

Manufacturers for antiCL and antiβ2GPI	In the thrombosis population			In the pregnancy morbidity population		
	With T	Without T	P value	With P	Without P	P value
HemosIL AcuStar®	169.0 CU (25.7-761.3)	63.7 CU (3.7-625.7)	0.084	200.2 CU (49.6-462.1)	65.0 CU (3.7-654.3)	0.179
BioPlex® 2200	156.1 CU (18.9-635.2)	11.8 CU (3.7-349.4)	**0.014**	191.2 CU (47.9-433.6)	13.8 CU (3.7-364.2)	**0.024**
Phadia®	202.5 CU (30.1-769.6)	25.1 CU (3.7-560.3)	**0.044**	200.2 CU (47.9-588.2)	66.4 CU (4.6-682.8)	0.238
QUANTA Lite ELISA®	229.0 CU (39.8-833.4)	66.4 CU (3.7-799.6)	0.236	308.2 CU (160.2-881.7)	87.4 CU (3.7-829.3)	0.285

β2GPI, β2glycoprotein I; CL, cardiolipin; CU, chemiluminescence units; DI, domain I; P, pregnancy morbidity; T, thrombosis.

Titers of antiDI IgG are expressed as the median (quartile(Q)25- Q75), Mann–Whitney U test was used to compare difference of two groups, the significant p values are shown in bold.

Chapter 4

Detection of anti-domain I antibodies by an in-house developed ELISA can improve the classification of antiphospholipid syndrome

Dongmei Yin, Hilde Kelchtermans, Philip G. de Groot, Bas de Laat, Katrien M. J. Devreese

In preparation

Chapter 5

Commentary on: Circulating plasmablasts contribute to antiphospholipid antibody production, associated with type I interferon upregulation

Dongmei Yin, Bas de Laat

Journal of Thrombosis and Haemostasis. 2019 May 21; 17:1030-1032.

The antiphospholipid syndrome has been an enigma for many years. The diagnosis of a patient is based on rather generally occurring clinical symptoms in combination with specific laboratory tests. In more detail, as stated in its criteria, it is a syndrome in which patients suffer from thrombosis and pregnancy morbidity[1] . Diagnosis should be made on these clinical criteria in combination with laboratory criteria. Patients are tested positive for either one of three tests: anti-beta2-glycoprotein I (β2GP1) IgM/IgG antibodies, anti-cardiolipin IgM/IgG antibodies, or a prolongation of a phospholipid-dependent coagulation time. Recently the group of Devreese together with our group initiated a multicentre study in order to investigate whether we can improve the diagnosis of the syndrome[2] .

Over the years, many groups have studied different populations of antiphospholipid antibodies and their effect on initiating a prothrombotic phenotype. Many mechanisms have been proposed. Antibodies have been shown in vitro and in vivo to affect almost any protein or cell that is involved in the occurrence of thrombosis (**Figure 1**)[3]. At present, the complement system is gaining attention as one of the major pathogenic mechanisms [4].

As many mechanisms have been proposed to be involved in the antiphospholipid syndrome, it is quite difficult to choose a protein or cell to target for treatment. With this in mind, the idea of the group of Hisada et al. is rather well thought out [4]. Their main study objective was to identify the mechanism of antiphospholipid antibody production. Study and identification of which cells are involved may make targeting of these for treatment an option. Interestingly, they found that the primary cells involved in antibody production were plasmablasts, which are the earliest plasma cells capable of producing antibodies. Characteristics of these cells are that they can divide rapidly, and that they are capable of internalisation antigens, presenting antigen to T-cell and producing antibodies. In fact, these cells are capable of producing high amounts of IgG antibodies.

In addition, the authors show that the subpopulation of isolated peripheral blood mononuclear cells (PBMCs) that are CD20-negative are more relevant regarding the production of antiphospholipid antibodies than PBMCs that are CD20-positive. Rituximab, a chimeric monoclonal antibody used as a treatment for autoimmune diseases, has been used within the antiphospholipid syndrome[5]. Rituximab binds CD20 and thereby silences CD20-positive B cells, preventing the production of autoimmune antibodies. Interestingly, trials with rituximab in patients with antiphospholipid syndrome have shown contrasting results. The failure of these trials might be partially related to the findings discussed above.

Attempting to stop antibody production instead of preventing symptoms is not a new idea, as already in 1998, the group of Iverson et al studied treatment options in this respect[6]. Antiphospholipid antibodies have been shown to predominantly react with beta2-glycoprotein I. The first domain of beta2-glycoprotein I was shown to interact with auto-antibodies that were highly associated with thrombosis. In the early years when anti-domain I antibodies were gaining popularity, La Jolla Pharmaceutical Company developed a treatment strategy based on domain I reactivity. It produced a domain I tetramer in order to produce immune tolerance at the B-cell level. Unfortunately, this treatment has not reached the clinic. This might be because the population of antiphospholipid antibodies is heterogeneous. Although only antibodies that bind

beta2GPI have been included in the official criteria used to diagnose APS, even within the subpopulation of anti-beta2GPI antibodies there is heterogeneity as several different non-continuous epitopes have been shown to react with antiphospholipid antibodies[7].

At present, different groups are working on different aspects of the antiphospholipid syndrome. More and more is being discovered about the mechanisms by which antiphospholipid antibodies induce thrombosis and pregnancy loss, although there is not yet absolute certainty regarding which mechanism is dominant. The recent multi-center study led by Devreese will, hopefully, further improves the diagnosis of antiphospholipid syndrome [2]. Also, the study of Hisada et al has provided new paths that can be further investigated to potentially improve treatment of patients suffering from antiphospholipid syndrome [4].

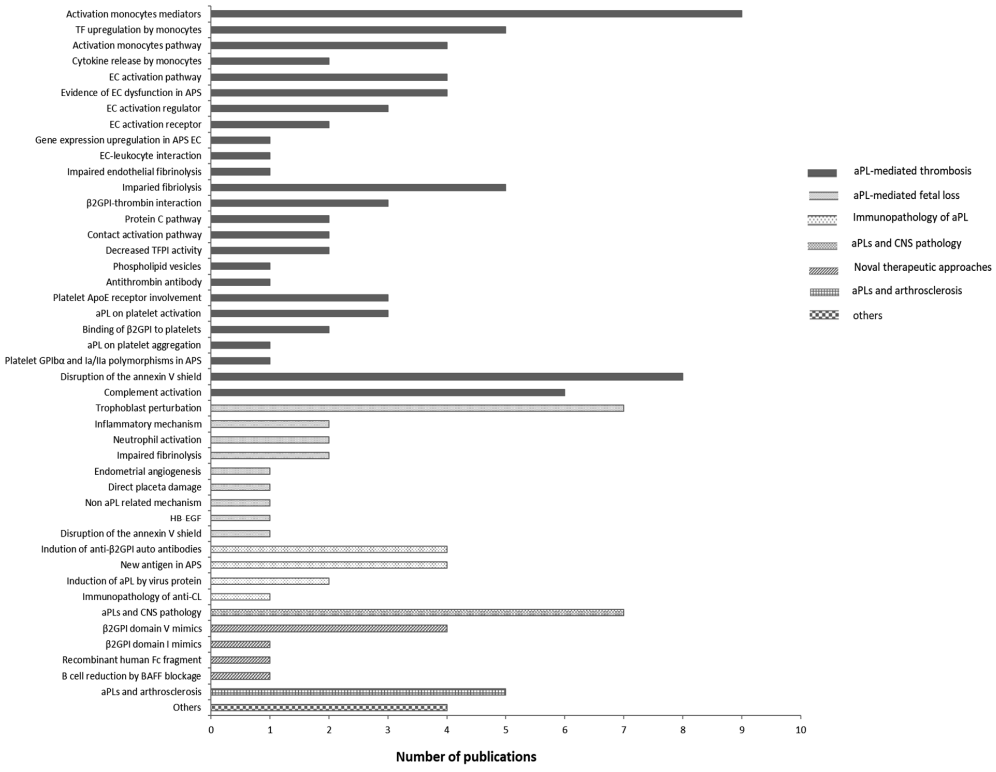

Figure 1. Mechanistical action of antiphospholipid antibodies (aPL): studies (2006 - March 2012). Adapted from Thromb. Res. 2013;132(3):319-326. CNS: central nervous system; EC: endothelial cells; HB-EGF: heparin-binding epidermal growth factor-like growth factor; BAFF: a tumor necrosis factor-like cytokine.

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). *Journal of thrombosis and haemostasis* 2006; **4**(2): 295-306.

2. Chayoua W, Kelchtermans H, Moore GW, et al. Identification of high thrombotic risk triple-positive antiphospholipid syndrome patients is dependent on anti-cardiolipin and anti-beta2glycoprotein I antibody detection assays. *Journal of thrombosis and haemostasis* 2018; **16**(10): 2016-23.

3. Du VX, Kelchtermans H, de Groot PG, de Laat B. From antibody to clinical phenotype, the black box of the antiphospholipid syndrome: pathogenic mechanisms of the antiphospholipid syndrome. *Thrombosis Research* 2013; **132**(3): 319-26.

4. Hisada R, Kato M, Sugawara E, et al. Circulating plasmablasts contribute to antiphospholipid antibody production, associated with type I interferon upregulation. *Journal of thrombosis and haemostasis* 2019; **17**(7): 1134-43.

5. Shanahan JC, Moreland LW, Carter RH. Upcoming biologic agents for the treatment of rheumatic diseases. *Current opinion in rheumatology* 2003; **15**(3): 226-63.

6. Iverson GM, Victoria EJ, DM. M. Anti-beta2 glycoprotein I (beta2GPI) autoantibodies recognize an epitope on the first domain of beta2GPI. *Proceedings of the National Academy of Sciences of the U S A* 1998; **95**: 15542-6.

7. Ioannou Y, Pericleous C, Giles I, Latchman DS, Isenberg DA, Rahman A. Binding of antiphospholipid antibodies to discontinuous epitopes on domain I of human beta(2)-glycoprotein I - Mutation studies including residues R39 to R43. *Arthritis & Rheumatology* 2007; **56**(1): 280-90.

Chapter 6

Is there an additional value in detecting anticardiolipin and anti-β2glycoprotein I IgA antibodies in the antiphospholipid syndrome?

Walid Chayoua, Dongmei Yin, Hilde Kelchtermans, Gary W. Moore, Jean-Christophe Gris, Jacek Musiał, Stéphane Zuily, Hugo ten Cate, Bas de Laat and Katrien M. J. Devreese

Thrombosis and Haemostasis. 2020 Nov;120(11):1557-1568.

Abstract

Background: Anti-cardiolipin (antiCL) and anti-β2glycoprotein I (aβ2GPI) IgA antiphospholipid antibodies (aPL) have shown to associate with thrombosis and pregnancy morbidity. However, inclusion of IgA aPL in the classification criteria of the antiphospholipid syndrome (APS) has been debated.

Objectives: We investigated the value of antiCL and antiβ2GPI IgA aPL in the detection of thrombosis and pregnancy morbidity in addition to the current aPL panel for APS.

Patients/Methods: We included 1068 patients from eight European medical centers: 259 thrombotic APS patients, 122 obstetric APS patients, 204 non-APS thrombosis patients, 33 non-APS obstetric patients, 60 APS patients with unspecified clinical manifestations, 196 patients with autoimmune diseases and 194 controls. antiCL and aβ2GPI IgG/M/A were detected with four commercial assays and LAC was determined by the local center.

Results: Positivity for IgA aPL was found in 17-26% of the patients with clinical manifestations of APS and in 6-13% of the control population. Both antiCL and aβ2GPI IgA were significantly associated with thrombosis and pregnancy morbidity. Isolated IgA positivity was rare in patients with clinical manifestations of APS (0.3-5%) and not associated with thrombosis and/or pregnancy morbidity. Addition of IgA to the current criteria panel did not increase odds ratios for thrombosis nor pregnancy morbidity.

Conclusions: antiCL and aβ2GPI IgA are associated with clinical manifestations of APS. However, isolated IgA positivity was rare and not associated with thrombosis or pregnancy morbidity. These data do not support testing for antiCL and antiβ2GPI IgA subsequent to conventional aPL assays in identifying patients with thrombosis or pregnancy morbidity.

Introduction

Clinical manifestations of the antiphospholipid syndrome (APS) include thrombosis and pregnancy morbidity [1]. However, these manifestations occur frequently and often independent of APS. Therefore, classification of APS predominantly relies on antiphospholipid (aPL) assays detecting lupus anticoagulant (LAC) by coagulation tests or detection of anti-cardiolipin (antiCL) and anti-β2glycoprotein I (antiβ2GPI) antibodies from the IgG or IgM isotype by solid phase assays [1,2]. Anti-cardiolipin and antiβ2GPI antibodies from the IgA isotype are not included in the current classification criteria [1,2]. Multiple studies have illustrated an association of antiCL and antiβ2GPI IgA antibodies with thrombosis and pregnancy morbidity [3-12]. Still, antiCL and aβ2GPI IgA are not included in the current guidelines [1,2]. The main reason is unavailability of data to show the clinical significance of antiCL or antiβ2GPI IgA with clinical manifestations of APS in addition to the current aPL-panel [1,13].

In a mouse model for thrombosis, IgA antibodies isolated from APS patients resulted in increased thrombus area, faster thrombus formation and decreased time of thrombus disappearance compared to control IgA [14]. Outcomes of clinical studies have been contradictory regarding the association of antiCL and/or antiβ2GPI IgA with clinical symptoms of APS and their role in identifying additional APS patients with thrombosis or pregnancy morbidity [3-12,15,16]. Multiple studies have shown that IgA is associated with thrombosis and pregnancy morbidity [3-12], other studies report no association of antiCL or antiβ2GPI IgA with thrombosis or pregnancy morbidity [15,16]. Isolated antiCL and/or antiβ2GPI IgA positivity was often found not to be associated with thrombosis or pregnancy morbidity [4,9,15-17]. However, some studies concluded that antiCL and/or antiβ2GPI IgA is an independent risk factor for thrombosis [6,10,18]. In addition to the clinical manifestations of APS, IgA positivity has also been associated with systemic lupus erythematosus (SLE), thrombocytopenia, heart valve disease, livedo reticularis and epilepsy [5,19]. Comparison of clinical studies is difficult as multiple study designs are used with a wide variety of aPL assays and aPL cut-off values. Furthermore, solid phase assays detecting antiCL and antiβ2GPI antibodies are poorly standardized and produce variable results in a head-to-head comparison [20-22].

In this multicenter study, we used four commercially available solid phase assays to detect antiCL and antiβ2GPI IgG/M/A, to assess for an association of antiCL and antiβ2GPI IgA and thrombosis or obstetric complications. 1068 patients were included of which 678 patients with clinical manifestations of APS and 390 patients that served as controls. We aimed to assess the added value of antiCL and antiβ2GPI IgA antibodies as a biomarker for thrombosis and pregnancy morbidity within the current aPL-panel consisting of LAC, antiCL IgG/M and antiβ2GPI IgG/M.

Materials and Methods

Study population

A total of 1068 patient samples were collected from eight medical European centers. The Sydney classification criteria were followed for the classification of thrombotic and obstetric APS (**Table 1**) [1]. Classification of thrombotic or obstetric APS was determined by the local center. In addition to APS patients, we included patients with an autoimmune disease other than APS (e.g. 54% SLE and 29% systemic sclerosis) without thrombotic complications (autoimmune disease (AID) controls); patients that were referred for aPL testing for other reasons (e.g. subfertility and prolonged activated partial thromboplastin time (aPTT)) than the clinical criteria of APS (controls); patients with a previous thrombotic event and negatively tested for aPL (non-APS thrombosis); and patients that experienced obstetric complications in the absence of aPL (non-APS obstetric). The study was approved by the local ethical committees.

Laboratory assays

Lupus anticoagulant

Lupus anticoagulant assays were performed by the local center, according to the International Society of Thrombosis and Haemostasis-Scientific Standardization Subcommittee (ISTH-SSC) guideline [23]. Briefly, citrated plasma was tested in a multiple step procedure (screening, mixing, confirmation step) with two test systems based on different principles (LA-sensitive aPTT and dilute Russell viper venom time) [1,23].

Solid phase assays

Commercially available solid phase assays were selected based on frequently used assays in the external quality control program of the ECAT (External quality Control of diagnostic Assays and Tests, Voorschoten, The Netherlands) and the willingness of manufacturers for providing the reagents. ACL and antiβ2GPI antibodies from the IgG, IgM and IgA isotype were detected at one occasion by four solid phase assays: BioPlex®2200 (Bio-Rad, Bio-Rad Laboratories, Hercules, USA), Phadia® (Thermo Fisher Scientific/Phadia, Uppsala, Sweden), HemosIL AcuStar® (Instrumentation Laboratories, Bedford, USA) and QUANTA Lite® ELISA (Inova Diagnostics, San Diego, USA) in the Ghent University Hospital (Ghent, Belgium). Detection of antiCL and antiβ2GPI antibodies was performed according to manufacturer's instructions. Manufacturer's recommended cut-off values were used upon confirmation in 20 healthy volunteers, in accordance with the ISTH-SSC guideline (**Supplemental Table 1**) [24].

Statistical analyses

Associations of antiCL and antiβ2GPI IgA positivity and clinical manifestations of APS were assessed by calculating odds ratios (ORs) with their respective 95% confidence intervals (CI). Thrombotic association with IgA aPL was assessed within patients with thrombotic APS, non-APS thrombosis, AID controls and controls (n=853). Obstetric association with IgA aPL was assessed within female patients with obstetric APS, non-APS obstetric APS, AID controls and controls

(n=483). The additional diagnostic value of antiCL and antiβ2GPI IgA antibodies was assessed by 2x2 contingency tables. Kruskal–Wallis test was used to evaluate differences in IgA titers. To compare numbers (percentages) of positive tests between systems, the comparison of two proportions (from independent samples) was used. Statistical analyses were performed using the statistical package for social sciences (SPSS 23.0; SPSS, Chicago, IL) and MedCalc Statistical Software version 17.7.2 (MedCalc Software bvba, Ostend, Belgium). Statistical significance was set at P value less than 0.05.

Results

Patient characteristics

We included 1068 patients of which 678 patients had clinical manifestations of APS and 390 controls (**Table 1**). In thrombotic APS, venous thrombosis was more prevalent than arterial thrombosis (160 (62%) versus 55 (21%) patients, respectively; **Table 1**). From the 204 non-APS thrombotic patients, 149 (73%) had a history of venous thrombosis and 47 (23%) had a history of arterial thrombosis (**Table 1**). From the 259 thrombotic APS patients, the majority (40%) received Vitamin K antagonists, 5% received Low-Molecular-Weight Heparin (LMWH), 4% Direct Oral Anticoagulants (DOAC), 10% antiplatelet therapy, 8% a combination of oral anticoagulants and anti-platelet therapy. Details on anticoagulant and antiplatelet therapy of the remaining 83 (32%) patients are not available. Patients classified as non-APS thrombosis and non-APS obstetric were negatively tested for criteria aPL by the local medical center. However, retesting of the 204 non-APS thrombosis patients resulted in 1.5 to 8% positives for antiCL and/or antiβ2GPI IgG/IgM, depending on the solid phase assay (▸**Table 2**). Re-testing of 33 non-APS obstetric patients resulted in 0 to 12% positives for antiCL and/or antiβ2GPI IgG/IgM, depending on the solid phase assay (▸**Table 2**).

Prevalence of (isolated) antiCL and/or aß2GPI IgA

Among patients with clinical manifestations of APS, 17-26% tested positive for antiCL and/or aβ2GPI IgA antibodies (**Table 2**). Positivity for antiCL and/or aβ2GPI IgA antibodies in thrombotic APS ranged between 26%-37%, dependent on the solid phase assay used to detect IgA antibodies. Within obstetric APS, positivity for antiCL and/or aβ2GPI IgA ranged between 16-34%, dependent on the solid phase assay. In the control group, consisting of AID patients and controls 6-8% were positive for antiCL and/or aβ2GPI IgA aPL. Isolated positivity for antiCL and/or aβ2GPI IgA (positive for IgA with negative LAC, IgG and IgM results) was rare in thrombotic and obstetric patients with a prevalence of 0%-3% and 1%-5%, respectively. Within AID and controls isolated antiCL and/or aβ2GPI IgA positivity ranged between 1%-4%. With two (BioPlex®2200 and HemosIL AcuStar®) out of the four tested solid phase assays, only 1% of the total study population was positive for IgA with negative LAC, IgG and IgM results. The other two solid phase assays detected more isolated IgA patients, 2% (Phadia®) and 3% (QUANTA Lite® ELISA).

Correlation of antiCL and/or aß2GPI IgA with criteria aPL

Within the total population consisting of 1068 patients, 408 (38%) were positive for LAC, thereby the most prevalent aPL (**Supplemental Table 2**). Immunoglobulin G was the most prevalent

antiCL or aβ2GPI antibody isotype in the total study population. Only 6% of the total patient population was positive for antiCL IgA, detected with QUANTA Lite® ELISA. By a comparison of two proportions, a significant difference (P<0.0001) was found for the number of patients positive for antiCL IgA and antiCL IgM, detected with either BioPlex®2200 or Phadia®. Detection of aβ2GPI IgM and IgA resulted in significant difference in number of positive samples with the BioPlex®2200 system only (P=0.0001). aβ2GPI IgG was more prevalent than aβ2GPI IgA antibodies, except when detected with BioPlex®2200 (P=0.0007). By a comparison of two proportions, significant differences were found between antiCL IgG and antiCL IgA for all solid phase assays. Prevelance of aPL in the subgroups (controls, AID, APS thrombosis, non-APS thrombosis, APS obstetric, non-APS obstetric and APS patients) are shown in **Supplemental Table 2**. Lupus anticoagulant and antiCL or aβ2GPI IgA were highly correlated, as 63%-80% IgA positive patients were also characterized by positive LAC results (data not shown). Similar, 72%-83% of the patients positive for antiCL IgG, were also positive for LAC. aβ2GPI IgG and LAC showed an overlap of 77%-87%. Positive results for antiCL IgM or aβ2GPI IgM were less correlated with LAC, 59%-74% and 72%-75%, respectively. Upon detection with BioPlex®2200, positivity for IgA aPL also highly correlated with triple positivity. Within the total population, the BioPlex®2200 identified 221 triple positive patients of which 162 (73%) were positive for antiCL and/or aβ2GPI IgA antibodies. Other solid phase assays tested showed less correlation between IgA aPL and triple positivity (Phadia® (49%), HemosIL AcuStar® (60%) and QUANTA Lite® ELISA (48%)).

Table 1. Demographic and clinical characteristics of the study population.

					Thrombosis				
	Patients (n)	**Female**	**Age [year,mean (range)]**	**Pregnancy morbidity**	**Venous**	**Arterial**	**Venous and arterial**	**Small vessel**	**Not specified**
APS thrombosis	259	164 (63%)	50 (17-87)	22	160	55	26	4	14
Non-APS thrombosis	204	116 (57%)	46 (19-85)	0	149	47	5	0	3
APS obstetric	122	122 (100%)	35 (19-61)	122	4	2	0	0	4
Non-APS obstetric	33	33 (100%)	33 (20-52)	33	NA	NA	NA	NA	NA
Autoimmune diseases	196	158 (81%)	46 (16-83)	NA	NA	NA	NA	NA	NA
Controls	194	170 (88%)	39 (18-82)	NA	NA	NA	NA	NA	NA
APS*	60	60 (100%)	48 (24-70)	Not available	Not available	Not available	Not available	Not available	Not available
Total population	1068	823 (77%)	44 (16-87)	177	313	104	31	4	21

*The medical center did not specify if the patient suffered from obstetric or thrombotic complications. NA, not applicable

Table 2. Additional diagnostic value of antiCL and antiβ2GPI IgA antibodies. Figures indicate the number of patients testing positive or negative for LAC, antiCL and antiβ2GPI IgG/M antibodies versus antiCL and antiβ2GPI IgA, for each solid phase assay.

			Overall population (n=1068)		**Non-APS thrombosis (n=204)**		**APS-thrombosis (n=259)**		**Non-APS obstetric (n=33)**		**APS-obstetric (n=122)**		**APS* (n=60)**		**AID (n=196)**		**Controls (n=194)**	
			LAC, antiCL IgG, antiβ2GPI IgG, antiCL IgM or antiβ2GPI IgM		**LAC, antiCL IgG, antiβ2GPI IgG, antiCL IgM or antiβ2GPI IgM**		**LAC, antiCL IgG, antiβ2GPI IgG, antiCL IgM or antiβ2GPI IgM**		**LAC, antiCL IgG, antiβ2GPI IgG, antiCL IgM or antiβ2GPI IgM**		**LAC, antiCL IgG, antiβ2GPI IgG, antiCL IgM or antiβ2GPI IgM**		**LAC, antiCL IgG, antiβ2GPI IgG, antiCL IgM or antiβ2GPI IgM**		**LAC, antiCL IgG, antiβ2GPI IgG, antiCL IgM or antiβ2GPI IgM**		**LAC, antiCL IgG, antiβ2GPI IgG, antiCL IgM or antiβ2GPI IgM**	
			+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
BioPlex® 2200	antiCL and/or antiβ2GPI IgA	+	197	10	1	2	97	0	0	1	39	2	31	1	22	3	7	1
		-	106	755	2	199	45	117	0	32	20	61	9	19	22	149	8	178
	Total		303	765	3	201	142	117	0	33	59	63	40	20	44	152	15	179
Phadia®	antiCL and/or antiβ2GPI IgA	+	121	26	2	7	60	4	1	2	17	4	21	1	17	7	3	3
		-	247	674	15	180	91	104	3	27	49	52	22	16	44	128	23	165
	Total		368	700	17	187	151	108	4	29	66	56	43	17	61	135	26	168
HemosIL AcuStar®	antiCL and/or antiβ2GPI IgA	+	147	6	0	0	77	0	0	0	24	1	26	1	15	4	5	0
		-	208	707	8	196	81	101	1	32	41	56	18	15	44	133	15	174
	Total		355	713	8	196	158	101	1	32	65	57	44	16	59	137	20	174
QUANTA Lite® ELISA	antiCL and/or antiβ2GPI IgA	+	102	34	1	7	62	5	0	1	14	6	15	1	8	10	2	4
		-	210	658	5	191	76	116	3	29	45	57	31	13	40	138	10	178
	Total		312	756	6	198	138	121	3	30	59	63	46	14	48	148	12	182

Abbreviations: antiβ2GPI, anti-β2 glycoprotein I; antiCL, anticardiolipin; AID, autoimmune diseases; APS, antiphospholipid syndrome; IgA, immunoglobulin A; IgM, immunoglobulin M; LAC, lupus anticoagulant.

*The medical center did not specify if the patient suffered from obstetric or thrombotic complications.

Association of antiCL and/or aß2GPI IgA with thrombosis and pregnancy morbidity

Both antiCL and aβ2GPI IgA positivity were significantly associated with thrombosis (**Table 3**). Positivity for antiCL IgA reached odds ratios (ORs) for thrombosis of 3.0 (95% confidence interval [CI], 1.9-4.9) to 9.9 (95% CI, 3.5-27.8). Anti-β2GPI IgA positivity showed ORs for thrombosis between 2.4 (95% CI, 1.5-3.9) to 3.1 (95% CI, 1.9-5.2). A stronger thrombotic association was found with antiCL IgA assays than the aβ2GPI IgA assay. Detection of antiCL IgA with QUANTA Lite® ELISA reached the highest OR for thrombosis (9.9 (95% CI, 3.5-27.8)). However, from the 47 patients positive for antiCL IgA, only 43 patients with a history of thrombosis were detected with this assay, while the antiCL IgA assay from BioPlex®2200 detected 130 patients of which 100 patients with a history of thrombosis. Positivity for antiCL and/or aβ2GPI IgA reached comparable ORs for thrombosis, between the tested assays with ORs between 2.2 (95% CI, 1.4-3.5) and 3.0 (2.0-4.5).

Positivity for both antiCL and aβ2GPI from the same isotype is considered to be more reliable for diagnosis of APS. Odds ratios for thrombosis did not increase in patients positive for antiCL and aβ2GPI IgA aPL, compared to patients positive for antiCL IgA or aβ2GPI IgA when detected with the BioPlex®2200 and HemosIL AcuStar. The other two tested solid phase assay showed an increase of OR for thrombosis in patients positive for antiCL and aβ2GPI IgA compared to ORs obtained from the antiCL and aβ2GPI IgA assay separately, most pronounced for QUANTA Lite ELISA. In comparison, patients positive for both antiCL and aβ2GPI IgG were characterized with a similar or slightly higher OR than positivity for antiCL IgG or aβ2GPI IgG. However, the increase in OR for double IgG positivity for QUANTA Lite ELISA was less pronounced compared to the increase observed in double IgA positivity for that platform. Also, for the IgM isotype similar results were obtained (**Supplemental Table 3**). Patients positive for both antiCL and aβ2GPI IgM antibodies were characterized with similar ORs for thrombosis than OR from an antiCL IgM or aβ2GPI IgM assay alone.

IgA aPL were significantly correlated with pregnancy morbidity, independent of the solid phase platform (**Table 3**). Similar to thrombosis, the antiCL IgA assay from QUANTA Lite® ELISA reached the highest OR (5.0 (95% CI, 1.5-16.5)) for pregnancy morbidity. Double positivity for IgG or IgM resulted in slightly higher OR for three out of the four platforms (**Supplemental Table 4**). Double positivity for IgA (**Table 3**) resulted in significant higher OR for one platform (QUANTA Lite ELISA), in agreement with the thrombosis results.

Association of antiCL and aβ2GPI IgA was calculated separately for venous and arterial thrombosis (**Supplemental Table 5**). ORs for venous thrombosis (n=309) ranged between 1.8 (95% CI, 1.1-2.8) to 7.0 (95% CI, 2.4-20.7) and 1.8 (95% CI, 1.1-3.0) to 2.4 (95% CI, 1.4-4.2), for antiCL and aβ2GPI IgA, respectively. ORs for arterial thrombosis (n=102) ranged between 2.2 (95% CI, 1.2-4.0) to 14.1 (95% CI, 4.5-44.3) and 2.9 (95% CI, 1.5-5.6) to 3.8 (95% CI, 1.9-7.4) for antiCL and aβ2GPI IgA, respectively.

Association of isolated antiCL and/or aß2GPI IgA with thrombosis and pregnancy morbidity

Isolated antiCL and/or aβ2GPI IgA positivity was not associated with thrombosis (**Table 4**). Odds Ratios varied between 0.4 (95% CI, 0.08-2.3) and 0.9 (95% CI, 0.4-2.2). In a more detailed analysis, no significant association was found for arterial or venous thrombosis and isolated IgA antiCL or aβ2GPI (**Supplemental Table 5**). Isolated antiCL or aβ2GPI IgA positivity was also not significantly associated with pregnancy morbidity with OR ranging between 0.7 (95% CI, 0.1-6.8) to 2.1 (95% CI, 0.4-10.7) (**Table 4**).

Table 3. Thrombotic and obstetric association of antiCL and antiβ2GPI IgA antibodies. Odds ratios for thrombosis are calculated in a subpopulation including controls, AID, non-APS thrombosis and APS thrombosis patients (n=853). Odds ratios for pregnancy morbidity are calculated in a female subpopulation including controls, AID, non-APS obstetric and APS obstetric patients (n=483).

		BioPlex®2200	Phadia®	HemosIL AcuStar®	QUANTA Lite® ELISA
Thrombosis	antiCL	3.3 (2.1-5.1)	3.1 (1.6-6.1)	3.0 (1.9-4.9)	9.9 (3.5-27.8)
	n	130	49	101	47
	antiβ2GPI	2.8 (1.8-4.3)	2.4 (1.5-3.9)	2.5 (1.5-4.0)	3.1 (1.9-5.2)
	n	124	94	88	91
	antiCL and/or antiβ2GPI	3.0 (2.0-4.5)	2.2 (1.4-3.5)	3.0 (1.9-4.9)	3.0 (1.8-4.8)
	n	133	103	101	99
	antiCL and antiβ2GPI	3.1 (2.0-4.8)	4.2 (1.8-9.6)	2.4 (1.5-4.0)	35.8 (4.8-254.5)
	n	121	40	88	39
Pregnancy morbidity	antiCL	3.6 (2.1-6.1)	3.5 (1.5-8.3)	2.4 (1.3-4.5)	5.0 (1.5-16.5)
	n	67	23	47	13
	antiβ2GPI	2.9 (1.7-4.9)	1.9 (1.0-3.5)	2.2 (1.2-4.1)	2.7 (1.4-5.3)
	n	65	42	45	37
	antiCL and/or antiβ2GPI	3.6 (2.1-5.9)	1.8 (1.0-3.4)	2.6 (1.4-4.7)	2.4 (1.3-4.6)
	n	73	49	48	41
	antiCL and antiβ2GPI	2.9 (1.7-5.0)	4.9 (1.7-14.5)	2.1 (1.1-3.9)	17.8 (2.2-143.6)
	n	59	16	44	9

Abbreviations: antiβ2GPI, anti-β2 glycoprotein I; antiCL, anticardiolipin; CI, confidence interval; n, number of patients positive for antiCL or antiβ2GPI IgA within the population; OR, odds ratio.

Note: Odds ratios for thrombosis are calculated in a subpopulation including controls, AID, non-APS thrombosis, and APS thrombosis patients (n= 853). Odds ratios for pregnancy morbidity are calculated in a female subpopulation including controls, AID, non-APS obstetric, and APS obstetric patients (n =483).

Table 4. Thrombotic and obstetric association of isolated antiCL and antiβ2GPI IgA antibodies. Odds ratios for thrombosis are calculated in a subpopulation including controls, AID, non-APS thrombosis and APS thrombosis patients (n=853). Odds ratios for pregnancy morbidity are calculated in a female subpopulation including controls, AID, non-APS obstetric and APS obstetric patients (n=483).

		BioPlex®2200	**Phadia®**	**HemosIL AcuStar®**	**QUANTA Lite® ELISA**
Thrombosis	antiCL	0.6 (0.1-3.4)	1.3 (0.4-4.5)	0.5 (0.4-0.5)	1.7 (0.2-18.7)
	n	5	10	4	3
	antiβ2GPI	0.5 (0.4-0.5)	1.1 (0.4-2.9)	0.5 (0.4-0.5)	0.6 (0.3-1.5)
	n	4	16	4	23
	antiCL and/or antiβ2GPI	0.4 (0.08-2.3)	0.9 (0.4-2.2)	0.5 (0.4-0.5)	0.7 (0.3-1.6)
	n	6	21	4	26
	antiCL and antiβ2GPI	0.5 (0.4-0.5)	3.4 (0.4-30.5)	0.5 (0.4-0.5)	-
	n	3	5	4	0
Pregnancy morbidity	antiCL	2.1 (0.3-15.3)	2.1 (0.4-10.7)	0.7 (0.1-6.8)	2.1 (0.1-34.2)
	n	4	6	4	2
	antiβ2GPI	2.1 (0.4-10.7)	1.7 (0.5-6.5)	0.7 (0.6-0.7)	1.5 (0.6-4.0)
	n	6	9	3	17
	antiCL and/or antiβ2GPI	2.1 (0.4-10.7)	1.1 (0.3-3.6)	0.7 (0.1-6.8)	1.4 (0.5-3.6)
	n	6	12	4	18
	antiCL and antiβ2GPI	2.1 (0.3-15.3)	0.3 (0.3-0.4)	0.7 (0.6-0.7)	0.3 (0.3-0.4)
	n	4	3	3	1

Abbreviations: antiβ2GPI, anti-β2 glycoprotein I; antiCL, anticardiolipin; CI, confidence interval; n, number of patients positive within the indicated subpopulation; OR, odds ratio.

Note: Odds ratios for thrombosis are calculated in a subpopulation including controls, AID, non-APS thrombosis, and APS thrombosis patients (n=853). Odds ratios for pregnancy morbidity are calculated in a female subpopulation including controls, AID, non-APS obstetric, and APS obstetric patients (n= 483).

Titers of isolated antiCL and/or aß2GPI IgA positive patients

Within the total population, the QUANTA Lite® ELISA assay detected the highest number of isolated IgA samples (n=34 patients). Only 12%, 47% and 6% of these 34 patients were also positive for isolated IgA with the BioPlex®2200, Phadia® and HemosIL AcuStar® assay, respectively. Titers of isolated IgA aPL patients were low (**Figure 1**). Isolated antiCL and aβ2GPI IgA titers of patients with clinical manifestation were similar to IgA titers in the control population. Of note, upon aPL detection with Phadia®, one patient with a history of thrombosis and one patient with known pregnancy morbidity had an antiCL and aβ2GPI IgA titer >100 AU, while all patients in the control population had titers <100 AU (**Figure 1C-D**).

antiCL and/or aß2GPI IgA titers of triple positive patients

The majority of triple positive patients (LAC, antiCL and aβ2GPI IgG/M positivity, from the same isotype) had a history of thrombosis and/or pregnancy morbidity (**Figure 2**). antiCL and aβ2GPI IgA titers were found to be significantly different between triple positives and non-triple positives, independent of the solid phase assay. Triple positives for the IgG isotype (LAC, antiCL IgG and aβ2GPI IgG) had higher antiCL and aβ2GPI IgA titers than non-triple positive patients, independent of the solid phase assay. antiCL and aβ2GPI IgA titers from triple positives for the IgM isotype (LAC, antiCL IgM and aβ2GPI IgM) were also significantly higher than non-triple positives, independent of the solid phase assay. antiCL and aβ2GPI IgA titers of triple positives for the IgG isotype and triple positives for the IgM isotype were not significantly different for all platforms.

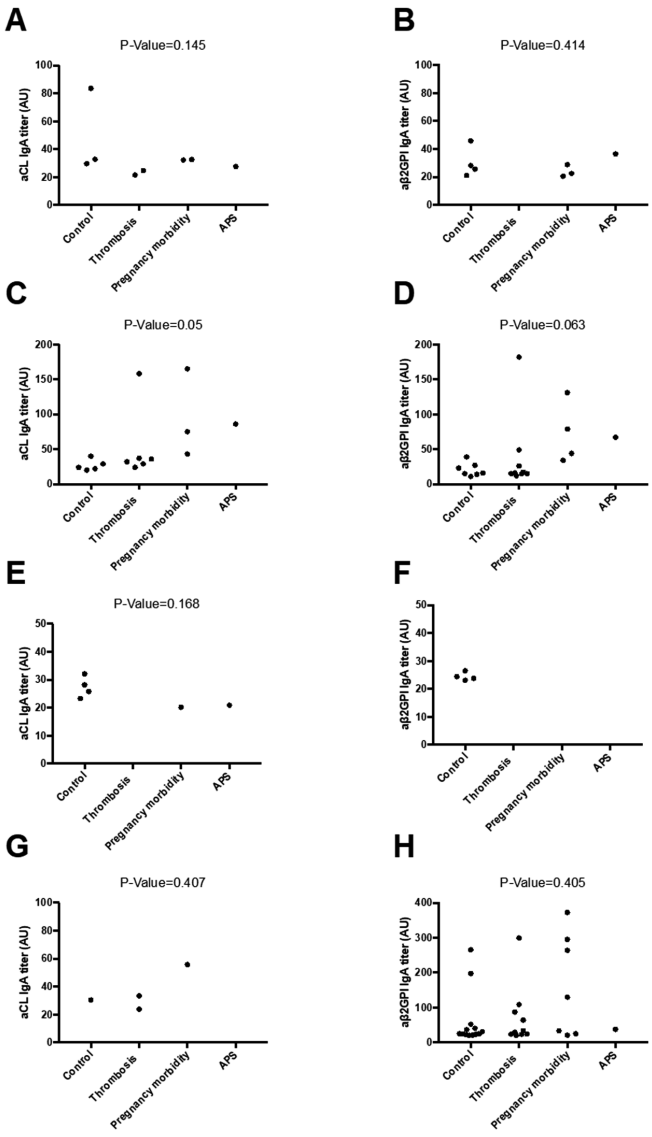

Figure 1. Isolated antiCL or antiβ2GPI IgA titers of controls and AID (control), APS thrombosis and non-APS thrombosis (thrombosis), and APS obstetric and non-APS obstetric (pregnancy morbidity). (A) Isolated antiCL IgA titers detected with BioPlex®2200; (B) Isolated antiβ2GPI IgA titers detected with BioPlex®2200; (C) Isolated antiCL IgA titers detected with Phadia®; (D) Isolated antiβ2GPI IgA titers detected with Phadia®; (E) Isolated antiCL IgA titers detected with HemosIL AcuStar®; (F) Isolated antiβ2GPI IgA titers detected with HemosIL AcuStar®; (G) Isolated antiCL IgA titers detected with QUANTA Lite® ELISA; (H) Isolated antiβ2GPI IgA titers detected with QUANTA Lite® ELISA. Represented P-values were calculated using the Kruskal–Wallis test. antiβ2GPI, anti-β2 glycoprotein I; antiCL, anticardiolipin; APS, antiphospholipid syndrome; IgA, immunoglobulin A.

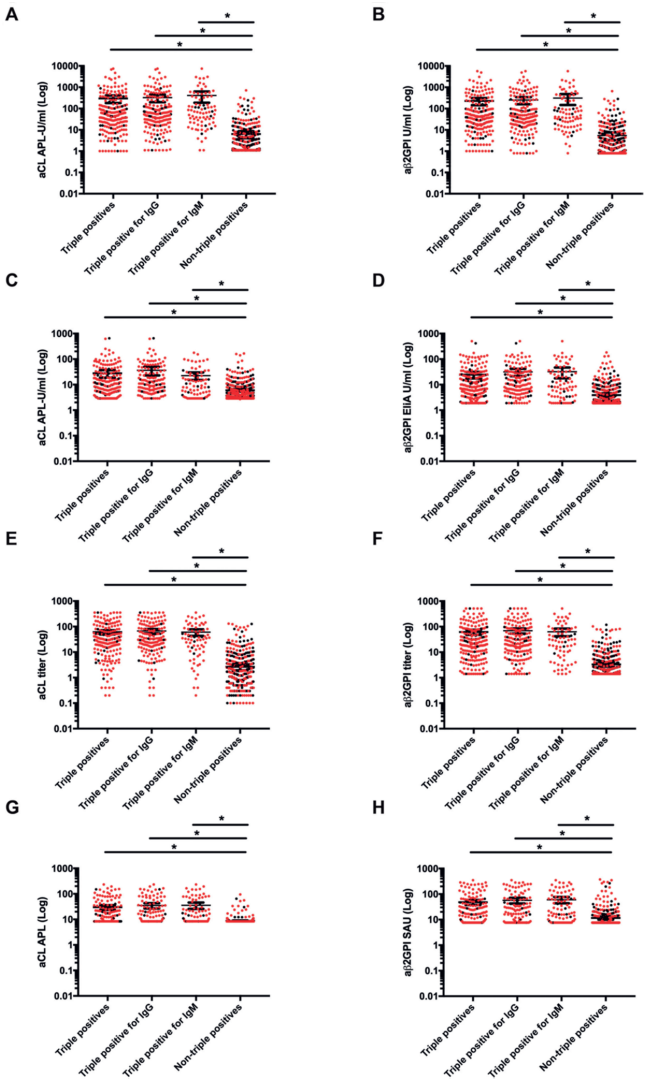

Figure 2. antiCL or antiβ2GPI IgA titers of triple positive patients and non-triple positive patients. Solid dots in black indicate antiCL or antiβ2GPI IgA titers of patients without clinical manifestations of APS. Solid dots in red indicated antiCL or antiβ2GPI IgA titers of patients with clinical manifestations of APS. (A) antiCL IgA titers detected with BioPlex®2200; (B) antiβ2GPI IgA titers detected with BioPlex®2200; (C) antiCL IgA titers detected with Phadia®; (D) antiβ2GPI IgA titers detected with Phadia®; (E) antiCL IgA titers detected with HemosIL AcuStar®; (F) antiβ2GPI IgA titers detected with HemosIL AcuStar®; (G) antiCL IgA titers detected with QUANTA Lite® ELISA; (H) antiβ2GPI IgA titers detected with QUANTA Lite® ELISA. Mean IgA titers with their 95% confidence intervals are shown. Significance was calculated using the Kruskal–Wallis test followed by Mann-Whitney U test. *P-value <0.0001. antiβ2GPI, anti-β2 glycoprotein I; antiCL, anticardiolipin; APS, antiphospholipid syndrome; IgA, immunoglobulin A.

Additional value in thrombosis of antiCL and aß2GPI IgA in the current aPL-panel

Inclusion of antiCL and aβ2GPI IgA antibodies in the current aPL-panel consisting of LAC, IgG and/or IgM antiCL and/or aβ2GPI did not increase ORs for thrombosis (**Table 5**). Replacement of IgM by IgA aPL resulted in similar ORs for thrombosis compared to the current classification criteria with most solid phase assays tested. However, an aPL panel consisting of LAC, antiCL IgG/M/A and aβ2GPI IgG/M/A resulted in similar ORs for thrombosis (2.4 (95% CI, 1.8-3.1)-2.8 (95% CI, 2.1-3.7)) in comparison to calculated OR with an aPL panel consisting of LAC, antiCL IgG/M and aβ2GPI IgG/M (2.4 (95% CI, 1.8-3.2)-2.9 (95% CI, 2.2-3.9)).

Table 5. Additional value of antiCL and antiβ2GPI IgA antibodies in thrombosis.

	LAC, antiCL IgG, antiβ2GPI IgG, antiCL IgM and/or antiβ2GPI IgM	**LAC, antiCL IgG, antiβ2GPI IgG, antiCL IgA and/or antiβ2GPI IgA**	**LAC, antiCL IgG, antiβ2GPI IgG, antiCL IgM, antiβ2GPI IgM, antiCL IgA and/or antiβ2GPI IgA**
	Odds ratio (95% CI)	**Odds ratio (95% CI)**	**Odds ratio (95% CI)**
BioPlex®2200	2.9 (2.2-3.9)	2.8 (2.1-3.7)	2.8 (2.1-3.7)
Phadia®	2.4 (1.8-3.2)	3.1 (2.3-4.2)	2.4 (1.8-3.1)
HemosIL AcuStar®	2.9 (2.1-3.8)	3.0 (2.3-4.1)	2.7 (2.0-3.6)
QUANTA Lite® ELISA	2.8 (2.1-3.7)	3.1 (2.3-4.2)	2.6 (2.0-3.5)

Abbreviations: antiβ2GPI, anti-β2 glycoprotein I; antiCL, anticardiolipin; CI, confidence interval; IgA, immunoglobulin A; IgM, immunoglobulin M; LAC, lupus anticoagulant.

Additional value in pregnancy morbidity of antiCL and aß2GPI IgA in the current aPL-panel

Positivity for at least one of the criteria aPL of APS resulted in significant ORs for pregnancy morbidity between 4.9 (95% CI, 3.2-7.4) and 6.6 (95% CI, 4.3-10.0), dependent on the solid phase assay (**Table 6**). An aPL panel for the classification of APS, consisting of LAC, IgG antiCL or aβ2GPI, antiCL or aβ2GPI IgA reached similar ORs, between 4.6 (95% CI, 3.0-6.9) and 5.1 (95% CI, 3.4-7.7). Positivity for LAC, IgG, IgM or IgA did not increase the OR for pregnancy morbidity as ORs between 4.6 (95% CI, 3.1-7.0) and 6.2 (95% CI, 4.1-9.4) were obtained.

Table 6. Additional value of antiCL and antiβ2GPI IgA antibodies in pregnancy morbidity.

	LAC, antiCL IgG, antiβ2GPI IgG, antiCL IgM and/or antiβ2GPI IgM	LAC, antiCL IgG, antiβ2GPI IgG, antiCL IgA and/or antiβ2GPI IgA	LAC, antiCL IgG, antiβ2GPI IgG, antiCL IgM, antiβ2GPI IgM, antiCL IgA and/or antiβ2GPI IgA
	Odds ratio (95% CI)	**Odds ratio (95% CI)**	**Odds ratio (95% CI)**
BioPlex®2200	5.1 (3.4-7.7)	4.6 (3.0-6.9)	5.0 (3.3-7.6)
Phadia®	4.9 (3.2-7.4)	4.7 (3.1-7.0)	4.6 (3.1-7.0)
HemosIL AcuStar®	5.3 (3.5-8.0)	4.8 (3.2-7.2)	5.2 (3.4-7.8)
QUANTA Lite® ELISA	6.6 (4.3-10.0)	5.1 (3.4-7.7)	6.2 (4.1-9.4)

Abbreviations: antiβ2GPI, anti-β2 glycoprotein I; antiCL, anticardiolipin; CI, confidence interval; IgA, immunoglobulin A; IgM, immunoglobulin M; LAC,lupus anticoagulant.

Discussion

In this study we evaluated the prevalence of antiCL and aβ2GPI IgA antibodies, tested with four solid phase platforms, in 1068 patients. We also investigated the added value of IgA aPL in APS classification.

Of the 1068 included patients 6% up to 19% tested positive for antiCL IgA antiCL and 12%-18% for aβ2GPI IgA antibodies, dependent on the solid phase assay used. LAC was by far the most prevalent aPL (38%) in our study population consisting of 1068 APS patients and controls. In comparison, a retrospective study included 472 patients with aPL testing and found a similar distribution of antiCL IgA and aβ2GPI IgA antibodies, 6% and 19% respectively [10]. Another study included 314 patients suspected for APS or related autoimmune diseases (e.g. SLE) and found 28% patients positive for aβ2GPI IgA using the QUANTA Lite® ELISA [15]. We only found 12% of the patients positive for aβ2GPI IgA in the total population using the QUANTA Lite® ELISA. The lower prevalence of aβ2GPI IgA antibodies might be due to a different patient population as we included a large number of patients with a history of thrombosis or pregnancy morbidity, but negative for any consensus aPL as control population. Another cross-sectional study included 156 patients that fulfilled the clinical criteria of APS and found a prevalence of 5% and 29% for antiCL IgA and aβ2GPI IgA positivity, respectively [8].

In our study, antiCL as well as aβ2GPI IgA antibodies were correlated with clinical manifestations of APS (thrombosis and pregnancy morbidity), independent of the solid phase platform used. In agreement with our results, multiple studies have shown an association between clinical manifestations of APS and antiCL and/or aβ2GPI IgA antibodies [3-12]. However, some studies were only able to show a clinical association for either antiCL or aβ2GPI IgA aPL detected with an in-house ELISA [3,9]. One retrospective study including 439 patient samples within a timespan of six years found an association between aβ2GPI IgA and thrombosis, but not for antiCL IgA [3]. Another

retrospective study including 130 SLE patients and 35 patients with primary APS (PAPS) demonstrated a correlation of antiCL IgA aPL with a history of thrombosis and recurrent fetal loss, but were unable to show any correlation of aβ2GPI IgA aPL with clinical manifestations of APS [9].

An external quality control program illustrated that antiCL and antiβ2GPI IgG/M assays produce variable results [20,25]. We have previously shown that even within commercially available solid phase assays, detection of antiCL and antiβ2GPI IgG/M differs between platforms [21]. Within our total population consisting of 1068 patients, 19.4%, 13.8%, 14.3% and 12.7% patients were found to be positive for antiCL and/or antiβ2GPI IgA antibodies detected with BioPlex®2200, Phadia®, HemosIL AcuStar® and QUANTA Lite® ELISA, respectively. These data indicate that the detection of IgA aPL also depends on the solid phase assay that is used. Other studies also showed the lack of standardization in IgA aPL detecting assays [26-28]. In a subpopulation from the PROMISSE cohort antiβ2GPI IgA was detected in 18.9% and 55.6% of the patients, detected with QUANTA Lite® ELISA and BioPlex®2200, respectively [26]. Taken together, these results indicate that the detection and association of IgA aPL is dependent on the solid phase assay and study population.

In agreement with a previous cross-sectional study, we confirmed the association of antiCL and antiβ2GPI IgA with arterial and venous thrombosis [8]. However, some studies found an association between IgA aPL and venous thrombosis, but were unable to show an association with arterial thrombosis and vice versa [17,18,29]. In contrast, a recent study could not demonstrate an association between antiβ2GPI IgA aPL and thrombosis [15]. Positivity for antiβ2GPI IgA aPL was found in 31% of the included APS patients [15], as well as a large portion (30%) of SLE patients was found to be positive for antiβ2GPI IgA aPL [15]. Upon exclusion of SLE patients, a significant association was found between antiβ2GPI IgA and venous thrombosis (OR 2.5 (95% CI, 1.1-4.9)) [15].

In our cohort, positive antiCL and/or antiβ2GPI IgA results were significantly associated with pregnancy morbidity, as defined in the Sydney criteria. However, the non-APS obstetric group was relative small (n=33), compared to obstetric APS patients (n=122) which might have affect the association of aPL and pregnancy morbidity. Limited data are available on the association between IgA aPL and obstetric complications [4,8,9,15]. Most studies found a value for antiCL or antiβ2GPI IgA testing in pregnancy morbidity [4,8,9]. However, a study that included 314 patients suspected from APS or related autoimmune diseases (e.g. SLE) did not found a significant association for antiCL or antiβ2GPI IgA aPL with pregnancy morbidity [15]. Interestingly, no association of antiCL IgG/M and antiβ2GPI IgG/M with pregnancy morbidity was found within the same study [15].

Pathogenicity of IgA aPL have been shown in animal models [14,18]. In mice, IgA antibodies from APS patients increased the mean thrombus area and mean thrombus disappearance time upon induced thrombus formation of a non-occlusive thrombus by pinch injury [14]. IgA antibodies were purified from two APS patients of which one was also positive for antiCL IgG and LAC. The other patient was positive for antiCL IgA, but negative for antiCL IgG, antiCL IgM and LAC [14]. However, the authors did not demonstrate that antiCL is solely responsible for the observed effect [14]. Another study also showed that IgA isolated from APS patients is pathogenic as thrombus formation and tissue factor activity in mice injected with IgA from APS was increased compared

to control IgA [18]. These data indicate that IgA aPL can be pathogenic [14,18]. However, pathogenic IgA aPL seem to correlate with LAC.

Despite the association of antiCL and/or antiβ2GPI IgA aPL with thrombosis shown in multiple studies, only some have suggested an added value for IgA testing in patients suspected of thrombotic APS [6,8,10,12,18,30]. Although an added value was suggested for so called 'isolated IgA' by some studies, many studies did not include LAC testing, hampering assessment of true isolated IgA aPL [8,12,18,30]. In a retrospective study testing 472 patients with suspected or confirmed thrombophilia, an autoimmune disease or pregnancy morbidity [10], IgA (antiCL, antiβ2GPI and/or anti-phosphatidylserine (antiPS)) aPL positivity was found to be an independent risk factor for thrombosis using a multivariate analysis [10] . Detection of antiCL and antiPS IgG, IgM and IgA antibodies were performed with another platform than detection of antiβ2GPI IgG, IgM and IgA antibodies [10]. Considering the high variability between solid phase platforms in antibody detection, classification of APS patients and the association of aPL might be affected. It is suggested to detect all aPL within the same system for the classification of APS patients [21]. In addition, the method used to determine the cut-off value for an aPL assay has also shown to affect between-assay performance [31]. Also, antibody heterogeneity has been suggested to attribute to variability in test results [32].

We defined isolated antiCL and/or antiβ2GPI IgA positivity for antiCL and/or antiβ2GPI IgA aPL, as negative for antiCL and/or antiβ2GPI IgG/M and/or LAC. Isolated IgA positivity was rare in the total population. Overall, the prevalence of isolated IgA was comparable between patients with clinical manifestations of APS and patients without a history of thrombosis or pregnancy morbidity. Positivity for isolated IgA aPL was not associated with thrombosis or pregnancy morbidity as calculated OR did not reach statistical significance. Titers of IgA positive patients were characterized by low antiCL or antiβ2GPI IgA titers, around the cut-off value. Other non-criteria aPL like anti- phosphatidylethanolamine (PE), negatively charged phospholipids (other than cardiolipin), anti-vimentin/cardiolipin, Annexin A5 and anti-phosphatidylserine/prothrombin (antiPS/PT) antibodies have been suggested to be clinical relevant in patients suspected of APS, but negatively tested for consensus aPL [33]. A recent systematic review showed a strong association between antiPS/PT aPL and clinical manifestations of APS, with a high association with LAC [34]. However, this needs to be validated in a large multicenter study.

In this study, we aimed to investigate the value of antiCL and antiβ2GPI IgA aPL in addition to the current aPL panel (LAC, antiCL IgG/M and antiβ2GPI IgG/M). Positivity for IgA aPL was found to be associated with thrombosis and pregnancy morbidity. However, isolated IgA positivity was rare and not correlated with clinical manifestations of APS. Our results do not support testing for antiCL and/or antiβ2GPI IgA in addition to conventional aPL for the identification of patients with clinical manifestations of APS.

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). *Journal of thrombosis and haemostasis* 2006; **4**(2): 295-306.
2. Devreese KMJ, Ortel TL, Pengo V, de Laat B, for the Subcommittee on Lupus Anticoagulant/Antiphospholipid A. Laboratory criteria for antiphospholipid syndrome: communication from the SSC of the ISTH. *Journal of Thrombosis and Haemostasis* 2018; **16**(4): 809-13.
3. Despierres L, Beziane A, Kaplanski G, et al. Contribution of anti-beta2glycoprotein I IgA antibodies to the diagnosis of anti-phospholipid syndrome: potential interest of target domains to discriminate thrombotic and non-thrombotic patients. *Rheumatology (Oxford)* 2014; **53**(7): 1215-8.
4. Carmo-Pereira S, Bertolaccini M, Escudero-Contreras A, Khamashta M, Hughes G. Value of IgA anticardiolipin and anti-β2-glycoprotein I antibody testing in patients with pregnancy morbidity. *Annals of the Rheumatic Diseases* 2003; **62**(6): 540-3.
5. Lakos G, Kiss E, Regeczy N, et al. Isotype distribution and clinical relevance of anti-β2-glycoprotein I (β2-GPI) antibodies: importance of IgA isotype. *Clinical and experimental immunology* 1999; **117**(3): 574-9.
6. Mattia E, Ruffatti A, Tonello M, et al. IgA anticardiolipin and IgA anti-β2 glycoprotein I antibody positivity determined by fluorescence enzyme immunoassay in primary antiphospholipid syndrome. *Clinical Chemistry and Laboratory Medicine* 2014; **52**(9): 1329-33.
7. Mehrani T, Petri M. Association of IgA Anti-beta2 glycoprotein I with clinical and laboratory manifestations of systemic lupus erythematosus. *The Journal of rheumatology* 2011; **38**(1): 64-8.
8. Ruiz-García R, Serrano M, Ángel Martínez-Flores J, et al. Isolated IgA anti-β2 glycoprotein I antibodies in patients with clinical criteria for antiphospholipid syndrome. *Journal of immunology research* 2014; **2014**: 704395.
9. Samarkos M, Davies K, Gordon C, Loizou S. Clinical significance of IgA anticardiolipin and anti-β 2-GP1 antibodies in patients with systemic lupus erythematosus and primary antiphospholipid syndrome. *Clinical Rheumatology*2006; **25**(2): 199-204.
10. Shen YM, Lee R, Frenkel E, Sarode R. IgA antiphospholipid antibodies are an independent risk factor for thromboses. *Lupus* 2008; **17**(11): 996-1003.
11. Sweiss NJ, Bo R, Kapadia R, et al. IgA anti-β2-glycoprotein I autoantibodies are associated with an increased risk of thromboembolic events in patients with systemic lupus erythematosus. *PloS one* 2010; **5**(8): e12280.
12. Tortosa C, Cabrera-Marante O, Serrano M, et al. Incidence of thromboembolic events in asymptomatic carriers of IgA anti ss2 glycoprotein-I antibodies. *PloS one* 2017; **12**(7): e0178889.
13. Bertolaccini M, Amengual O, Atsumi T, et al. 'Non-criteria' aPL tests: report of a task force and preconference workshop at the 13th International Congress on Antiphospholipid Antibodies, Galveston, TX, USA, April 2010. *Lupus* 2011; **20**: 191-205.

14. Pierangeli SS, Liu XW, Barker JH, Anderson G, Harris EN. Induction of thrombosis in a mouse model by IgG, IgM and IgA immunoglobulins from patients with the antiphospholipid syndrome. *Thrombosis and Haemostasis* 1995; **74**(05): 1361-7.
15. Vlagea A, Pascual-Salcedo D, Doforno RÁ, et al. IgA anti-β2 glycoprotein I antibodies: Experience from a large center. *Thrombosis Research* 2018; **162**: 38-43.
16. Selva-O'Callaghan A, Ordi-Ros J, Monegal-Ferran F, Martinez N, Cortes-Hernandez F, Vilardell-Tarres M. IgA anticardiolipin antibodies-relation with other antiphospholipid antibodies and clinical significance. *Thrombosis and Haemostasis* 1998; **79**(02): 282-5.
17. Bertolaccini ML, Atsumi T, Contreras AE, Khamashta MA, Hughes G. The value of IgA antiphospholipid testing for diagnosis of antiphospholipid (Hughes) syndrome in systemic lupus erythematosus. *The Journal of rheumatology* 2001; **28**(12): 2637-43.
18. Murthy V, Willis R, Romay-Penabad Z, et al. Value of Isolated IgA Anti-β2-Glycoprotein I Positivity in the Diagnosis of the Antiphospholipid Syndrome. *Arthritis & Rheumatology* 2013; **65**(12): 3186-93.
19. Lopez LR, Santos ME, Espinoza LR, La Rosa FG. Clinical significance of immunoglobulin A versus immunoglobulins G and M anti-cardiolipin antibodies in patients with systemic lupus erythematosus: correlation with thrombosis, thrombocytopenia, and recurrent abortion. *American journal of clinical pathology* 1992; **98**(4): 449-54.
20. Favaloro EJ, Wheatland L, Jovanovich S, Roberts-Thomson P, Wong RC. Internal quality control and external quality assurance in testing for antiphospholipid antibodies: Part I—Anticardiolipin and anti-β2-glycoprotein I antibodies. *Seminars in thrombosis and hemostasis* 2012; 38(04): 390-403.
21. Chayoua W, Kelchtermans H, Moore GW, et al. Detection of Anti-Cardiolipin and Anti-beta2glycoprotein I Antibodies Differs between Platforms without Influence on Association with Clinical Symptoms. *Thrombosis and Haemostasis* 2019; **119**(05): 797-806.
22. Devreese K. Standardization of antiphospholipid antibody assays.Where do we stand? *Lupus* 2012; **21**: 718-21.
23. Pengo V, Tripodi A, Reber G, et al. Update of the guidelines for lupus anticoagulant detection. *Journal of Thrombosis and haemostasis* 2009; **7**(10): 1737-40.
24. Devreese KM, Pierangeli SS, de Laat B, et al. Testing for antiphospholipid antibodies with solid phase assays: guidance from the SSC of the ISTH. *Journal of thrombosis and haemostasis* 2014; **12**(5): 792-5.
25. Favaloro EJ, Silvestrini R, Mohammed A. Clinical utility of anticardiolipin antibody assays: high inter-laboratory variation and limited consensus by participants of external quality assurance programs signals a cautious approach. *Pathology* 1999; **31**(2): 142-7.
26. Tebo AE, Willis R, Jaskowski TD, et al. Clinical significance and correlations between anti-beta2 glycoprotein I IgA assays in antiphospholipid syndrome and/or systemic lupus erythematosus. *Clinica chimica acta; international journal of clinical chemistry* 2016; **460**: 107-13.
27. Martinez-Flores JA, Serrano M, Alfaro J, et al. Heterogeneity between diagnostic tests for IgA anti-beta2 glycoprotein I: explaining the controversy in studies of association with vascular pathology. *Analytical Chemistry* 2013; **85**(24): 12093-8.

28. Perez D, Martínez-Flores J, Serrano M, et al. Evaluation of three fully automated immunoassay systems for detection of IgA anti-beta 2-glycoprotein I antibodies. *International journal of laboratory hematology* 2016; **38**(5): 560-8.
29. Akhter E, Shums Z, Norman GL, Binder W, Fang H, Petri M. Utility of Antiphosphatidylserine/Prothrombin and IgA Antiphospholipid Assays in Systemic Lupus Erythematosus. *The Journal of Rheumatology*2013; **40**(3): 282-6.
30. Greco T, Amos MD, Conti-Kelly A, Naranjo J, Ijdo J. Testing for the antiphospholipid syndrome: importance of IgA anti-beta 2-glycoprotein I. *Lupus* 2000; **9**(1): 33-41.
31. Vanoverschelde L, Kelchtermans H, Musial J, de Laat B, Devreese KM. Influence of anticardiolipin and anti-β2 glycoprotein I antibody cutoff values on antiphospholipid syndrome classification. *Research and Practice in Thrombosis and Haemostasis* 2019; **3**(3): 515-27.
32. Devreese KM. Antiphospholipid antibody testing and standardization. *International journal of laboratory hematology* 2014; **36**(3): 352-63.
33. Pignatelli P, Ettorre E, Menichelli D, Pani A, Violi F, Pastori D. Seronegative antiphospholipid syndrome: refining the value of “non-criteria” antibodies for diagnosis and clinical management. *Haematologica* 2020; **105**(3): 562-72.
34. Radin M, Foddai SG, Cecchi I, et al. Antiphosphatidylserine/Prothrombin Antibodies: An Update on Their Association with Clinical Manifestations of Antiphospholipid Syndrome. *Thrombosis and Haemostasis* 2020; **120**(4): 592-8.

Supplemental information

Supplemental Table 1. Cut-off values of antiCL IgG/M/A and antiβ2GPI IgG/M/A detected with BioPlex®2200, Phadia®, HemosIL AcuStar® and QUANTA Lite® ELISA.

	antiCL IgG	antiβ2GPI IgG	antiCL IgM	antiβ2GPI IgM	antiCL IgA	antiβ2GPI IgA
BioPlex®2200	20	20	20	20	20	20
Phadia®	10	10	10	10	20	10
HemosIL AcuStar®	20	20	20	20	20	20
QUANTA Lite® ELISA	20	20	20	20	20	20

Supplemental Table 2. Prevalence of LAC, antiCL and antiβ2GPI IgG/M/A in the total population (n=1068).

	LAC	IgG		IgM		IgA	
		antiCL	antiβ2GPI	antiCL	antiβ2GPI	antiCL	antiβ2GPI
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
	408 (38)						
BioPlex®2200		248 (23)	252 (24)	118 (11)	131 (12)	200 (19)	187 (18)
Phadia®		215 (20)	202 (19)	202 (19)	138 (13)	174 (16)	131 (12)
HemosIL AcuStar®		222 (21)	284 (27)	169 (16)	113 (11)	151 (14)	132 (12)
QUANTA Lite® ELISA		191 (18)	142 (13)	170 (16)	152 (14)	67 (6)	126 (12)

Supplemental Table 3. Thrombotic association of antiCL and antiβ2GPI IgG/M antibodies. Odds ratios are calculated in a subpopulation including controls, AID, non-APS thrombosis and APS thrombosis patients (n=853).

	BioPlex®2200	Phadia®	HemosIL AcuStar®	QUANTA Lite® ELISA
antiCL IgG	3.2 (2.2-4.7)	4.2 (2.7-6.5)	4.1 (2.7-6.3)	4.5 (2.8-7.4)
N	163	137	144	121
antiβ2GPI IgG	2.8 (1.9-4.1)	3.5 (2.3-5.4)	2.8 (1.9-3.9)	5.4 (3.0-9.6)
n	166	134	193	91
antiCL and antiβ2GPI IgG	3.3 (2.2-5.0)	4.1 (2.5-6.7)	4.3 (2.8-6.8)	5.3 (2.9-9.6)
n	157	113	140	84
antiCL IgM	2.5 (1.5-4.1)	1.6 (1.1-2.3)	1.7 (1.1-2.5)	2.0 (1.3-3.1)
n	82	147	119	105
antiβ2GPI IgM	2.7 (1.6-4.4)	2.2 (1.4-3.4)	2.6 (1.5-4.5)	2.0 (1.3-3.2)
n	86	94	74	97
antiCL and antiβ2GPI IgM	2.7 (1.6-4.7)	2.7 (1.6-4.6)	2.7 (1.5-4.7)	2.4 (1.4-4.2)
n	76	79	67	74

Supplemental Table 4. Obstetric association of antiCL and antiβ2GPI IgG/M antibodies. Odds ratios are calculated in a female subpopulation including controls, AID, non-APS obstetric and APS obstetric patients (n=483).

Pregnancy morbidity	**BioPlex®2200**	**Phadia®**	**HemosIL AcuStar®**	**QUANTA Lite® ELISA**
antiCL IgG	3.5 (2.2-5.7)	5.1 (3.0-8.7)	4.5 (2.7-7.6)	5.2 (2.9-9.2)
n	89	71	74	59
antiβ2GPI IgG	3.3 (2.1-5.3)	3.6 (2.1-6.1)	2.8 (1.8-4.4)	4.2 (2.1-8.6)
n	91	69	102	36
antiCL and antiβ2GPI IgG	3.7 (2.3-6.1)	4.6 (2.6-8.2)	4.6 (2.7-7.7)	3.8 (1.8-7.8)
n	85	58	72	34
antiCL IgM	3.0 (1.6-5.7)	2.0 (1.2-3.3)	2.0 (1.2-3.4)	2.9 (1.7-5.0)
n	41	81	69	61
antiβ2GPI IgM	3.1 (1.7-5.8)	3.2 (1.8-5.8)	3.6 (1.8-6.9)	2.7 (1.5-4.7)
n	46	51	40	55
antiCL and antiβ2GPI IgM	3.2 (1.6-6.2)	3.3 (1.6-6.5)	3.7 (1.8-7.5)	2.9 (1.5-5.6)
n	40	36	36	38

Supplemental Table 5. Association of antiCL and antiβ2GPI IgA with venous or arterial thrombosis. Odds ratios are calculated with their respective 95% confidence intervals for each solid phase assay. Significant odds ratios are shown in bold.

	Venous (n=309)				**Arterial (n=102)**			
	IgA OR (95% CI)		Isolated IgA OR (95% CI)		IgA OR (95% CI)		Isolated IgA OR (95% CI)	
	antiCL	antiβ2GPI	antiCL	antiβ2GPI	antiCL	antiβ2GPI	antiCL	antiβ2GPI
BioPlex®2200	**2.7 (1.7-4.3)**	**2.2 (1.4-3.5)**	0.4 (0.04-4.0)	0.6 (0.5-0.6)	**3.7 (2.0-6.7)**	**2.9 (1.6-5.3)**	1.3 (0.1-12.4)	0.8 (0.8-0.8)
Phadia®	**1.8 (1.1-2.8)**	**1.8 (1.1-3.0)**	0.8 (0.4-1.6)	1.5 (0.5-4.1)	**2.2 (1.2-4.0)**	**3.0 (1.6-5.7)**	0.7 (0.2-2.0)	0.8 (0.8-0.8)
HemosIL AcuStar®	**2.3 (1.4-4.0)**	**1.8 (1.1-3.2)**	0.6 (0.5-0.6)	0.6 (0.5-0.6)	**3.5 (1.8-6.7)**	**2.9 (1.5-5.6)**	0.8 (0.8-0.8)	0.8 (0.8-0.8)
QUANTA Lite® ELISA	**7.0 (2.4-20.7)**	**2.4 (1.4-4.2)**	2.5 (0.2-28.1)	0.7 (0.3-1.7)	**14.1 (4.5-44.3)**	**3.8 (1.9-7.4)**	0.8 (0.8-0.8)	0.3 (0.04-2.2)

OR, Odds ratio; CI, Confidence interval

Chapter 7

The clinical relevance of isolated lupus anticoagulant positivity in patients with thrombotic antiphospholipid syndrome

Dongmei Yin, Philip G. de Groot , Marisa Ninivaggi, Katrien M. J. Devreese , Bas de Laat

Thrombosis and Haemostasis. 2020 Dec 31. doi: 10.1055/a-1344-4271. Epub ahead of print. PMID: 33385181.

Abstract

Background: Patients positive for all three types of antiphospholipid antibodies (aPLs) (triple positivity) have been identified for having a high risk for thrombotic events. However, the clinical significance of isolated lupus anticoagulant (LAC) positivity is debated.

Objectives: To investigate the clinical relevance of isolated LAC.

Patients/Methods 456 patients were enrolled in this study; 66 antiphospholipid syndrome patients and 390 control patients. The control group existed of autoimmune patients (n=91), patients with thrombosis but without aPLs (n=127) and normal controls (n=172). LAC, anti-cardiolipin (antiCL) and anti-beta2glycoprotein I (antiβ2GPI) IgG and IgM were determined according to the ISTH guidelines. antiCL and antiβ2GPI was measured by four different solid phase platforms to overcome variability between test systems. The non-criteria IgA antiCL and antiβ2GPI, anti-domain I (antiDI) of β2GPI IgG and anti-phosphatidylserine/prothrombin antibodies (anti-PS/PT) IgG and IgM were detected according to the ISTH guidelines for solid phase assays.

Results: 70 patients were positive for LAC, of which 44 were negative for both antiβ2GPI and antiCL antibodies. We found that isolated LAC proved to be strongly associated with vascular thrombosis (Odds ratio (OR) (95% CI) 7.3 (3.3-16.1)), even better than triple positive samples (OR 4.3 (1.6-12.2)). The titers of the anti-PS/PT IgG and IgM were significantly higher in triple positivity samples compared to samples with isolated LAC positivity. The majority of single LAC positives were anti-PS/PT negative. We observed that LAC positivity was weaker in isolated LAC positive patients compared to LAC activity in triple positive patients.

Conclusions: Isolated LAC was highly associated with thrombosis. The presence of anti-PS/PT antibodies could not explain LAC positivity in isolated LAC. Isolated LAC showed a weaker LAC activity compared to triple positive patients.

Introduction

Lupus anticoagulant (LAC) is a functional assay that measures phospholipid-dependent prolongation of the clotting time. LAC requires a three-step procedure including a screening, a mixing and a confirmatory step, as advised by the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) [1-5]. Currently, LAC together with anti-cardiolipin (antiCL) IgG/IgM antibodies and anti-beta2glycoprotein I (antiβ2GPI) IgG/IgM antibodies are included in the international criteria to classify the antiphospholipid syndrome (APS) [5-7]. Patients positive for the combination of LAC, antiβ2GPI and antiCL antibodies, the so-called triple positive patients, exhibit a high risk for developing a first thrombotic event and recurrence of thrombosis [8,9].

Although a positive LAC is considered to be a strong risk factor for thrombosis in APS [10,11], the clinical relevance of isolated LAC positivity, in the absence of antiCL and antiβ2GPI antibodies has been widely debated. Recent studies showed a poor predictive value for a first thrombotic event for isolated LAC [12,13], however, other studies suggested a strong predictive value. A large multicenter, population-based case-control study showed that LAC positivity predicts acute myocardial infarction and ischaemic stroke, whereas the presence of any other antiphospholipid antibodies (aPLs) did not show any clinical association [14]. More recently, a prospective observational study found that in a LAC positive population the association between occurrence of thrombosis and inferior survival was independent of the detection of antiCL and antiβ2GPI antibodies [15]. Data from 158 stroke cases and 369 controls indicated that LAC is a strong risk factor for stroke, especially in young and middle-aged individuals. On the contrary, the presence of other aPLs showed very low association with increased risk for stroke [16].

The aim of this study is to investigate the clinical relevance of isolated LAC positivity, and explore whether non-criteria aPLs are responsible for LAC activity in the absence of antiCL and antiβ2GPI antibodies.

Methods

Samples

In total, 456 patients were included in this study. The study was approved by the Ethics Committee of the Ghent University Hospital and conducted according to the Declaration of Helsinki (2013). Blood (9 volumes) was drawn aseptically in vacutainer tubes (Greiner Bio-One) containing 3.2% sodium citrate (1 volume), from the antecubital vein. Immediately after blood drawing, platelet poor plasma was prepared by centrifuging the blood twice for ten minutes at 2630 × g. The plasma samples were stored at -80°C until further use.

Lupus anticoagulant and antiphospholipid syndrome

Lupus anticoagulant was determined using the STA-R Evolution®Coagulation Analyzer (Diagnostica Stago, Asnieres, France) according to ISTH guidelines [1] applying dilute Russell's viper venom time (dRVVT Screen and dRVVT Confirm, Diagnostica Stago, Asnières-sur-Seine, France) and a sensitive activated partial thromboplastin time (aPTT) with silica as an activator (PTT-LA, Diagnostica Stago, Asnières-sur-Seine, France). Patient samples were considered positive when at least one test system was positive, based on in-house calculated cut-off values for screening, mixing and confirmation tests, all expressed as normalized ratio. In the dRVVT system, the confirmation step was expressed as a ratio of Screen/Confirm. In the aPTT system, the confirmatory step for aPTT is expressed as a difference in clotting time between two aPTTs with and without hexagonal phase II phospholipids. Diagnosis of APS was based on the Sydney criteria [6].

Solid phase assays

AntiCL IgG, IgM and IgA and antiβ2GPI IgG, IgM and IgA were detected by four different solid phase assays: HemosIL AcuStar® (Instrumentation Laboratories, Bedford, USA), BioPlex®2200 (Bio-Rad, Bio-Rad Laboratories, Hercules, USA), Phadia® (Thermo Fisher Scientific/Phadia, Uppsala, Sweden) and QUANTA Lite® ELISA (Inova Diagnostics, San Diego, USA). Anti- β2GPI IgG antibodies recognizing the first domain (antiDI) were detected by the chemiluminescence QUANTA Flash® β2GPI domain I assay (Inova Diagnostics, San Diego, USA) on the ACL AcuStar® platform. Anti-phosphatidylserine /prothrombin (anti-PS/PT) IgG and IgM antibodies were determined by QUANTA Lite antiPS/PT ELISA assay (Inova Diagnostics, San Diego, USA). Results were expressed as arbitrary units. All tests were performed following the instructions of the manufacturer. The cut-off values from the manufacturers were transferred after confirmation in 20 healthy individuals according to the guidelines [17]. Based on the cut-off values positive samples were identified.

Statistics

Statistical analysis was performed using IBM SPSS® Statistics (IBM SPSS, New York, USA). Association between the presence of the antibodies and the thrombosis risk was investigated by calculating Odds ratios (ORs) with the 95% confidence interval (CI). The Chi-squared test was used for comparison of prevalence, while the Mann-Whitney U test for comparison of continuous variables between two different antibody groups. P-values less than 0.05 (two-tailed) were considered to be statistically significant.

Results

Antibody profile and patient characteristics

In total, 456 patients were included in this study. The demographic data and the patient characteristics are summarized in **Table 1**. The mean age of patients was 44 years (±SD, ±13) and the male/female prevalence in this group was 27% versus 73%. The population of patients consisted of 66 APS patients, 91 autoimmune disease (AID) patients, 127 non-APS thrombosis

patients and 172 normal controls. The AID control patients had autoimmune diseases other than APS, such as systemic lupus erythematosus (n=21) and systemic sclerosis (n=55), without thrombotic or pregnancy morbidity complications. Non-APS thrombosis patients were patients that had experienced thrombosis in the past, but tested negative for the laboratory criteria for APS. The normal controls were patients that were referred for aPL antibody testing for other reasons than the clinical criteria of APS, including subfertility (n=115) and prolonged activated partial thromboplastin time (n=7). Seventy patients tested positive for LAC and 386 patients were LAC negative, of which 11 (3%) were classified as APS patients based on a positive antiCL and/or antiβ2GPI IgG/IgM assay. The LAC positive patients were tested positive on 2 separate occasions according to the ISTH guidelines [5]. Fifty-five out of these 70 patients were diagnosed with APS, 13 were AID patients and 2 were normal controls.

When the isolated LAC patients were compared to the triple positive patient group, the age and male/female ratio were balanced (p=0.11 and p=0.74, respectively), as well as their clinical characteristics (**Table 2**). Twenty out of 70 LAC positive patients were triple-positive (LAC, antiCL IgG or IgM, anti- β2GPI IgG or IgM), in combination with antiCL and/or anti- β2GPI antibody positivity in at least one of the four platforms. Out of these 20 triple positive patients 14 were APS patients, 4 were AID controls and 1 was a normal control. Of the 15 APS patients, 7 patients had a history of venous thrombosis, 1 patient had a history of arterial thrombosis and 7 patients had a combination of venous/arterial thrombosis and pregnancy morbidity. Forty-four patients were isolated LAC positive and tested negative for antiCL IgG, antiCL IgM, antiβ2GPI IgG and antiβ2GPI IgM on all four platforms used to detect the presence of these antibodies. Thirty-six (82%) out of 44 were diagnosed as APS patients, 7 were AID patients and 1 was a normal control. Of these 36 APS patients, 28 had a history of venous thrombosis, six had a history of arterial thrombosis and 2 had a combination of venous thrombosis and arterial thrombosis or pregnancy morbidity. Six out of 70 LAC positive patients were LAC positive with antiCL or anti- β2GPI antibodies and 4 (67%) of them were APS patients.

Non-criteria aPL antibodies, including antiβ2GPI and antiCL IgA antibodies, anti-domain I IgG antibodies and anti-PS/PT IgG and IgM antibodies, were studied for their association with both isolated LAC and triple positivity (**Table 3**). For IgA we found a lower prevalence of antiCL and antiβ2GPI IgA in the isolated LAC group compared to triple positive patient group (antiCL: 0-5% versus 30-55% and antiβ2GPI: 0% versus 30-55%). For anti-domain I antibodies we found no patients positive with an isolated LAC compared to 55% in the group of patients with triple positivity. Anti-PS/PT IgG (7% versus 40%) and anti-PS/PT IgM (11% versus 80%) were less prevalent in the isolated LAC group compared to the triple positive group. Both the anti-PS/PT IgG and anti-PS/PT IgM titers were significantly lower in the isolated LAC group compared to the triple positive group, with a median anti-PS/PT IgG titer of 6.8 U/ml (interquartile range (IQR) 5.9-8.6) versus 13.0 U/ml (IQR 6.2-77.9) (Mann–Whitney U test, P = 0.006), and a median anti-PS/PT IgM titer of 13.0 U/ml (IQR 8.3-20.5) versus 77.3 U/ml (IQR 34.4-324.5) (Mann–Whitney U test, P < 0.0001) (**Figure 1A**). For the anti-PS/PT IgG and anti-PS/PT IgM positive samples (> 30 U/ml), the anti-PS/PT IgG or anti-PS/PT IgM titers did not differ between the isolated LAC positive group and the triple positive group (**Figure 1B**).

Table 1: Patient characteristics. The patients are divided into four groups: APS, autoimmune disease, non-APS and a normal control group. The presence of the antiCL and antiβ2GPI antibodies was tested with four solid phase assays (HemosIL AcuStar®, BioPlex®2200, Phadia® and QUANTA Lite® ELISA). Other antibodies were detected with the antiPS/PT ELISA or the CLIA anti-domainI (Inova diagnostics).

	Total n (%)
Patients	456
Female	333 (73)
Age [years, mean ± SD]	44 ± 13
APS group	66 (14)
Primary APS	60 (13)
Secondary APS	6 (1)
APS-thrombosis only	58 (13)
Venous thrombosis	42 (9)
Arterial thrombosis	14 (3)
Both venous thrombosis and arterial thrombosis	2 (0.4)
APS-both thrombosis and pregnancy morbidity	8 (2)
Autoimmune disease group	91 (20)
Systemic lupus erythematosus	21 (5)
Systemic sclerosis	55 (12)
Other autoimmune disease	15 (3)
Non-APS thrombosis group	127 (28)
Venous thrombosis	88 (19)
Arterial thrombosis	34 (7)
Both venous thrombosis and arterial thrombosis	5 (1)
Normal control group	172 (38)
Subfertility/infertility	115 (25)
Prolonged activated partial thromboplastin time	7 (2)
non-APS related clinical features	50 (11)
Antiphospholipid antibodies	
Lupus anticoagulant	70 (15)
Criteria aPL antibodies (antiCL and/or antiβ2GPI IgG/M)	
HemosIL AcuStar®	56 (12)
BioPlex®2200	36 (8)
Phadia®	73 (16)
QUANTA Lite® ELISA	47 (10)
Non-criteria aPL antibodies	
antiCL and/or antiβ2GPI IgA	
HemosIL AcuStar®	17 (4)
BioPlex®2200	26 (6)
Phadia®	45 (10)
QUANTA Lite® ELISA	20 (4)
anti-Domain I IgG	18 (4)
anti-PS/PT IgG	59 (13)
anti-PS/PT IgM	66 (14)

Secondary APS: APS related to autoimmune disease. Abbreviations: APS, antiphospholipid syndrome; aPL, antiphospholipid antibodies; antiCL, anti-cardiolipin; antiβ2GPI, anti-beta2glycoprotein I; anti-PS/PT, anti-phosphatidylserine/prothrombin.

Table 2: Clinical characteristics of patients with isolated LAC and triple positivity.

	Isolated LAC n (%)	Triple positivity n (%)	P-value
Patients	44	20	
Female	21 (48)	14 (70)	0.113
Age [years, median (range)]	47 ± 16	48 ± 15	0.798
APS group	36 (82)	15 (75)	0.523
Primary APS	36 (82)	12 (60)	0.117
Secondary APS	0	3 (15)	
APS-related clinical features	36 (82)	15 (75)	
Venous thrombosis	28 (64)	7 (35)	0.057
Arterial thrombosis	6 (14)	1 (5)	0.419
Both venous thrombosis and arterial thrombosis	1 (2)	0	
Venous thrombosis and pregnancy morbidity	1 (2)	3 (15)	
Arterial thrombosis and pregnancy morbidity	0	3 (15)	
Both venous and arterial thrombosis and pregnancy morbidity	0	1 (5)	
Autoimmune disease group	7 (16)	4 (20)	0.728
Systemic lupus erythematosus	2(5)	2 (10)	0.583
Systemic sclerosis	3 (7)	2 (10)	0.644
Other autoimmune disease	2 (5)	0	
Normal control group	1 (2)	1 (5)	0.531
Superficial phlebitis	1 (2)	0	
Venous insufficiency	0	1 (5)	

Secondary APS is APS related to autoimmune disease. Abbreviations: APS, antiphospholipid syndrome; LAC, lupus anticoagulant.

Table 3. Antiphospholipid antibody characteristics of LAC-positive patients. The presence of the antiCL and antiβ2GPI antibodies was tested with four solid phase assays (HemosIL AcuStar®, BioPlex®2200, Phadia® and QUANTA Lite® ELISA). Other antibodies were detected with the antiPS/PT ELISA or the CLIA anti-domain I (Inova diagnostics).

	Isolated LAC positivity	**Triple positivity**
Patients (n)	44	20
Antiphospholipid antibodies n (%)		
Criteria aPL antibodies (antiCL/antiβ2GPI IgG/M)		
HemosIL AcuStar®	0	18 (90)
BioPlex®2200	0	17 (85)
Phadia®	0	19 (95)
QUANTA Lite® ELISA	0	18 (90)
Non-criteria aPL antibodies		
antiCL IgA		
HemosIL AcuStar®	0	8 (40)
BioPlex®2200	0	11 (55)
Phadia®	2 (5)	11 (55)
QUANTA Lite® ELISA	0	6 (30)
antiβ2GPI IgA		
HemosIL AcuStar®	0	7 (35)
BioPlex®2200	0	11 (55)
Phadia®	0	6 (30)
QUANTA Lite® ELISA	0	6 (30)
anti-Domain I IgG	0	11 (55)
anti-PS/PT IgG	3 (7)	8 (40)
anti-PS/PT IgM	5 (11)	16 (80)

aPL, antiphospholipid; antiCL, anti-cardiolipin; antiβ2GPI, anti-beta2glycoprotein I; LAC, lupus anticoagulant; anti-PS/PT, anti-phosphatidylserine/ prothrombin.

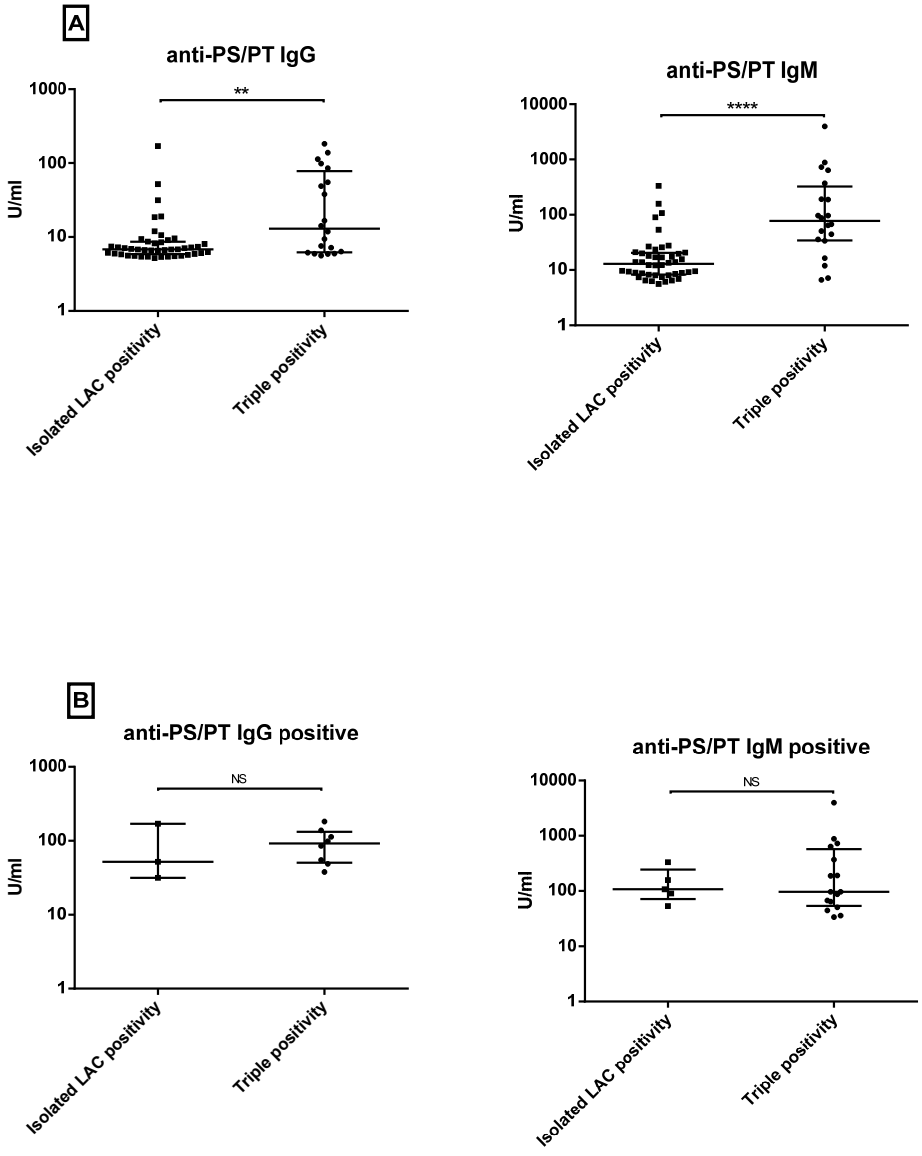

Figure 1: Comparison of anti-PS/PT IgG and IgM titers between patients with isolated LAC positivity and patients with triple positivity. The titers of anti-PS/PT antibodies (A) and the titers of anti-PS/PT positive antibodies (B) are expressed as the median with interquartile ranges. **P < 0.01, ****P < 0.0001. Abbreviations: anti-PS/PT, anti-phosphatidylserine/prothrombin antibodies; LAC, lupus anticoagulant, NS: not significant.

Association of isolated LAC and triple positivity with thrombosis

The association between isolated LAC or triple positivity with thrombosis resulted in an OR (95% CI) of 7.3 (3.3-16.1) and 4.3 (1.6-12.2), respectively (**Table 4A**). To avoid a possible bias, we re-calculated the association of isolated LAC with thrombosis after removing the triple positive samples and we re-calculated the association of triple-positivity with thrombosis after removing the samples with isolated LAC. The OR for developing thrombosis of the isolated LAC remained higher than the OR of triple-positivity (7.9 (3.6-17.5) versus 5.3 (1.9-14.8)) (**Table 4A**). The association between isolated LAC or triple positivity and APS showed overall higher ORs (**Table 4B**), also higher in the isolated LAC group compared to the triple positive group.

Table 4: Association of isolated LAC and triple positivity with thrombosis. OR of isolated LAC and triple positive patients are given for thrombosis versus non-thrombosis patients (A) and for APS versus non-APS patients (B). The given OR are either regarding the whole population or by excluding single or triple positive patients as indicated.

A. Thrombosis versus non-thrombosis patients

Patient group		**OR**	**95%CI**
Isolated LAC positivity	in total population	7.3	3.3-16.1
Triple positivity	in total population	4.3	1.6-12.2
Isolated LAC positivity	in population with taking out triple positivity	7.9	3.6-17.5
Triple positivity	in population with taking out isolated LAC positivity	5.3	1.9-14.8

B. APS versus non-APS patients

Patient group		**OR**	**95%CI**
Isolated LAC positivity	in total population	57.3	24.5-134.3
Triple positivity	in total population	22.6	7.9-64.9
Isolated LAC positivity	in population with taking out triple positivity	113.1	44.9-284.8
Triple positivity	in population with taking out isolated LAC positivity	75.4	24.2-234.8

LAC, lupus anticoagulant; OR, Odds ratio; 95%CI, 95% confidence interval.

Strength of LAC activity; isolated LAC versus LAC in triple positive patients

Isolated positive LAC patients showed weaker LAC activity compared to triple positive patients, although there were differences between the aPTT and the dRVVT (**Figure 2**). There was no difference between the prolongation of the dRVVT Screen in isolated LAC samples compared to triple positive samples 1.70 (1.47 - 2.13) versus 1.96 (1.47 - 2.74, P = 0.118). When we analyzed the confirmation step, we observed lower Screen/Confirm ratios in the isolated LAC group (1.39 (1.33 - 1.50)) compared to the triple positive group (1.60 (1.38 - 1.96), p = 0.007) (**Figure 2A**). The aPTT-Screen appeared to be lower in the isolated LAC group compared to the triple positive group 1.38 (1.21 - 1.55) versus 1.80 (1.34 - 2.62), P = 0.004), while the confirmation results did not show significant differences between the two groups 1.45 (-0.58 - 11.50) seconds versus 4.85 (1.65 - 12.73) seconds, P = 0.118) (**Figure 2B**).

For the mixing step, both dRVVT and aPTT showed lower mix ratios in the isolated LAC group compared to the triple positive samples, for the dRVVT the median was 1.17 (1.13 - 1.27) versus 1.35 (1.21 - 1.66; P = 0.0005) and for the aPTT 1.07 (1.00 - 1.16) versus 1.27 (1.17 - 1.89; P < 0.0001).

In the isolated LAC group, the majority of patients were dRVVT positive (98%) and only one patient was positive in the aPTT system only. This patient had a high level of C-reactive protein (CRP) (100 mg/L). Similar results for the triple positive group were found as 100% was positive in the dRVVT and no patient was positive in the aPTT system only. In addition, 25% of the patients with isolated LAC and 33% of patients with triple positivity were positive for both assays.

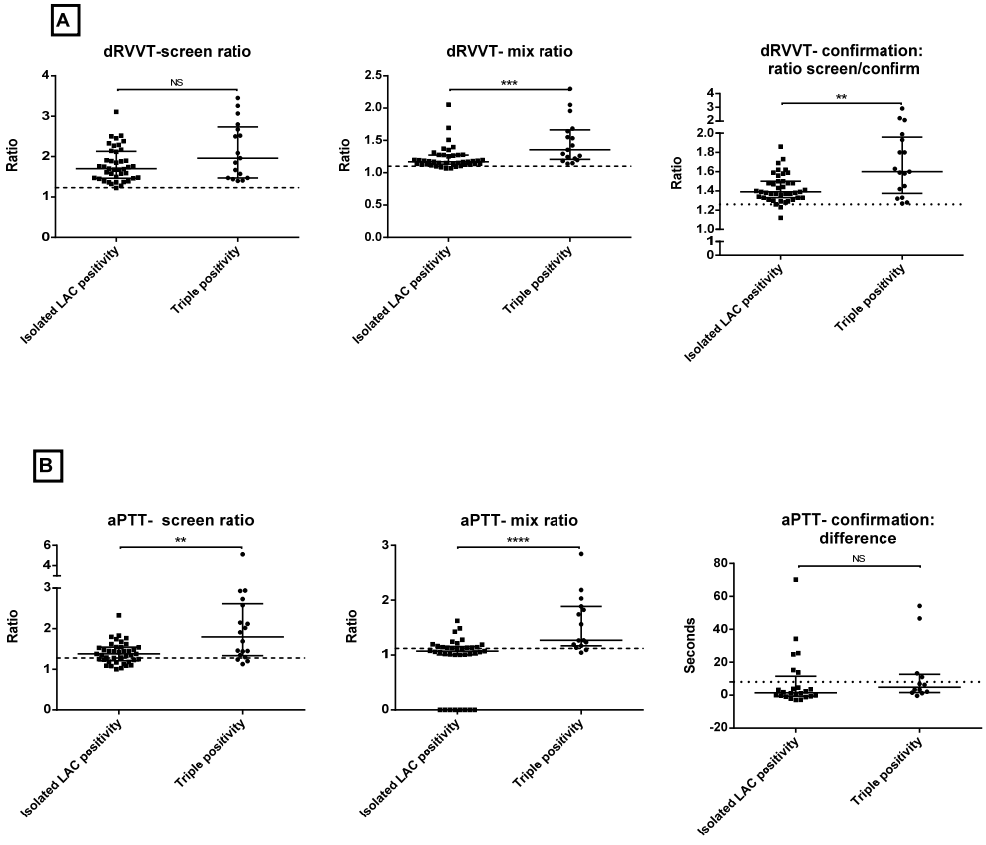

Figure 2. Comparison of LAC activity between the patients with isolated LAC positivity and patients with triple positivity. LAC activity was shown as detected with dRVVT-Screen ratio, mix ratio and confirmation (A) and aPTT-Screen ratio, mix ratio and confirmation (B). Results are expressed as the median with interquartile ranges; dashed lines indicate the cut-off value of each step test (1.23 for dRVVT-Screen ratio, 1.10 for dRVVT-mix ratio and 1.26 for dRVVT-confirmation; 1.28 for aPTT-Screen ratio, 1.12 for aPTT-mix ratio and 8 seconds for aPTT-confirmation). **P < 0.01, ***P < 0.001, ****P < 0.0001. Abbreviations: dRVVT, dilute Russell's viper venom time; aPTT, activated partial thromboplastin time; LAC, lupus anticoagulant, NS: not significant.

Discussion

In the present study we investigated the clinical significance of an isolated LAC and compared it to triple positivity in a cohort of APS and non-APS patients. We defined a plasma sample as isolated LAC when tested positive in a dRVVT and/or an aPTT based assay, and tested negative for antiCL and antiβ2GPI IgG and IgM antibodies applying four different solid phase platforms. This was done to overcome the variability between solid phase assays [18,19]. Triple positivity was defined as LAC positive and positive in both assays for antiCL antibodies or antiβ2GPI antibodies, at least by one solid phase platform. Although patients with isolated LAC positivity and triple positivity differed by the presence of antiCL and antiβ2GPI antibodies, they were comparable with respect to age, gender and history of thrombosis.

It is generally accepted that patients with isolated LAC have a lower risk profile in APS [20,21] and patients positive for all three aPL antibodies identify individuals at high risk for a first clinical event and for recurrence [8,9]. Isolated LAC is often observed in absence of clinical symptoms, in elderly patients, on a first occasion not confirmed after 12 weeks. Nonetheless the persistence of LAC in triple positive patients is very high (up to 100% of patients, also single LAC positives remain positive after twelve weeks in 40% of patients [22]. Others found no significant lower persistence in the single positive patients (93.3%) compared to the double and triple positive patients (96.8% and 97.9%, respectively) [23]. Moreover, an isolated LAC is an independent risk factor for myocardial infarction and ischemic stroke [14]. A prospective study showed that in a LAC positive population the association between occurrence of thrombosis and mortality was independent of antiCL and antiβ2GPI antibodies [15]. In our study, triple positivity was strongly associated with thrombosis, which is consistent with previous studies [19,24]. Interestingly, our study showed that an isolated LAC was also strongly correlated with a history of thrombosis with an even higher OR.

LAC can be caused by a heterogeneous subset of inhibitors. Both β2GPI and prothrombin have been studied for their role as target for LAC inducing antibodies. Only 8 of the 44 individuals with single LAC positivity were positive for anti-PS/PT antibodies, leaving 36 samples with LAC activity independent of β2GPI or prothrombin as co-factor. Interestingly, none of the isolated LAC samples were positive for anti-domain I antibodies, indicating that this group of samples is completely different from the triple positive group. In single LAC positive samples, negative for antiβ2GPI and anti-PS/PT antibodies, the antibody is not directed towards the known antigens but might be directed against other plasma proteins [25,26]. Indeed, other antibodies against protein C/S and annexin V have also been shown to interfere with coagulation[27,28]. aPL binding through other co-factors, such as complement C4 or factor H, may also be responsible for the LAC positivity [29-31] . Also cofactor independent antibodies have been shown relevant, although clinical data are rare [32,33].

It is interesting to note that in this study the dRVVT seems to be a more sensitive to pick up LAC than the aPTT. In international external quality programs the dRVVT performs better and with less variation than an aPTT in identifying APS positive samples [34]. This study supports the dRVVT as the preferred assay to detect LAC [1]. The presence of high levels of CRP can influence the LAC

results, especial in the aPTT assay [32]. In our study only a single patient was positive for the aPTT only, this patient had a higher CRP level and could be a false positive result, as described in the literature [32,33].

International External Quality Assessment Programmes have shown significant inter-laboratory variation, especially for weaker LAC samples [2 4 7]. Cut-off values in our study were calculated by the 99th percentile on 120 healthy donors [5] and LAC was performed according to the ISTH guidelines [1,35]. Each value above the cut-off should be regarded as positive and the final conclusion of LAC positivity can only be made if the three steps of the test procedure (screening, mixing, and confirmation) are positive, which was the case in our cohort. We did observe that patients with isolated LAC showed significantly less prolongation in the dRVVT and aPTT screening tests compared to patients with triple positivity, as previously reported [36]. However, guidance on categorising LAC according to strength does not seem to be appropriate, since there is no established LAC standard available in sufficient quantity. Furthermore, no evidence exists on the association of “stronger” LAC with clinical symptoms. But it is likely that the “weaker” positives fluctuate and “low positive” results might not be persistently positive. Therefore positive results have to be repeated after 12 weeks. In this cohort, all single LAC positive samples were confirmed after 12 weeks.

In conclusion, our study shows that patients with isolated LAC have a comparable or even higher risk of thrombosis compared to patients with triple positivity. As samples were negative for antibodies against β2GPI and prothrombin, further studies are needed to identify the target antigen responsible for isolated LAC.

References

1. Pengo V, Tripodi A, Reber G, et al. Update of the guidelines for lupus anticoagulant detection. *Journal of Thrombosis and haemostasis* 2009; **7**(10): 1737-40.
2. Devreese K. Standardization of antiphospholipid antibody assays.Where do we stand? *Lupus* 2012; **21**: 718-21.
3. Moore GW. Recent guidelines and recommendations for laboratory detection of lupus anticoagulants. Seminars in thrombosis and hemostasis 2014:163-71.
4. Devreese KM. Antiphospholipid antibody testing and standardization. *International journal of laboratory hematology* 2014; **36**(3): 352-63.
5. Devreese KMJ, Ortel TL, Pengo V, de Laat B, for the Subcommittee on Lupus Anticoagulant/Antiphospholipid A. Laboratory criteria for antiphospholipid syndrome: communication from the SSC of the ISTH. *Journal of Thrombosis and Haemostasis* 2018; **16**(4): 809-13.
6. Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *Journal of thrombosis and haemostasis* 2006; **4**(2): 295-306.
7. Bertolaccini ML, Amengual O, Andreoli L, et al. 14th International Congress on Antiphospholipid Antibodies Task Force. Report on antiphospholipid syndrome laboratory diagnostics and trends. *Autoimmunity reviews* 2014; **13**(9): 917-30.
8. Pengo V, Ruffatti A, Legnani C, et al. Clinical course of high-risk patients diagnosed with antiphospholipid syndrome. *Journal of Thrombosis and Haemostasis* 2010; **8**(2): 237-42.
9. Pengo V, Ruffatti A, Legnani C, et al. Incidence of a first thromboembolic event in asymptomatic carriers of high-risk antiphospholipid antibody profile: a multicenter prospective study. *Blood, The Journal of the American Society of Hematology* 2011; **118**(17): 4714-8.
10. Monica Galli DL, Guido Bertolini, and Tiziano Barbui. Lupus anticoagulants are stronger risk factors for thrombosis than anticardiolipin antibodies in the antiphospholipid syndrome: a systematic review of the literature. *Blood* 2003; **101**: 1827-32.
11. Reynaud Q, Lega J-C, Mismetti P, et al. Risk of venous and arterial thrombosis according to type of antiphospholipid antibodies in adults without systemic lupus erythematosus: a systematic review and meta-analysis. *Autoimmunity reviews* 2014; **13**(6): 595-608.
12. Pengo V, Testa S, Martinelli I, et al. Incidence of a first thromboembolic event in carriers of isolated lupus anticoagulant. *Thrombosis research* 2015; **135**(1): 46-9.
13. Mustonen P, Lehtonen K, Javela K, Puurunen M. Persistent antiphospholipid antibody (aPL) in asymptomatic carriers as a risk factor for future thrombotic events: a nationwide prospective study. *Lupus* 2014; **23**(14): 1468-76.
14. Urbanus RT, Siegerink B, Roest M, Rosendaal FR, de Groot PG, Algra A. Antiphospholipid antibodies and risk of myocardial infarction and ischaemic stroke in young women in the RATIO study: a case-control study. *The Lancet Neurology* 2009; **8**(11): 998-1005.
15. Gebhart J, Posch F, Koder S, et al. Increased mortality in patients with the lupus anticoagulant: the Vienna Lupus Anticoagulant and Thrombosis Study (LATS). *Blood* 2015; **125**(22): 3477-83.

16. de Mast Q, Molhoek JE, van der Ven AJ, et al. Antiphospholipid Antibodies and the Risk of Stroke in Urban and Rural Tanzania: A Community-Based Case-Control Study. *Stroke* 2016; **47**(10): 2589-95.
17. Devreese KM, Pierangeli SS, de Laat B, et al. Testing for antiphospholipid antibodies with solid phase assays: guidance from the SSC of the ISTH. *Journal of thrombosis and haemostasis* 2014; **12**(5): 792-5.
18. Chayoua W, Kelchtermans H, Moore GW, et al. Detection of Anti-Cardiolipin and Anti-beta2glycoprotein I Antibodies Differs between Platforms without Influence on Association with Clinical Symptoms. *Thrombosis and Haemostasis* 2019; **119**(05): 797-806.
19. Chayoua W, Kelchtermans H, Moore GW, et al. Identification of high thrombotic risk triple-positive antiphospholipid syndrome patients is dependent on anti-cardiolipin and anti-beta2glycoprotein I antibody detection assays. *Journal of thrombosis and haemostasis* 2018; **16**(10): 2016-23.
20. Devreese KM. Antiphospholipid antibodies: evaluation of the thrombotic risk. *Thrombosis Research* 2012; **130 Suppl 1**: S37-40.
21. Pengo V, Testa S, Martinelli I, et al. Incidence of a first thromboembolic event in carriers of isolated lupus anticoagulant. *Thrombosis Research* 2015; **135**(1): 46-9.
22. Pengo V, Ruffatti A, Del Ross T, et al. Confirmation of initial antiphospholipid antibody positivity depends on the antiphospholipid antibody profile. *Journal of thrombosis and haemostasis* 2013; **11**(8): 1527-31.
23. Devignes J, Smaïl-Tabbone M, Hervé A, et al. Extended persistence of antiphospholipid antibodies beyond the 12-week time interval: Association with baseline antiphospholipid antibodies titres. *International journal of laboratory hematology* 2019; **41**(6): 726-30.
24. Pengo V, Banzato A, Denas G, et al. Correct laboratory approach to APS diagnosis and monitoring. *Autoimmunity reviews* 2013; **12**(8): 832-4.
25. Rampazzo P, Biasiolo A, Garin J, et al. Some patients with antiphospholipid syndrome express hitherto undescribed antibodies to cardiolipin-binding proteins. *Thrombosis and Haemostasis* 2001; **85**(01): 57-62.
26. Yin D, de Laat B. Commentary on: Circulating plasmablasts contribute to antiphospholipid antibody production, associated with type I interferon upregulation. *Journal of thrombosis and haemostasis* 2019; **17**(7): 1030-2.
27. de Laat B, Eckmann CM, van Schagen M, Meijer AB, Mertens K, van Mourik JA. Correlation between the potency of a beta2-glycoprotein I-dependent lupus anticoagulant and the level of resistance to activated protein C. *Blood coagulation & fibrinolysis* 2008; **19**(8): 757-64.
28. de Laat B, Wu XX, van Lummel M, Derksen R, de Groot PG, Rand JH. Correlation between antiphospholipid antibodies that recognize domain I of beta 2-glycoprotein I and a reduction in the anticoagulant activity of annexin A5. *Blood* 2007; **109**(4): 1490-4.
29. Pengo V, Denas G. Diagnostics and treatment of thrombotic antiphospholipid syndrome (APS): A personal perspective. *Thrombosis Research* 2018; **169**: 35-40.
30. Devreese K, Ortel T, Pengo V, de Laat B. Laboratory criteria for antiphospholipid syndrome: reply. *Journal of thrombosis and haemostasis* 2018; **16**: 2117-9.

31. de Groot PG, de Laat B, Rand J. et al. Natural proteins involved in antiphospholipid syndrome. *In: Erkan D, Lockshin M. eds.* Antiphospholipid Syndrome. Cham: Springer; 2017: 15-17.
32. Schouwers SM, Delanghe JR, Devreese KM. Lupus Anticoagulant (LAC) testing in patients with inflammatory status: Does C-reactive protein interfere with LAC test results? *Thrombosis Research* 2010; **125**(1): 102-4.
33. Devreese KMJ, Verfaillie CJ, De Bisschop F, Delanghe JR. Interference of C-reactive protein with clotting times. *Clinical Chemistry and Laboratory Medicine* 2015; **53**(5): 6141-5.
34. Pengo V, Biasiolo A, Rampazzo P, Brocco T. dRVVT is more sensitive than KCT or TTI for detecting lupus anticoagulant activity of anti-β2-glycoprotein I autoantibodies. *Thrombosis and Haemostasis* 1999; **81**(02): 256-8.
35. Katrien M.J.Devreese, Philip G. de Groot, Bas de Laat, et al. Guidance from the Scientific and Standardization Committee for lupus anticoagulant/antiphospholipid antibodies of the International Society on Thrombosis and Haemostasis: Update of the guidelines for lupus anticoagulant detection and interpretation. *Journal of Thrombosis and Haemostasis* 2020; **18**: 2828-39.
36. Pengo V, Del Ross T, Ruffatti A, et al. Lupus anticoagulant identifies two distinct groups of patients with different antibody patterns. *Thrombosis research* 2018; **172**: 172-8.

Chapter 8

General discussion

General discussion

The Solid phase aPLs assays that are included in the laboratory criteria for the diagnosis of APS, lack standardization which is limiting their utility in clinical practice. The lack of standardization is due to the variability observed between centers, manufactures and research groups. The uniformity is also compromised by methodological shortcomings and the heterogeneity of aPLs antibodies [1,2]. Currently, only antibodies that bind β2GPI have been included in the official criteria used to APS diagnosis. However, this is a heterogenous population of antiβ2GPI antibodies. Several different non-continuous epitopes on domain I (DI) of β2GPI have been shown to react with aPL antibodies [3]. More and more evidence suggests that detection of a subset of aPLs antibodies that are reactive against DI of β2GPI is a promising risk stratification tool in APS [4,5]. Nowadays, various assays that specifically measure antiDI antibodies have been developed and been reported in the literature.

History of antiDI assay

As summarized **in chapter 2**, Iverson et al. showed in 1998 that aPL antibodies are able to recognize epitopes on DI of β2GPI [6]. In 2000 Reddel et al. demonstrated that inducing a charge altering mutation of Gly-to-Glu at position 40 and of Arg-to-Gly at position 43 of DI, resulted in a decreased β2GPI antigen binding [7]. In 2002 there were two studies that confirmed that the charged surface patch defined by residues 40-43 on β2GPI was a dominant target epitope for autoantibodies[8,9]. Based on this information, de Laat et al. developed the first antiDI assay in 2005 [10]. In this assay, DI is coated on a hydrophobic and a hydrophilic plate. The arbitrary binding of DI on the hydrophobic plate will ensure enough exposure of the G40-R43 epitope. On the contrary, the binding of DI on a hydrophilic plate will make the positive epitope G40-R43 bind strongly to the plate, masking the epitope for binding by antibodies. In 2006 a simple direct antiDI ELISA was developed and used to confirm that the G40-R43 epitope and the adjacent arginine 39 (R39) residue play a major role in the binding to antibodies by using site-directed mutants of DI [11]. In 2010, a chemically synthesized DI was used to inhibit the binding of antibodies to whole β2GPI immobilized on a 96 wells plate in a competitive inhibition ELISA assay [12]. In 2014, by using the same recombinant DI of β2GP1 obtained from the baculovirus expression system, INOVA Diagnostics developed an ELISA [13] and a chemiluminescence (CLIA) assay for the measurement of antiDI antibodies by coupling DI to paramagnetic beads[14] (**Table 1**).

Table1. Overview of assays used for the detection of antibodies against domain I of β2GPI (antiDI).

Assay	Principle of the method	Source of DI	Solid phase	Surface capacity	Exposure of the epitope G40-R43
In house two step ELISA	Antibodies are detected against DI coated on a hydrophilic versus hydrophobic plate by an in-house ELISA	Baculovirus expression system	Hydrophobic and hydrophilic ELISA plate	Normal	Yes
Direct ELISA	Antibodies are detected against DI coated on a nickel plate by an in-house ELISA	Bacteria expression system	Nickel chelate ELISA plate	Normal	NS
Competitive inhibition ELISA	The percentage inhibition is calculated when IC50 of DI in solution is used to inhibit antibodies binding to β2GPI coated on a flexible plate by an in-house ELISA	Chemical synthesis	ELISA plate	Normal	NS
Commercial developed ELISA	Antibodies are detected against DI coated on ELISA plates by a commercially developed ELISA	Baculovirus expression system	ELISA plate	Normal	NS
Commercial CLIA	Antibodies are detected against DI coated on paramagnetic beads by a CLIA	Baculovirus expression system	Paramagnetic beads	Higher	NS

CLIA: chemiluminescence immunoassay; ELISA: enzyme-linked immunosorbent assay; NS: not specified

Clinical role of various antiDI assays

As antiDI antibodies represent a specific pathogenic population of aPLs antibodies, the representative assays were expected to improve the classification and risk stratification of APS compared to conventional aPLs criteria tests. However, when reviewing the laboratory and clinical performance characteristics of the different antiDI assays (**chapter 2**), we found that there was no consensus regarding the added value of measuring antiDI antibodies in the classification criteria of APS. Some studies applying antiDI assays have demonstrated a higher correlation with thrombosis compared to the traditional antiβ2GPI assays, whereas other studies have failed to show an added value of the antiDI assays. Regarding the different antiDI assays, it is the two-step antiDI IgG ELISA in particular that showed high odds ratios (ORs) rather than IgG antibodies targeting other domains of β2GPI [10,15]. The commercially available CLIA is currently the most widely used method to detect antiDI IgG antibodies. However, the results regarding the added clinical value of antiDI IgG measured by the CLIA assay are not consistent. Some studies showed an added value of the addition of the detection of antiDI antibodies to the aPL antibody panel[16-19], whereas other studies failed [20-22]. None of the remaining three assays (i.e. the direct antiDI ELISA, the competitive inhibition ELISA and the commercial developed INOVA antiDI ELISA) showed an added value of detecting antiDI IgG compared to other antiβ2GPI IgG antibodies. Taken together, the observed inconsistency probably explains why antiDI antibodies have not yet been included in the laboratory criteria [2]. The inconsistent results might be due to differences in

non-assay related factors, such as different patient population and sample handling protocols, as well as the assays used to detect antibodies against DI.

To further investigate the added value of measuring antiDI antibodies on top of the current APS classification criteria we initiated a multicenter study. In this multicenter study, antiCL and antiβ2GPI antibodies were detected by four different commercially available platforms in a large cohort of APS and non-APS patients (**chapter 3**). We were able to demonstrate that the detection of antiDI IgG by antiDI CLIA was less sensitive, but more specific compared to the current laboratory criteria assay. This resulted in higher ORs for developing thrombosis and pregnancy morbidity. Moreover, antiDI IgG antibodies are mainly present in high-risk triple positive patients. The combination of antiDI antibodies and triple positivity, confers a higher risk for clinical symptoms compared to triple positivity. In contrast to our expectation, the addition of antiDI or the replacement of antiβ2GPI IgG by antiDI hardly improved the ORs for developing thrombosis or pregnancy morbidity. We were unable to demonstrate the added value of measuring antiDI IgG on top of the laboratory criteria, independent of the platform used to measure antiCL and antiβ2GPI antibodies. We hypothesized that the reduced exposure of the pathogenic DI epitope in the automated CLIA assay was a possible explanation for the lack of added value. This hypothesis came from our previously published results, where we demonstrated the large variability in commercially available antiCL and antiβ2GPI assays. Part of the variability can be explained by the variable exposure of the pathogenic G40-R43 epitope on DI of β2GPI, which could lead to incorrect patient classification [23].

In chapter 1, we demonstrated the importance of coating β2GPI on a hydrophilic surface, as this results in the conformational change of β2GPI which leads to the exposure of cryptic epitopes. It has been shown that antibodies directed against this cryptic epitope G40-R43 on DI are pathogenic. Hence, the results obtained by the antiβ2GPI assays depend on the conformation of the coated β2GPI (i.e. whether epitope G40-R43 is exposed), which depends of the type of solid phase surface used to immobilize β2GPI as well as the source of the protein. By coating only DI of β2GPI, this issue does not exist. However, as epitope G40-R43 is positively charged, the charge of the coating surface could also influence the availability of the G40-R43 epitope. De Laat et al.[10] showed that the use of a neutral coating surface results in an arbitrary orientation of DI including orientations in which epitope G40-R43 is exposed. When a negative surface is used, the positive epitope will bind to the surface thereby preventing its availability for antibodies present in the sample. It cannot be excluded that the beads used in the CLIA assays are charged, which makes the exposure of epitope G40-R43 uncertain. To verify our hypothesis we used two human-derived monoclonal antibodies (P1-117 and P2-6) to determine whether the pathogenic epitope is exposed in the antiDI CLIA assay. Antibody P1-117 recognizes epitope G40-R43 and can only bind to β2GPI when it is coated in its open conformation, while antibody is able to recognize DI regardless of its conformation [24]. Our data showed that only the P2-6 antibody could bind to the DI of the CLIA assay. Contrary to our expectation, this would mean that the CLIA antiDI assay does not expose epitope G40-R43 on the surface of the beads, thereby missing the patient samples with antibodies towards this epitope. In addition, a high agreement was observed between antiβ2GPI and antiDI in the same CLIA device, suggesting that both assays measure almost the

same antibody population directed against DI excluding the antibodies that bind to epitope G40-R43.

Detection of antiDI IgG by a hydrophobic ELISA

Other methods than the CLIA assay are available to detect antiDI antibodies. In 2005, an in-house two-step ELISA was developed that ensures the exposure of the cryptic epitope. By the use of this assay we demonstrated that the presence of antiDI IgG antibodies could identify APS patients at high risk for developing thrombosis and/or pregnancy morbidity [10,15]. This two-step ELISA is performed by coating DI of β2GPI on a hydrophilic and a hydrophobic ELISA plate. The difference in type of plate used to immobilize DI resulted in a different exposure of the pathogenic DI epitope (**Figure 1**). We used our in-house assay (**chapter 4)** and compared it to the commercial antiDI CLIA assay in a large cohort of APS and non-APS patients. When DI was coated on the hydrophilic plate, no antibodies could be detected. However, when DI was coated on the hydrophobic plate, the antibodies against DI could be detected, resulting in significantly higher optical density (OD) values in the APS samples compared to the control group. In addition, we observed that 18 out of 101 samples with a history of thrombosis were negative in the antiDI CLIA assay, but positive in the in-house antiDI hydrophobic ELISA. This would indicate that the in-house assay is more sensitive for the detection of pathogenic samples than the commercial antiDI CLIA assay. Moreover, the in-house antiDI hydrophobic ELISA was able to detect more samples with clinical manifestations of APS.

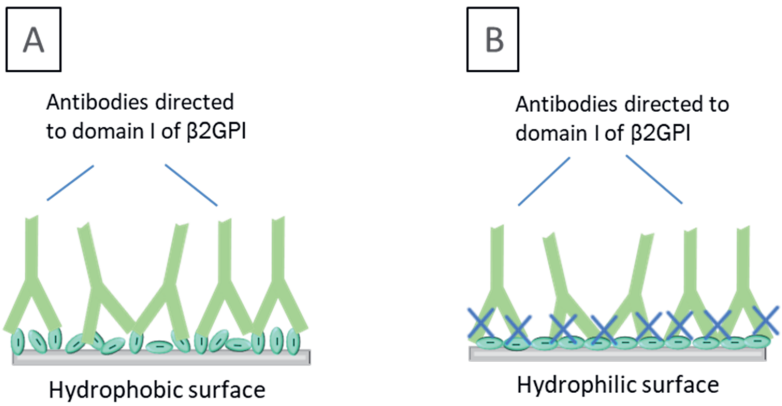

Figure 1. The two types of solid phase assays for detection of antiDI IgG antibodies. (A) When DI is coated on a hydrophobic surface, the arbitrary orientation of immobilized DI allows exposure of the cryptic epitope G40-R43. Antibodies against this pathological epitope will be able to bind to the epitope. (B) when DI is coated on a hydrophilic surface, the positively charged G40-R43 epitope will bind strongly to the hydrophilic surface, thereby preventing the binding of antibodies to this epitope.

Many antiDI assays are available, but they have the same problems as the full-length antiβ2GPI assays. As with antiβ2GPI assays, standardization of the antiDI assay is of utmost importance. An important part of this standardization process is to ensure enough exposure of the G40-R43

epitope, to ascertain that at least one specific pathogenic antibody population can be measured. The charge of the solid phase surfaces used to immobilize DI will influence the exposure of the epitope G40-R43 of DI. Although CLIA can provide a large surface for antibody binding to DI, our data revealed an impaired exposure of the pathogenic epitope in CLIA. In addition, we found that when DI was coated on a hydrophilic suface, no antibodies could be detected. On the contrary, our in-house antiDI hydrophobic ELISA was able to detect more samples with clinical manifestations of APS compared to the antiDI IgG CLIA assay. Therefore, it may be interesting to verify correct exposure of the cryptic epitope in all available antiDI assays, which will also help to improve the standardization process of the antiDI assays.

Clinical value in detecting non-criteria antiCL and antiβ2GPI IgA antibodies in APS

The interpretation of immunoassays must take into consideration the isotype of the detected antibodies. Current criteria recommend increased levels of antiCL and antiβ2GPI IgG and IgM for the diagnosis of APS, whereas the detection of antiCL and antiβ2GPI IgA antibodies is not included in these criteria [2,25] . The pathogenicity of IgA aPL antibodies has been shown in animal models, however, the authors did not demonstrate that the IgA antibodies were solely responsible for the observed effect [26]. The role of IgA remains controversial in clinical events related to APS. Multiple studies have shown that antiCL and antiβ2GPI IgA antibodies are associated with thrombosis and pregnancy morbidity [27-37]. Nevertheless, a recent systematic review indicated that several studies failed to demonstrate utility of adding antiCL and antiβ2GPI IgA testing. This was probably due to either the low prevalence of these antibodies and their association with other aPL antibodies, or because of the lack of improved diagnostic accuracy of IgA antibodies [38]. Data proving the clinical significance of antiCL or antiβ2GPI IgA testing in addition to the current aPL-panel is still lacking [25,39] .

Therefore, we set up a multicenter study (**chapter 6**) and used four commercially available solid phase assays to detect antiCL and antiβ2GPI IgG/IgM/IgA, to assess for a possible association of antiCL and antiβ2GPI IgA with thrombosis or obstetric complications. In our study cohort, we included APS patients and non-APS patients that served as controls. Our study showed that antiCL and antiβ2GPI IgA antibodies did correlate with the clinical manifestations of APS (thrombosis and pregnancy morbidity), independently of the solid phase platform used. An external quality control program illustrated that the different antiCL and antiβ2GPI IgG/IgM assays produced variable results [40 41]. Our study also demonstrated that by the use of different solid phase assays a different proportion of APS patients was positive for antiCL and/or antiβ2GPI IgA antibodies. These data indicate that the detection of IgA aPL antibodies also depends on the solid phase assay used, as well as the study population. Although an added value was suggested for so called 'isolated IgA' positivity by some studies[30,32,34,36,37,42], many of these studies did not include LAC testing, thereby hampering the assessment of true isolated IgA. We defined isolated antiCL and/or antiβ2GPI IgA positivity as positivity for antiCL and/or antiβ2GPI IgA and negativity for antiCL and antiβ2GPI IgG/IgM and LAC. Isolated IgA positivity was rare in the total population.

Overall, the prevalence of isolated IgA was comparable between patients with clinical manifestations of APS and patients without a history of thrombosis or pregnancy morbidity. Positivity for isolated IgA aPL was not associated with thrombosis or pregnancy morbidity as the calculated OR did not reach statistical significance. Our results do not support antiCL and/or antiβ2GPI IgA testing in addition to the conventional aPL testing in identifying patients with clinical manifestations of APS.

The clinical relevance of isolated lupus anticoagulant positivity in patients with thrombotic APS

Currently, the presence of LAC together with antiCL and/or antiβ2GPI IgG/IgM antibodies are included in the international criteria for the classification of APS [2,25,43,44]. Triple positive patients (i.e. positive for the combination of LAC, antiβ2GPI and antiCL IgG/IgM antibodies), exhibit a high risk for thrombotic and/or obstetric manifestations [44-47]. However, the clinical relevance of an isolated LAC has been debated in the past. Although both retrospective and prospective studies suggested that LAC has a strong predictive value for thrombosis and adverse pregnancy outcome [48-55], recent studies showed a poor predictive value of isolated LAC positivity for the first thrombotic event [56,57]. Therefore, we repeated a study to investigate the correlation of isolated LAC with thrombosis and compared it to triple positivity in a cohort of APS and non-APS patients (**chapter 7).** In order to overcome the variability between solid phase assays [58,59], we defined a plasma sample as isolated LAC when tested positive in a dRVVT and/or an aPTT based assay, and tested negative for antiCL and antiβ2GPI IgG and IgM antibodies applying four different solid phase platforms. Triple positivity was defined as being positive for LAC, antiCL antibodies and antiβ2GPI antibodies, by at least one solid phase platform. In our study, triple positivity was strongly associated with thrombosis (OR 5.3(1.9-14.8)), which is consistent with previous studies [59,60]. Interestingly, we found that an isolated LAC also correlated strongly with thrombosis with an even higher OR (7.6 (3.6-17.5)).

Many possible mechanisms for the pathogenicity of APS are described **in chapter 5.** Different proteins have been identified to be involved in the pathogenesis of APS. The LAC assay can be affected by a heterogeneous group of autoantibodies that are binding to the negatively charged phospholipids present in the assay. Both β2GPI and prothrombin have been studied for their role as a target for LAC-inducing antibodies [10,24,61-63]. **In Chapter 2,3 and 4,** we showed that antiDI IgG antibodies are mainly present in patients with triple positivity, independent of the assays used to measure these antibodies. Moreover, this is also independent of the platform used to measure antiCL and antiβ2GPI IgG/IgM antibodies. We also demonstrated that 55% of the triple positive patients tested also positive for antiDI IgG, whereas none of the isolated LAC patients were positive for antiDI IgG. In addition, in **chapter 6** we showed that only very few patients with clinical manifestations of APS were positive for isolated antiCL/antiβ2GPI IgA. Most of the IgA positive patients were also positive for antiCL and/or antiβ2GPI IgG/IgM. This was also shown in **chapter 7**, hardly any of the isolated LAC patients were also positive for antiCL and/or antiβ2GPI IgA, compared to the triple positive patient group (i.e. 55%). These results indicated that this

group of isolated LAC positive patients differed from the triple positive patient group. Studies have shown that antibodies against phosphatidylserine/prothrombin (anti-PS/PT) are frequently present in APS patients and are strongly associated with LAC [64,65]. We found that 18 out of 20 triple positive patients were also positive for anti-PS/PT antibodies (mainly of the IgM isotype). On the contrary, only 8 of the 44 individuals with single LAC positivity were also positive for anti-PS/PT antibodies (**Table 2**).

Table 2. Antiphospholipid antibody characteristics and the Odds ratios (95% confidence interval for thrombosis of two antibody patterns: isolated LAC and triple positivity.

	LAC	AntiCL IgG/M	Antiβ2G PI IgG/M	AntiCL/antiβ2G PI IgA	AntiDI IgG	AntiPS/PT IgG/M	OR for thrombosis
Isolated LAC positivity	Positive	Negative	Negative	Negative	Negative	<20%	7.6 (3.6-17.5)
Triple positivity	Positive	Positive	Positive	Half is positive	Half is positive	>80%	5.3 (1.9-14.8)

As mentioned previously, isolated LAC correlated significantly with thrombosis. This would indicate that other phospholipids-binding proteins (other than β2GPI and prothrombin) could be responsible of the development of thrombotic events [66,67]. In fact, antibodies against protein C/S and annexin V have also been shown to interfere with coagulation [68,69]. aPLs antibody binding through other co-factors, such as complement C4 or factor H, may also be responsible for LAC positivity [70-72] . Also cofactor-independent antibodies have been shown to be relevant, although clinical data demonstrating this are rare [73,74]. The presence of high C-reactive protein (CRP) plasma levels can influence LAC results as well, especially in the aPTT assay [73]. In our study only one single patient tested positive for only the aPTT, however, this patient had also a high CRP plasma level and could therefore be false positive [73,74]. Further studies are needed to identify the target antigen responsible for isolated LAC activity in the absence of antiPS/PT and antiβ2GPI antibodies.

Isolated LAC positive patients showed weaker LAC activity compared to triple positive patients. The International External Quality Assessment Programmes have shown significant inter-laboratory variation, especially for weaker LAC samples [43,75,76]. Cut-off values in our study were calculated by the 99th percentile of the results of 120 healthy donors [2] and LAC was performed according to the ISTH guidelines [77]. Each value above the cut-off should be regarded as positive and the final conclusion of LAC positivity can only be made if the three steps of the test procedure (screening, mixing, and confirmation) are positive. We did observe that patients with isolated LAC showed a significantly less prolonged dRVVT and aPTT screening tests compared to triple positive patients [64]. However, guidance on categorising LAC according to strength does not seem to be appropriate, since there is not an established LAC standard available in sufficient quantity. Furthermore, no evidence exists on the association of “stronger” LAC with the risk of the clinical symptoms. But it is likely that the “weaker” positive patients fluctuate in their results and that

"low positive" results might not be persistently positive. Therefore, consensus was reached that the positive results should be repeated after 12 weeks to confirm their positivity. In our cohort, all single LAC positive samples were retested and reconfirmed after 12 weeks. [78,79].

In conclusion, patients with isolated LAC have a comparable or even higher risk of developing thrombosis compared to triple positive patients. Further studies are needed to identify the target antigen responsible for the pathogenicity in isolated LAC.

Conclusions

The clinical symptoms of the APS occur frequently irrespectively of the syndrome, making the diagnosis of APS difficult and predominantly depends on the laboratory criteria. Currently, the laboratory diagnosis of APS is still challenging due to the lack of standardization and the heterogeneity of aPLs antibodies. We showed that it is important to verify the correct exposure of the cryptic epitope in all available antiDI assays to improve standardization of the antiDI assays. Furthermore, isolated IgA was rare and did not correlate with the clinical manifestations of APS. However, patients with isolated LAC have a comparable or even higher risk of thrombosis compared to patients with triple positivity. Further studies are needed to address the implications of our findings.

References

1. Devreese KM, Pierangeli SS, de Laat B, et al. Testing for antiphospholipid antibodies with solid phase assays: guidance from the SSC of the ISTH. *Journal of thrombosis and haemostasis* 2014; **12**(5): 792-5.
2. Devreese KMJ, Ortel TL, Pengo V, de Laat B, for the Subcommittee on Lupus Anticoagulant/Antiphospholipid A. Laboratory criteria for antiphospholipid syndrome: communication from the SSC of the ISTH. *Journal of Thrombosis and Haemostasis* 2018; **16**(4): 809-13.
3. Ioannou Y, Pericleous C, Giles I, Latchman DS, Isenberg DA, Rahman A. Binding of antiphospholipid antibodies to discontinuous epitopes on domain I of human beta(2)-glycoprotein I - Mutation studies including residues R39 to R43. *Arthritis & Rheumatology* 2007; **56**(1): 280-90.
4. Arachchillage DRJ, Laffan M. Pathogenesis and management of antiphospholipid syndrome. *British journal of haematology* 2017; **178**(2): 181-95.
5. Kelchtermans H, Chayoua W, Laat B. The Significance of Antibodies against Domain I of Beta-2 Glycoprotein I in Antiphospholipid Syndrome. *Seminars in thrombosis and hemostasis* 2018; **44**(5): 458-65.
6. Iverson GM, Victoria EJ, DM. M. Anti-beta2 glycoprotein I (beta2GPI) autoantibodies recognize an epitope on the first domain of beta2GPI. *Proceedings of the National Academy of Sciences of the U S A* 1998; **95**: 15542-6.
7. Stephen W. Reddel YXW, Yong Hua Sheng,Steven A. Krilis. Epitope studies with anti-beta 2-glycoprotein I antibodies from autoantibody and immunized sources. *Journal of Autoimmunity* 2000; **15**(2): 91-6.
8. Iverson GM, Reddel S, Victoria EJ, et al. Use of Single Point Mutations in Domain I of 2-Glycoprotein I to Determine Fine Antigenic Specificity of Antiphospholipid Autoantibodies. *The Journal of Immunology* 2002; **169**(12): 7097-103.
9. Iverson GM, Matsuura E, Victoria EJ, Cockerill KA, Linnik MD. The Orientation of β2GPI on the Plate is Important for the Binding of Anti-β2GPI Autoantibodies by ELISA. *Journal of Autoimmunity* 2002; **18**(4): 289-97.
10. 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**(4): 1540-5.
11. Ioannou Y, Giles I, Lambrianides A, et al. A novel expression system of domain I of human beta2 glycoprotein I in Escherichia coli. *BMC biotechnology* 2006; **6**: 8.
12. Banzato A, Pozzi N, Frasson R, et al. Antibodies to Domain I of beta(2)Glycoprotein I are in close relation to patients risk categories in Antiphospholipid Syndrome (APS). *Thrombosis Research* 2011; **128**(6): 583-6.
13. Andreoli L, Chighizola CB, Nalli C, et al. Clinical characterization of antiphospholipid syndrome by detection of IgG antibodies against beta2 -glycoprotein i domain 1 and domain 4/5: ratio of anti-domain 1 to anti-domain 4/5 as a useful new biomarker for antiphospholipid syndrome. *Arthritis & Rheumatology* 2015; **67**(8): 2196-204.

14. Mahler M, Radice A, Yang W, et al. Development and performance evaluation of novel chemiluminescence assays for detection of anti-PR3 and anti-MPO antibodies. *Clinica chimica acta; international journal of clinical chemistry* 2012; **413**(7-8): 719-26.
15. de Laat B, Pengo V, Pabinger I, et al. The association between circulating antibodies against domain I of beta2-glycoprotein I and thrombosis: an international multicenter study. *Journal of thrombosis and haemostasis* 2009; **7**(11): 1767-73.
16. Mahler M, Albesa R, Zohoury N, et al. Autoantibodies to domain 1 of beta 2 glycoprotein I determined using a novel chemiluminescence immunoassay demonstrate association with thrombosis in patients with antiphospholipid syndrome. *Lupus* 2016; **25**(8): 911-6.
17. Zhang S, Wu Z, Chen S, et al. Evaluation of the diagnostic potential of antibodies to beta2-glycoprotein 1 domain 1 in Chinese patients with antiphospholipid syndrome. *Scientific reports* 2016; **6**: 23839.
18. Lee JS, Gu J, Park HS, Yoo HJ, Kim HK. Coexistence of anti-beta2-glycoprotein I domain I and anti-phosphatidylserine/prothrombin antibodies suggests strong thrombotic risk. *Clinical Chemistry and Laboratory Medicine* 2017; **55**(6): 882-9.
19. Mondejar R, Gonzalez-Rodriguez C, Toyos-Saenz de Miera FJ, et al. Role of antiphospholipid score and anti-beta2-glycoprotein I Domain I autoantibodies in the diagnosis of antiphospholipid syndrome. *Clinica chimica acta; international journal of clinical chemistry* 2014; **431**: 174-8.
20. De Craemer AS, Musial J, Devreese KM. Role of anti-domain 1-beta2 glycoprotein I antibodies in the diagnosis and risk stratification of antiphospholipid syndrome. *Journal of thrombosis and haemostasis* 2016; **14**(9): 1779-87.
21. Marchetti T, de Moerloose P, Gris JC. Antiphospholipid antibodies and the risk of severe and non-severe pre-eclampsia: the NOHA case-control study. *Journal of Thrombosis and Haemostasis* 2016; **14**(4): 675-84.
22. Iwaniec T, Kaczor MP, Celinska-Lowenhoff M, Polanski S, Musial J. Clinical significance of anti-domain 1 beta2-glycoprotein I antibodies in antiphospholipid syndrome. *Thrombosis Research* 2017; **153**: 90-4.
23. Pelkmans L, Kelchtermans H, de Groot PG, et al. Variability in exposure of epitope G40-R43 of domain i in commercial anti-beta2-glycoprotein I IgG ELISAs. *PloS one* 2013; **8**(8): e71402.
24. Dienava-Verdoold I, Boon-Spijker MG, de Groot PG, et al. Patient-derived monoclonal antibodies directed towards beta(2) glycoprotein-1 display lupus anticoagulant activity. *Journal of Thrombosis and Haemostasis* 2011; **9**(4): 738-47.
25. Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *Journal of thrombosis and haemostasis* 2006; **4**(2): 295-306.
26. Galli M, Barbui T, Comfurius P, et al. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. *The Lancet* 1990; **335**(8705): 1544-7.
27. Despierres L, Beziane A, Kaplanski G, et al. Contribution of anti-β2glycoprotein I IgA antibodies to the diagnosis of anti-phospholipid syndrome: potential interest of target domains to discriminate thrombotic and non-thrombotic patients. *Rheumatology* 2014; **53**(7): 1215-8.

28. Carmo-Pereira S, Bertolaccini M, Escudero-Contreras A, Khamashta M, Hughes G. Value of IgA anticardiolipin and anti-β2-glycoprotein I antibody testing in patients with pregnancy morbidity. *Annals of the Rheumatic Diseases* 2003; **62**(6): 540-3.
29. Lakos G, Kiss E, Regeczy N, et al. Isotype distribution and clinical relevance of anti-β2-glycoprotein I (β2-GPI) antibodies: importance of IgA isotype. *Clinical and experimental immunology* 1999; **117**(3): 574-9.
30. Mattia E, Ruffatti A, Tonello M, et al. IgA anticardiolipin and IgA anti-β2 glycoprotein I antibody positivity determined by fluorescence enzyme immunoassay in primary antiphospholipid syndrome. *Clinical Chemistry and Laboratory Medicine* 2014; **52**(9): 1329-33.
31. Mehrani T, Petri M. Association of IgA anti-ß2 glycoprotein i with clinical and laboratory manifestations of systemic lupus erythematosus. *The Journal of rheumatology* 2011; **38**(1): 64-8.
32. Ruiz-García R, Serrano M, Ángel Martínez-Flores J, et al. Isolated IgA anti-β2 glycoprotein I antibodies in patients with clinical criteria for antiphospholipid syndrome. *Journal of immunology research* 2014; **2014**: 704395.
33. Samarkos M, Davies K, Gordon C, Loizou S. Clinical significance of IgA anticardiolipin and anti-β 2-GP1 antibodies in patients with systemic lupus erythematosus and primary antiphospholipid syndrome. *Clinical Rheumatology* 2006; **25**(2): 199-204.
34. Shen YM, Lee R, Frenkel E, Sarode R. IgA antiphospholipid antibodies are an independent risk factor for thromboses. *Lupus* 2008; **17**(11): 996-1003.
35. Sweiss NJ, Bo R, Kapadia R, et al. IgA anti-β2-glycoprotein I autoantibodies are associated with an increased risk of thromboembolic events in patients with systemic lupus erythematosus. *PloS one* 2010; **5**(8): e12280.
36. Tortosa C, Cabrera-Marante O, Serrano M, et al. Incidence of thromboembolic events in asymptomatic carriers of IgA anti ss2 glycoprotein-I antibodies. *PloS one* 2017; **12**(7): e0178889.
37. Murthy V, Willis R, Romay-Penabad Z, et al. Value of isolated IgA anti-beta2 -glycoprotein I positivity in the diagnosis of the antiphospholipid syndrome. *Arthritis & Rheumatology* 2013; **65**(12): 3186-93.
38. Meijide H, Sciascia S, Sanna G, Khamashta MA, Bertolaccini ML. The clinical relevance of IgA anticardiolipin and IgA anti-beta2 glycoprotein I antiphospholipid antibodies: a systematic review. *Autoimmunity reviews* 2013; **12**(3): 421-5.
39. Bertolaccini M, Amengual O, Atsumi T, et al. 'Non-criteria' aPL tests: report of a task force and preconference workshop at the 13th International Congress on Antiphospholipid Antibodies, Galveston, TX, USA, April 2010. *Lupus* 2011; **20**: 191-205.
40. Favaloro EJ, Bonar R, Marsden K. Internal Quality Control and External Quality Assurance in Testing for Antiphospholipid Antibodies: Part II-Lupus Anticoagulant. *Seminars in thrombosis and hemostasis* 2012; **38**(4): 404-11.
41. Favaloro EJ, Silvestrini R, Mohammed A. Clinical utility of anticardiolipin antibody assays: high inter-laboratory variation and limited consensus by participants of external quality assurance programs signals a cautious approach. *Pathology* 1999; **31**(2): 142-7.
42. Greco T, Amos MD, Conti-Kelly A, Naranjo J, Ijdo J. Testing for the antiphospholipid syndrome: importance of IgA anti-beta 2-glycoprotein I. *Lupus* 2000; **9**(1): 33-41.

43. Bertolaccini ML, Amengual O, Andreoli L, et al. 14th International Congress on Antiphospholipid Antibodies Task Force. Report on antiphospholipid syndrome laboratory diagnostics and trends. *Autoimmunity reviews* 2014; **13**(9): 917-30.
44. Ruffatti A, Tonello M, Visentin MS, et al. Risk factors for pregnancy failure in patients with anti-phospholipid syndrome treated with conventional therapies: a multicentre, case-control study. *Rheumatology (Oxford)* 2011; **50**(9): 1684-9.
45. Pengo V, Ruffatti A, Legnani C, et al. Clinical course of high-risk patients diagnosed with antiphospholipid syndrome. *Journal of thrombosis and haemostasis* 2010; **8**(2): 237-42.
46. Pengo V, Ruffatti A, Legnani C, et al. Incidence of a first thromboembolic event in asymptomatic carriers of high-risk antiphospholipid antibody profile: a multicenter prospective study. *Blood* 2011; **118**(17): 4714-8.
47. Lazzaroni M-G, Fredi M, Andreoli L, et al. Triple antiphospholipid (aPL) antibodies positivity is associated with pregnancy complications in aPL carriers: a multicentre study on 62 pregnancies. *Front Immunol* 2019; **10**: 1948.
48. Urbanus RT, Siegerink B, Roest M, Rosendaal FR, de Groot PG, Algra A. Antiphospholipid antibodies and risk of myocardial infarction and ischaemic stroke in young women in the RATIO study: a case-control study. *The Lancet Neurology* 2009; **8**(11): 998-1005.
49. Gebhart J, Posch F, Koder S, et al. Increased mortality in patients with the lupus anticoagulant: the Vienna Lupus Anticoagulant and Thrombosis Study (LATS). *Blood, The Journal of the American Society of Hematology* 2015; **125**(22): 3477-83.
50. de Mast Q, Molhoek JE, van der Ven AJ, et al. Antiphospholipid Antibodies and the Risk of Stroke in Urban and Rural Tanzania: A Community-Based Case-Control Study. *Stroke* 2016; **47**(10): 2589-95.
51. Lockshin MD, Kim M, Laskin CA, et al. Prediction of adverse pregnancy outcome by the presence of lupus anticoagulant, but not anticardiolipin antibody, in patients with antiphospholipid antibodies. *Arthritis & Rheumatism* 2012; **64**(7): 2311-8.
52. Ruffatti A, Del Ross T, Ciprian M, et al. Risk factors for a first thrombotic event in antiphospholipid antibody carriers: a prospective multicentre follow-up study. *Annals of the Rheumatic Diseases* 2011; **70**(6): 1083-6.
53. Monica Galli DL, Guido Bertolini, and Tiziano Barbui. Lupus anticoagulants are stronger risk factors for thrombosis than anticardiolipin antibodies in the antiphospholipid syndrome: a systematic review of the literature. *Blood* 2003; **101**: 1827-32.
54. Reynaud Q, Lega J-C, Mismetti P, et al. Risk of venous and arterial thrombosis according to type of antiphospholipid antibodies in adults without systemic lupus erythematosus: a systematic review and meta-analysis. *Autoimmunity reviews* 2014; **13**(6): 595-608.
55. Gebhart J, Posch F, Koder S, et al. Increased mortality in patients with the lupus anticoagulant: the Vienna Lupus Anticoagulant and Thrombosis Study (LATS). *Blood* 2015; **125**(22): 3477-83.
56. Pengo V, Testa S, Martinelli I, et al. Incidence of a first thromboembolic event in carriers of isolated lupus anticoagulant. *Thrombosis research* 2015; **135**(1): 46-9.
57. Mustonen P, Lehtonen K, Javela K, Puurunen M. Persistent antiphospholipid antibody (aPL) in asymptomatic carriers as a risk factor for future thrombotic events: a nationwide prospective study. *Lupus* 2014; **23**(14): 1468-76.

58. Chayoua W, Kelchtermans H, Moore GW, et al. Detection of Anti-Cardiolipin and Anti-beta2glycoprotein I Antibodies Differs between Platforms without Influence on Association with Clinical Symptoms. *Thrombosis and Haemostasis* 2019; **119**(05): 797-806.
59. Chayoua W, Kelchtermans H, Moore GW, et al. Identification of high thrombotic risk triple-positive antiphospholipid syndrome patients is dependent on anti-cardiolipin and anti-beta2glycoprotein I antibody detection assays. *Journal of thrombosis and haemostasis* 2018; **16**(10): 2016-23.
60. Pengo V, Banzato A, Denas G, et al. Correct laboratory approach to APS diagnosis and monitoring. *Autoimmunity reviews* 2013; **12**(8): 832-4.
61. Sakai Y, Atsumi T, Ieko M, et al. The effects of phosphatidylserine-dependent antiprothrombin antibody on thrombin generation. *Arthritis & Rheumatology* 2009; **60**(8): 2457-67.
62. Litvinova E, Darnige L, Kirilovsky A, Burnel Y, de Luna G, Dragon-Durey M-A. Prevalence and significance of non-conventional antiphospholipid antibodies in patients with clinical APS criteria. *Front Immunol* 2018; **9**: 2971.
63. Chinnaraj M, Planer W, Pengo V, Pozzi N. Discovery and characterization of 2 novel subpopulations of aPS/PT antibodies in patients at high risk of thrombosis. *Blood Advances* 2019; **3**(11): 1738-49.
64. Pengo V, Del Ross T, Ruffatti A, et al. Lupus anticoagulant identifies two distinct groups of patients with different antibody patterns. *Thrombosis Research* 2018; **172**: 172-8.
65. Radin M, Foddai SG, Cecchi I, et al. Antiphosphatidylserine/Prothrombin Antibodies: An Update on Their Association with Clinical Manifestations of Antiphospholipid Syndrome. *Thrombosis and Haemostasis* 2020; **120**(4): 592-8.
66. Rampazzo P, Biasiolo A, Garin J, et al. Some patients with antiphospholipid syndrome express hitherto undescribed antibodies to cardiolipin-binding proteins. *Thrombosis and Haemostasis* 2001; **85**(01): 57-62.
67. Yin D, de Laat B. Commentary on: Circulating plasmablasts contribute to antiphospholipid antibody production, associated with type I interferon upregulation. *Journal of thrombosis and haemostasis* 2019; **17**(7): 1030-2.
68. de Laat B, Eckmann CM, van Schagen M, Meijer AB, Mertens K, van Mourik JA. Correlation between the potency of a beta2-glycoprotein I-dependent lupus anticoagulant and the level of resistance to activated protein C. *Blood coagulation & fibrinolysis* 2008; **19**(8): 757-64.
69. de Laat B, Wu XX, van Lummel M, Derksen R, de Groot PG, Rand JH. Correlation between antiphospholipid antibodies that recognize domain I of beta 2-glycoprotein I and a reduction in the anticoagulant activity of annexin A5. *Blood* 2007; **109**(4): 1490-4.
70. Pengo V, Denas G. Diagnostics and treatment of thrombotic antiphospholipid syndrome (APS): A personal perspective. *Thrombosis Research* 2018; **169**: 35-40.
71. Devreese K, Ortel T, Pengo V, de Laat B. Laboratory criteria for antiphospholipid syndrome: reply. *Journal of thrombosis and haemostasis* 2018; **16**: 2117-9.
72. de Groot PG, de Laat B, Rand J. et al. Natural proteins involved in antiphospholipid syndrome. *In: Erkan D, Lockshin M. eds*. Antiphospholipid Syndrome. Cham: Springer; 2017: 15-17..

73. Schouwers SM, Delanghe JR, Devreese KM. Lupus Anticoagulant (LAC) testing in patients with inflammatory status: Does C-reactive protein interfere with LAC test results? *Thrombosis Research* 2010; **125**(1): 102-4.
74. Devreese KMJ, Verfaillie CJ, De Bisschop F, Delanghe JR. Interference of C-reactive protein with clotting times. *Clinical Chemistry and Laboratory Medicine* 2015; **53**(5): 6141-5.
75. Devreese K. Standardization of antiphospholipid antibody assays.Where do we stand? *Lupus* 2012; **21**: 718-21.
76. Devreese KM. Antiphospholipid antibody testing and standardization. *International journal of laboratory hematology* 2014; **36**(3): 352-63.
77. Pengo V, Tripodi A, Reber G, et al. Update of the guidelines for lupus anticoagulant detection. *Journal of Thrombosis and haemostasis* 2009; **7**(10): 1737-40.
78. Pengo V, Ruffatti A, Legnani C, et al. Incidence of a first thromboembolic event in asymptomatic carriers of high-risk antiphospholipid antibody profile: a multicenter prospective study. *Blood, The Journal of the American Society of Hematology* 2011; **118**(17): 4714-8.
79. Pengo V, Ruffatti A, Legnani C, et al. Clinical course of high-risk patients diagnosed with antiphospholipid syndrome. *Journal of Thrombosis and Haemostasis* 2010; **8**(2): 237-42.

Chapter 9

Nederlandse samenvatting

Nederlandse samenvatting

Het antifosfolipidensyndroom (APS) wordt gediagnosticeerd wanneer een patiënt lijdt aan een trombose of zwangerschapsmorbiditeit met de persistente aanwezigheid antifosfolipidenantilichamen (aFLs) [1]. Aangezien vasculaire trombose en zwangerschapsgerelateerde morbiditeit vaak voorkomen in de algemene bevolking en niet altijd gerelateerd zijn aan APS, hangt de diagnose van APS voornamelijk af van de laboratoriummethode die gebruikt wordt voor de bepaling van aFLs. Doordat patiënten met APS vaak een intensievere antistollingstherapie nodig hebben, rust er een zware last op de testen die worden gebruikt om de aanwezigheid van circulerende aFLs te detecteren.

Laboratoriumdiagnose van APS: de huidige problemen

De huidige herziene laboratoriumcriteria voor APS-classificatie bestaan uit een combinatie van verschillende laboratoriumtesten om aFLs te detecteren. Deze laboratoriumcriteria omvatten één functionele stollingstest die bekend staat als lupus anticoagulans (LAC), en twee immunologische testen die immunoglobuline (Ig) G en/of IgM meten met bèta2-glycoproteïne I (antiβ2GPI) als antigeen, de anticardiolipin antistofstest en de anti- β2GPI antistof test. Om vals-positieve testen door infecties te voorkomen, moeten positieve testen worden herhaald met een interval van ten minste 12 weken [1].

Momenteel vertoont de detectie van aFLs grote verschillen tussen de verschillende assays en tussen de laboratoria[2,3]. Er zijn namelijk problemen bij de standaardisatie van deze testen, wat de laboratoriumdiagnose van APS moeilijker maakt. De huidige criteria voor de detectie van aFLs betreft een heterogene populatie antilichamen. Het is nog niet bekend welke eiwitten en factoren bijdragen aan de productie van aFLs en hoe deze antilichamen trombose en zwangerschapsmorbiditeit kunnen veroorzaken. Daarom moet er op verschillende manieren worden onderzocht of een nieuwe aFLs-assay mogelijk nuttig kan zijn bij de diagnose van APS: ten eerste moet men aantonen dat de antilichamen pathogeen zijn in APS-diermodellen, ten tweede moeten de antilichamen een sterke associatie vertonen met de klinische manifestaties van APS (trombose of zwangerschapscomplicaties). Als dit voldaan is, dan kan het gebruik van deze testen niet alleen de diagnose van APS verbeteren, maar zou het ook kunnen helpen bij de risicostratificatie van de patiënten.

Is er een toegevoegde waarde van het detecteren van anti-domein I van β2GPI IgG bovenop de huidige APS laboratoriumcriteria?

De antilichamen gericht tegen het eerste domein (DI) van β2GPI blijken pathogeen te zijn in een diermodel[4,5]. DI bevat een belangrijk pathogeen epitoop (G40-R43) dat cryptisch is en alleen

wordt blootgesteld wanneer β2GPI zich in zijn open conformatie bevindt [6-8]. Er is een hoge variatie aan blootstelling van dit epitoop in de anti-β2GPI IgG-assays, waardoor er DI-reactieve stalen gemist worden in deze assays [3].

Klinische rol van antiDI-assays

Recent zijn er testen ontwikkeld die specifiek antiDI-antilichamen detecteren. Echter, er is nog geen consensus bereikt over de toegevoegde waarde van antiDI-antilichamen in de classificatiecriteria van APS (**hoofdstuk 2**). Sommige onderzoeken toonden een hogere correlatie aan met trombose, terwijl andere geen toegevoegde waarde van antiDI-assays konden bewijzen. Van alle testen die antiDI IgG detecteren, was het vooral de tweestaps antiDI-ELISA die aanzienlijk hogere Odd Ratio's (OR's) voor antiDI IgG vertoonde dan de antilichamen die gericht zijn op andere domeinen van het eiwit. De chemiluminescentie-immunoassay (CLIA) is momenteel de meest gebruikte methode om antiDI IgG antilichamen te detecteren. AntiDI IgG gemeten door CLIA blijken te correleren met de klinische manifestaties van APS, maar de toegevoegde klinische waarde is echter niet consistent. Sommige studies konden wel de toegevoegde waarde van antiDI aantonen, terwijl andere studies niet. Geen van de overige drie assays, namelijk de directe antiDI ELISA, de competitieve inhibitie ELISA en de commercieel ontwikkelde INOVA antiDI ELISA, kon een toegevoegde waarde van antiDI IgG aantonen in vergelijking met de andere antiβ2GPI IgG antilichamen. De inconsistente resultaten kunnen te wijten zijn aan verschillen in niet-assay gerelateerde factoren (zoals verschillende onderzoekspopulaties en, behandelingsprotocollen), maar ook aan factoren die verband houden met de methodologie om antiDI, antiCL en anti-β2GPI antilichamen te detecteren.

Detectie van antiDI IgG door CLIA

Op basis van de literatuurresultaten hebben we data van een multicenter studie gebruikt om te onderzoeken of er een meerwaarde is van het meten van antiDI-antilichamen met de CLIA aanvullend op de huidige APS-classificatiecriteria. Dit werd getest bij een grote cohort van APS- en niet-APS-patiënten, waarbij er dan ook gekeken werd naar antiCL en antiβ2GPI die gemeten werden met vier verschillende commercieel beschikbare assays (**hoofdstuk 3**). In deze multicenterstudie vonden we dat de detectie van antiDI IgG door CLIA minder gevoelig, maar wel specifieker was in vergelijking met de huidige laboratoriumcriteria aFL-tests. Dit resulteerde in een hogere OR voor trombose en zwangerschapsmorbiditeit. Bovendien zijn anti-DI IgG voornamelijk aanwezig bij triple-positieve patiënten met een hoog risico en die ook hogere concentraties aFL's vertonen. Bovendien geeft de combinatie van antiDI antilichamen met een drievoudige positiviteit een hoger risico op klinische symptomen dan alleen drievoudige positiviteit. Echter, in tegenstelling tot onze verwachting, verbeterde de toevoeging van antiDI of de vervanging van antiβ2GPI IgG door antiDI, de OR's voor trombose of zwangerschapsmorbiditeit nauwelijks. Daarom kon onze studie geen toegevoegde waarde aantonen van het meten van antiDI IgG bovenop de huidige laboratoriumcriteria. Dit bleek ook onafhankelijk te zijn van type assay dat gebruikt werd om antiCL en antiβ2GPI antilichamen te meten. Een mogelijke verklaring hiervoor kan zijn dat de CLIA assay een verminderde blootstelling heeft van het pathogene DI-epitoop, wat mogelijk implicaties geeft voor de juiste patiënt classificatie [3].

In **hoofdstuk 1** hebben we het belang aangetoond van het gebruik van een hydrofiel oppervlak om β2GPI op te coaten, waardoor de conformationele verandering wordt geïnduceerd die resulteert in de blootstelling van het cryptisch pathogeen epitoop op DI van β2GPI. Zoals hierboven beschreven, blijken de antilichamen die gericht zijn tegen het cryptische epitoop G40-R43 op DI pathogeen te zijn in vivo en vitro studies. De resultaten die met antiβ2GPI-assays worden verkregen, zijn dus afhankelijk van de conformatie van het gecoate β2GPI (d.w.z. de blootstelling van het epitoop G40-R43). Dit wordt dus beïnvloed door het type oppervlak dat gebruikt wordt om β2GPI te immobiliseren. Aangezien in antiDI-assays DI wordt gecoat in plaats van het volledige β2GPI eiwit, werden vergelijkbare problemen met deze assay niet verwacht. Echter, dient er ook rekening te worden gehouden met de positieve lading van het epitoop G40-R43. Hierdoor kan de lading van het coatingoppervlak mogelijk ook de beschikbaarheid van het G40-R43 epitoop beïnvloeden. Er wordt namelijk verondersteld dat het gebruik van een neutraal coatingsoppervlak resulteert in een willekeurige oriëntatie van DI, en dus ook in de blootstelling van epitoop G40-R43. Maar wanneer men een negatief oppervlak gebruikt, dan gaat het positieve epitoop daarop binden, waardoor het niet beschikbaar is voor de antilichamen. Bij de CLIA-assay kan een mogelijke lading van de gebruikte beads niet worden uitgesloten, waardoor de blootstelling van het epitoop G40-R43 onzeker is. Om dit na te gaan hebben we twee monoklonale antilichamen (P1-117 en P2-6) gebruikt in de CLIA. Antilichaam P1-117, dat epitoop G40-R43 herkent, kan alleen binden op DI wanneer β2GPI zich in zijn open conformatie bevindt, terwijl P2-6 DI herkent ongeacht de conformatie van β2GPI [9]. Onze data toonden aan dat P2-6 kon worden gedetecteerd, terwijl er geen signaal werd verkregen voor P1-117 in deze antiDI CLIA. In tegenstelling tot de verwachting, is het epitoop G40-R43 in deze antiDI-assay niet beschikbaar, waardoor patiëntenstalen met antilichamen die het epitoop G40-R43 herkennen dus worden gemist. De commercieel verkrijgbare antiDI CLIA detecteert dus antilichamen tegen DI, met uitzondering van de meer specifieke antiDI-antilichaampopulatie gericht tegen het epitoop G40-R43. Bovendien kwamen de resultaten van anti-β2GPI en antiDI gemeten op hetzelfde platform met elkaar overeen, wat suggereert dat beide testen bijna dezelfde antilichaampopulatie meten die dus gericht zijn tegen DI, maar niet tegen het G40-R43-epitoop.

Detectie van antiDI IgG door een hydrofobe ELISA

Afgezien van de antiDI CLIA-assay, zijn er andere methoden beschikbaar om antiDI-antilichamen te detecteren. Al in 2005 gaf een in-huis ELISA aan dat het testen van antiDI IgG de identificatie mogelijk maakt van APS patiënten met het hoogste risico op het ontwikkelen van trombose of zwangerschapsmorbiditeit [10,11]. Hierbij werd nogmaals bevestigd dat de juiste blootstelling van het G40-R43-epitoop van uitermate belang is [11]. In deze ELISA word DI zowel op een hydrofiele ELISA-plaat, als een hydrofobe ELISA-plaat gecoat, wat resulteert in een verschil in blootstelling van het pathogene DI-epitoop.

In **hoofdstuk 4** hebben we deze in-huis test vergeleken met de commerciële antiDI CLIA in een groot cohort van APS- en niet-APS-patiënten. Wanneer DI werd gecoat op een hydrofiele plaat, werden er geen antilichamen gedetecteerd in alle stalen. Echter, wanneer DI op een hydrofobe plaat werd gecoat, konden de antilichamen wel worden gedetecteerd en waren de waarden van de optische dichtheid (OD) van de APS-stalen significant hoger dan die van de controlegroepen. Daarnaast hebben we vastgesteld dat 18 van de 66 stalen met trombose negatief waren met de

antiDI CLIA, maar positief met de in-huis antiDI hydrofobe ELISA. Dit resulteerde in een hogere gevoeligheid van de in-huis antiDI hydrofobe ELISA in vergelijking met de commerciële antiDI CLIA. Bovendien detecteerde de in-huis antiDI-hydrofobe ELISA meer stalen met klinische manifestaties van APS in vergelijking met de antiDI IgG-CLIA.

Samengevat suggereren deze resultaten dat de antiDI hydrofobe ELISA een meer specifieke antiDI-antilichaampopulatie kan meten die gericht is tegen G40-R43, vergeleken met de commercieel verkrijgbare antiDI CLIA die alle antilichamen tegen DI detecteert, met uitzondering van antilichamen gericht tegen het epitoop G40-R43. Onze gegevens benadrukken het belang van het meten van antistoffen gericht tegen het cryptische epitoop G40-R43, niet alleen in de antiβ2GPI-assays, maar ook in de antiDI-assays om er zeker van te zijn dat in ieder geval deze pathogene antilichaampopulatie niet wordt gemist.

Kortom, net als bij de antiβ2GPI-assay, is standaardisatie van antiDI-assays van het grootste belang. Deze standaardisatie omvat de bevestiging van een bevredigende blootstelling van het G40-R43 epitoop om er zeker van te zijn dat ten minste één specifieke pathogene antilichaampopulatie wordt gemeten. Hierbij is de lading van het oppervlak dat gebruikt wordt om DI te immobiliseren van belang, aangezien het de blootstelling van het G40-R43 epitoop kan beïnvloeden. Het is dus interessant om de blootstelling van dit epitoop in alle beschikbare antiDI-assays te verifiëren, wat ook de standaardisatie van antiDI-assays kan verbeteren.

Is er een meerwaarde om de IgA-isotype antiCL-antilichamen en antiβ2GPI-antilichamen in APS te detecteren?

De huidige criteria zeggen dat men IgG en IgM antiCL en antiβ2GPI moet detecteren om de diagnose van APS te bevestigen, en zijn de IgA antilichamen niet opgenomen in de huidige classificatiecriteria. In **hoofdstuk 6** onderzochten we of er een toegevoegde waarde is van het meten van antiCL en antiβ2GPI IgA antilichamen naast het huidige aFLs paneel bestaande uit LAC, antiCL en antiβ2GPI IgG/M. We vonden dat positiviteit voor antiCL en/of antiβ2GPI IgA antilichamen inderdaad geassocieerd was met trombose en zwangerschapsmorbiditeit. Echter, een geïsoleerde IgA positiviteit was zeldzaam en correleerde niet met klinische manifestaties van APS. Onze resultaten ondersteunen dus niet de meting van antiCL- en/of antiβ2GPI-IgA naast de conventionele aFLs bij het identificeren van patiënten met klinische manifestaties van APS.

Is een geïsoleerde LAC in afwezigheid van antiCL- en anti-β2GPI-antilichamen klinisch relevant?

LAC detecteert een functioneel effect van een heterogene groep aFLs antilichamen. De klinische relevantie van een geïsoleerde LAC, in afwezigheid van antiCL en antiβ2GPI IgG en IgM, is discutabel. Daarom hebben we in **hoofdstuk 7** gekeken naar de correlatie van een geïsoleerde aanwezigheid van LAC met trombose, en dit vergeleken met triple positiviteit in een cohort van

APS- en niet-APS-patiënten. We ontdekten dat een geïsoleerde LAC een zwakkere LAC-activiteit vertoonde in vergelijking met triple positieve patiënten. Patiënten met een geïsoleerde LAC hebben echter een vergelijkbaar of zelfs hoger risico op trombose in vergelijking met patiënten met triple positiviteit. De aanwezigheid van anti-PS/PT-antilichamen kon de LAC-positiviteit niet verklaren in alle stalen met geïsoleerde LAC. Aangezien de stalen negatief waren voor antilichamen tegen β2GPI en antiPS/PT zijn verdere studies nodig om het antigeen te identificeren dat verantwoordelijk is voor een geïsoleerde LAC.

Conclusie

De klinische symptomen van APS komen vaak voor, ongeacht het syndroom, waardoor de diagnose van APS moeilijk is en voornamelijk afhankelijk is van de laboratoriumcriteria. Momenteel is de laboratoriumdiagnose van APS nog steeds een uitdaging vanwege het gebrek aan standaardisatie en de heterogeniteit van aFLs-antilichamen. We toonden aan dat het belangrijk is om de juiste blootstelling van het cryptische epitoop in alle beschikbare antiDI-assays te verifiëren om de standaardisatie van de antiDI-assays te verbeteren. Bovendien was een geïsoleerde IgA zeldzaam en correleerde het niet met de klinische manifestaties van APS. Patiënten met een geïsoleerde LAC hebben echter een vergelijkbaar of zelfs hoger risico op trombose in vergelijking met patiënten met triple positiviteit. Verdere studies zijn nodig om de implicaties van onze bevindingen uit te werken.

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). *Journal of thrombosis and haemostasis* 2006; **4**(2): 295-306.

2. Favaloro EJ, Wheatland L, Jovanovich S, Roberts-Thomson P, Wong RC. Internal quality control and external quality assurance in testing for antiphospholipid antibodies: Part I—Anticardiolipin and anti-β2-glycoprotein I antibodies. *Seminars in thrombosis and hemostasis* 2012; 38(04): 390-403.

3. Pelkmans L, Kelchtermans H, de Groot PG, et al. Variability in exposure of epitope G40-R43 of domain i in commercial anti-beta2-glycoprotein I IgG ELISAs. *PloS one* 2013; **8**(8): e71402.

4. Chiara Agostinis, Paolo Durigutto, Daniele Sblattero, et al. A non-complement-fixing antibody to beta 2 glycoprotein I as a novel therapy for antiphospholipid syndrome. *Blood* 2014; **123**(22): 3478-87.

5. Pericleous C, Ruiz-Limon P, Romay-Penabad Z, et al. Proof-of-concept study demonstrating the pathogenicity of affinity-purified IgG antibodies directed to domain I of beta2-glycoprotein I in a mouse model of anti-phospholipid antibody-induced thrombosis. *Rheumatology (Oxford)* 2015; **54**(4): 722-7.

6. de Laat B, Derksen R, van Lummel M, Pennings MTT, de Groot PG. Pathogenic anti-beta(2)-glycoprotein I antibodies recognize domain I of beta(2)-glycoprotein I only after a conformational change. *Blood* 2006; **107**(5): 1916-24.

7. Çetin. A, van Os GMA, Morgelin M, et al. beta(2)-Glycoprotein I can exist in 2 conformations: implications for our understanding of the antiphospholipid syndrome. *Blood* 2010; **116**(8): 1336-43.

8. de Laat B, van Berkel M, Urbanus RT, et al. Immune responses against domain I of beta(2)-glycoprotein I are driven by conformational changes: domain I of beta(2)-glycoprotein I harbors a cryptic immunogenic epitope. *Arthritis & Rheumatology* 2011; **63**(12): 3960-8.

9. Dienava-Verdoold I, Boon-Spijker MG, de Groot PG, et al. Patient-derived monoclonal antibodies directed towards beta(2) glycoprotein-1 display lupus anticoagulant activity. *Journal of Thrombosis and Haemostasis* 2011; **9**(4): 738-47.

10. 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**(4): 1540-5.

11. de Laat B, Pengo V, Pabinger I, et al. The association between circulating antibodies against domain I of beta2-glycoprotein I and thrombosis: an international multicenter study. *Journal of thrombosis and haemostasis* 2009; **7**(11): 1767-73.

Appendices

Appendix Ⅰ

中文总结

中文总结

当患者患有血栓形成或妊娠并发症并伴有持续性抗磷脂抗体（aPLs）时，可诊断为抗磷脂综合征（APS）[1]。因为血管血栓形成和与妊娠相关的疾病在一般人群中也很普遍，并不总是跟 APS 相关，诊断 APS 主要取决于实验室检测 aPLs。由于 APS 患者通常需要加强的抗凝治疗，因此检测循环 aPLs 负担沉重。

APS 的实验室诊断：当前的问题

当前修订的 APS 分类实验室标准要求结合不同的实验室方法来检测 aPLs。这些实验室标准包括一种名称为狼疮抗凝剂（LAC）的功能性凝血检测以及两种免疫学检测。免疫学检测分别以心磷脂（CL）和 β2-糖蛋白 I (β2GPI) 作为抗原来检测免疫球蛋白 (Ig) G 和/或 IgM，命名为抗心磷脂 (antiCL) 抗体检测和抗 β2GPI 抗体检测。为避免与感染相关的假阳性测试，应至少间隔 12 周重复进行阳性测试 [1]。

目前，aPLs 的检测结果在不同的检测方法和实验室之间显示较大的差异 [2,3]。这些检测方法的标准化存在问题，使 APS 的实验室诊断变得更加困难。当前检测 aPLs 的标准涉及抗磷脂抗体的异质群体。目前尚不清楚哪些蛋白质和蛋白因子有助于 aPLs 的产生，以及这些抗体是如何诱导血栓形成和妊娠发病的。 因此，以多种方式研究新的 aPLs 检测方法是否可能对 APS 的诊断有用是有必要的：首先，必须证明抗体在 APS 动物模型中具有致病性，其次，抗体必须与 APS 的临床表现（血栓形成或妊娠并发症）有很强相关性。如果满足这几点，使用这些测试不仅可以改善 APS 的诊断，还可以帮助对患者进行风险分层。

在当前 APS 实验室标准之上，检测抗 β2GPI 结构域 I（DI）IgG 抗体是否有附加价值？

针对 β2GPI 第一个结构域 (DI) 的抗体在动物模型中呈现致病性 [4,5]。 DI 包含一个主要的致病表位 (G40-R43)，该表位是隐蔽的，仅当 β2GPI 处于其开放构象时才会暴露 [6-8]。 在抗 β2GPI IgG 检测方法中，该表位是否暴露存在很大差异，导致有些抗 β2GPI IgG 检测方法会漏掉对 DI 有反应的样本 [3]。

抗 DI 抗体检测方法的临床应用

最近，已经开发出用于检测抗 DI 抗体的检测方法。然而，APS 分类标准中抗 DI 抗体是否有附加价值尚未达成共识（**第 2 章**）。一些研究表明检测到的抗 DI 抗体与血栓形成有更高的相关性，而其他的研究则未能证明检测到的抗 DI 抗体相对于已有的标准检测方法有附加价值。在检测抗 DI IgG 的所有方法中，特别是用两步抗 DI ELISA 检测抗 DI IgG 与血栓形成的比值比(OR)显着高于靶向 β2GPI 其他结构域的抗体 [9,10]。化学发光免疫分析（CLIA）是目前使用最广泛的检测抗 DI IgG 抗体的方法。通过 CLIA 测量的抗 DI IgG 与 APS 的临床表现相关，但是否有附加的临床价值研究结果并不一致：一些研究能够证明 antiDI 的附加价值，而另一些研究则显示没有附加价值。其他三种检测方法，即直接抗 DI ELISA、竞争性抑制 ELISA 和商业开发的 INOVA 抗 DI ELISA，均不能证明抗 DI IgG 比其他抗 β2GPI IgG 抗体

有更高的价值。这些不一致的结果可能是由于非检测相关因素的差异引起（例如不同的研究人群和样本处理程序），也有可能是由于与抗 DI、抗 CL 和抗 β2GPI 抗体的检测方法相关的因素引起。

通过 CLIA 检测抗 DI IgG 抗体

基于文献结果，我们使用来自多中心的数据来研究在当前的 APS 分类标准以外，采用商业 CLIA 检测抗 DI 抗体是否具有附加的临床价值。检测在 APS 患者和非 APS 对照组患者中进行，通过四种不同的商业平台检测 antiCL 和 antiβ2GPI 抗体（**第 3 章**）。在这项多中心研究中，我们发现与当前的实验室标准 aPLs 检测相比， CLIA 检测抗 DI IgG 的灵敏度较低，但具有更高的特异性，从而导致与血栓形成和妊娠发病率的相关性（OR 值）更高。此外，抗 DI IgG 主要存在于高风险的三重阳性患者中，这些患者也表现出更高的抗 DI IgG 水平。而且，与单独三重阳性相比，抗 DI 抗体阳性联合三重阳性具有更高的发生临床症状的风险。然而，与我们的预期相反，添加抗 DI IgG 或用抗 DI 替代抗 β2GPI IgG 几乎没有改善血栓形成或妊娠发病率的 OR 值。因此，我们的研究无法证明在当前的实验室标准之上，用 CLIA 检测 antiDI IgG 抗体的附加的临床价值，并且此结果与用于检测 antiCL 和 antiβ2GPI 的实验平台无关。对此的可能解释是，用 CLIA 检测方法，致病性 DI 表位的暴露减少了，这可能对正确的患者分类产生影响 [3]。

在**第 1 章**中，我们阐述了使用亲水性表面包覆 β2GPI 的重要性，亲水性表面能诱导 β2GPI 构象变化，使 β2GPI 的 DI 中的隐性病原性表位暴露。如前所述，针对 DI 上隐蔽表位 G40-R43 的抗体在体内和体外研究中显示是致病性的。因此，用抗 β2GPI 检测方法检测 aPLs 获得的结果取决于包被于检测平面的 β2GPI 的构象（即表位 G40-R43 是否充分暴露），这些会受到用于固定 β2GPI 的固相表面类型的影响。由于在抗 DI 检测中是 DI 被包被，而不是整个 β2GPI 蛋白被包被，因此我们期待抗 DI 检测不会出现类似的问题。然而，考虑到表位 G40-R43 是带正电荷的，检测平台涂层表面的电荷也可能影响 G40-R43 表位的暴露。事实上，如果涂层表面是中性的（不带任何电荷），包被 DI 的方向将会是随机的，因此表位 G40-R43 可能会有一个充足的暴露。但是，当使用带有负电荷的表面时，带有阳性电荷的表位 G40-R43 会与其结合（向下），从而使抗体无法与之结合。在 CLIA 检测中，不能排除所用磁珠的电荷，因此表位 G40-R43 的暴露是不确定的。为了验证这一点，我们在 CLIA 中使用了两种单克隆抗体（P1-117 和 P2-6）。P1-117 抗体能识别表位 G40-R43，仅在 β2GPI 处于其开放构象时才能与 DI 结合；而 P2-6 识别 DI，无论 β2GPI 的构象如何 [11]。我们的数据显示在该抗 DI CLIA 中可以检测到 P2-6，而未能检测到抗体 P1-117 抗体 。表位 G40-R43 在该抗 DI CLIA 中不暴露，因此可能漏检具有表位 G40-R43 抗体的患者样本。因此，商业抗 DI CLIA 可以检测针对 DI 的抗体，但是可能漏检针对表位 G40-R43 的更特异的抗 DI 抗体群。此外，在同一平台上测量的抗 β2GPI 和抗 DI 的结果高度一致，这表明这两种分析检测的抗体群几乎相同，是靶向 DI，而不是靶向 G40-R43 表位。

通过疏水 ELISA 检测抗 DI IgG

除了抗 DI CLIA 检测以外，还有其他方法可用于检测抗 DI 抗体。早在 2005 年，一项实验室开发的 ELISA 检测抗 DI IgG 能够识别出具有血栓形成或妊娠发病高风险的患者 [9,10]。这重申了 G40-R43 表位的正确暴露至关重要。在该 ELISA 中，DI 被包被在亲水性 ELISA 板和疏水性 ELISA 板上，导致致病性 DI 表位暴露的差异。 在**第 4 章**中，我们纳入 APS 和非 APS 患者，将此种抗 DI ELISA 检测与商业化抗 DI CLIA 检测进行了比较。当 DI 被包被在亲水板上时，在所有样品中均未检测到任何抗体。然而，当 DI 被包被在疏水板上时，可以检测到

抗体，并且 APS 样品的光密度 (OD) 值显着高于对照组。此外，我们发现 66 份具有血栓形成症状的患者样本中有 18 份用抗 DI CLIA 检测抗体呈阴性，但用此抗 DI 疏水 ELISA 检测抗体呈阳性，与商业抗 DI CLIA 相比，抗 DI 疏水 ELISA 的灵敏度更高，能检测到更多具有 APS 临床表现的样本。 综上所述，这些结果表明，与检测除针对表位 G40-R43 的抗体以外的所有 抗 DI 抗体的商业化抗 DI CLIA 相比，抗 DI 疏水 ELISA 可以测量到更多的针对 G40-R43 的抗 DI 抗体群。我们的数据强调了测量针对隐蔽表位 G40-R43 的抗体的重要性，不仅在抗 β2GPI 测定中，而且在抗 DI 测定中，以确保至少不会遗漏这种致病性抗体群。

总之，与抗 β2GPI 抗体检测一样，抗 DI 抗体检测的标准化至关重要。 这种标准化包括确认 G40-R43 表位的暴露是否令人满意，以确保至少测量到该种特定的致病性抗体群。在抗体检测中，用于包被固定 DI 的固相表面的电荷很重要，因为它会影响 G40-R43 表位的暴露。 因此，在所有可用的抗磷脂抗体检测中验证该表位的暴露是很有趣的，这也可能提高抗磷脂抗体检测的标准化。

检测 APS 中 IgA 同种型抗 CL 抗体和抗 β2GPI 抗体是否有附加的临床价值？

目前的标准必须检测 IgG 和 IgM 抗 CL 和抗 β2GPI 抗体才能确诊 APS，而 IgA 抗体不包括在目前的分类标准中。 在**第 6 章**中，我们研究了除了由 LAC、抗 CL 和抗 β2GPI IgG/M 组成的当前 aPLs 谱之外，检测抗 CL 和抗 β2GPI IgA 抗体是否有附加的临床价值。 我们发现抗 CL 和/或抗 β2GPI IgA 抗体的阳性确实与血栓形成和妊娠发病率有关。 然而，单独的 IgA 阳性很少见，并且与 APS 的临床表现无关。 因此，我们的结果不支持在识别具有 APS 临床表现的患者时，除常规 aPL 外还测量抗 CL 和/或抗 β2GPI IgA。

在没有抗 CL 和抗 β2GPI 抗体的情况下单独的 LAC 是否具有临床相关性？

LAC 检测一组异质性的 aPLs 抗体的功能效应。 在缺乏抗 CL 和抗 β2GPI IgG 和 IgM 的情况下，单独 LAC 阳性的临床相关性是有争议的。 因此，在**第 7 章**中，我们研究了单独存在的 LAC 阳性与血栓形成的相关性，并在包含 APS 和非 APS 患者的队列研究中将其与三重阳性进行了比较。 我们发现，与三重阳性患者相比，孤立的 LAC 表现出较弱的 LAC 活性。 然而，与三重阳性患者相比，单独 LAC 阳性患者的血栓形成风险相当甚至更高。 抗磷脂酰丝氨酸/凝血酶原抗体 (antiPS/PT) 的存在无法解释所有单独 LAC 阳性样品中的 LAC 阳性。由于样品中针对 β2GPI 和凝血酶原的抗体呈阴性，因此需要进一步研究以确定导致单独 LAC 阳性的抗原。

结论

APS 的临床症状血栓形成于妊娠并发症经常与 APS 不相关，这使得 APS 的诊断很困难，主要依赖于实验室标准。目前，由于检测缺乏标准化以及 aPLs 抗体的异质性，APS 的实验室诊断仍然具有挑战性。我们的研究表明，在所有可用的 aPLs 抗体检测中验证隐蔽表位的

正确暴露以提高抗体检测的标准化非常重要。 此外，单独的 IgA 阳性很少见，并且与 APS 的临床表现无关。 然而，与三重阳性患者相比，单独的 LAC 阳性患者的血栓形成风险更高。 需要进一步的研究来阐述我们发现的启示。

参考文献

1. Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *Journal of thrombosis and haemostasis* 2006; **4**(2): 295-306.

2. Favaloro EJ, Wheatland L, Jovanovich S, Roberts-Thomson P, Wong RC. Internal quality control and external quality assurance in testing for antiphospholipid antibodies: Part I—Anticardiolipin and anti-β2-glycoprotein I antibodies. *Seminars in thrombosis and hemostasis* 2012; 38(04): 390-403.

3. Pelkmans L, Kelchtermans H, de Groot PG, et al. Variability in exposure of epitope G40-R43 of domain i in commercial anti-beta2-glycoprotein I IgG ELISAs. *PloS one* 2013; **8**(8): e71402.

4. Chiara Agostinis, Paolo Durigutto, Daniele Sblattero, et al. A non-complement-fixing antibody to beta 2 glycoprotein I as a novel therapy for antiphospholipid syndrome. *Blood* 2014; **123**(22): 3478-87.

5. Pericleous C, Ruiz-Limon P, Romay-Penabad Z, et al. Proof-of-concept study demonstrating the pathogenicity of affinity-purified IgG antibodies directed to domain I of beta2-glycoprotein I in a mouse model of anti-phospholipid antibody-induced thrombosis. *Rheumatology (Oxford)* 2015; **54**(4): 722-7.

6. de Laat B, Derksen R, van Lummel M, Pennings MTT, de Groot PG. Pathogenic anti-beta(2)-glycoprotein I antibodies recognize domain I of beta(2)-glycoprotein I only after a conformational change. *Blood* 2006; **107**(5): 1916-24.

7. Çetin. A, van Os GMA, Morgelin M, et al. beta(2)-Glycoprotein I can exist in 2 conformations: implications for our understanding of the antiphospholipid syndrome. *Blood* 2010; **116**(8): 1336-43.

8. de Laat B, van Berkel M, Urbanus RT, et al. Immune responses against domain I of beta(2)-glycoprotein I are driven by conformational changes: domain I of beta(2)-glycoprotein I harbors a cryptic immunogenic epitope. *Arthritis & Rheumatology* 2011; **63**(12): 3960-8.

9. 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**(4): 1540-5.

10. de Laat B, Pengo V, Pabinger I, et al. The association between circulating antibodies against domain I of beta2-glycoprotein I and thrombosis: an international multicenter study. *Journal of thrombosis and haemostasis* 2009; **7**(11): 1767-73.

11. Dienava-Verdoold I, Boon-Spijker MG, de Groot PG, et al. Patient-derived monoclonal antibodies directed towards beta(2) glycoprotein-1 display lupus anticoagulant activity. *Journal of Thrombosis and Haemostasis* 2011; **9**(4): 738-47.

Appendix II

Impact

Impact

The main purpose of this thesis was to explore the optimization of the laboratory diagnosis of the antiphospholipid syndrome (APS). The detection of antiphospholipid antibodies (aPLs) is problematic due to the lack of standardization. Moreover, we showed the results of a multicenter study in which we investigated a newly developed aPLs-assay that might be a useful tool in the diagnosis and management of APS.

APS: a societal and economic burden

APS patients suffer from a systemic autoimmune disease characterized by recurrent venous or arterial thrombosis and/or pregnancy morbidity most likely caused by persistent aPLs [1]. APS affects approximately 1 on 2000 people[2], and is therefor one of the most common causes of acquired hypercoagulability and miscarriages in people under 50 years of age. The median age of disease onset is 31 years [3]. APLs-related vascular events exert a strong clinical impact in terms of morbidity and mortality. aPLs positivity was observed in patients with deep vein thrombosis, myocardial infarction, stroke and pregnancy morbidity [4]. Other non-criteria clinical manifestations are also frequently reported, as e.g. thrombocytopenia, autoimmune haemolytic anaemia, livedo reticularis, superficial thrombophlebitis, nephropathy, cognitive dysfunction, skin ulcers, epilepsy and cardiac valve dysfunction. Moreover, the catastrophic variant of APS (CAPS) is a serious, life-threatening aPLs-related manifestation characterized by the acute development of extensive thrombosis, leading to failure of three or more organs in less than a week. Despite treatment, mortality rate is still high and ranges between 30% to 50% [5]. Such epidemiological evidence implies that APS diagnosis and management carries an enormous social and economic costs.

Improving the diagnostic laboratory for APS

Laboratory diagnosis of APS: current problems

As the clinical symptoms of APS occur frequently irrespectively of the syndrome, classification of APS predominantly depends on a combination of different laboratory assays measuring the presence or function of aPLs. These laboratory criteria include one functional coagulation assay, known as lupus anticoagulant (LAC), and two immunological assays measuring anti-cardiolipin (antiCL) or anti-beta2-glycoprotein I (antiβ_2GPI) immunoglobulin (Ig) G and/or IgM. Positive tests should be repeated and reconfirmed at least 12 weeks apart [1]. Currently, the detection of aPLs show large inter-platform and -laboratory variation. The heterogeneity of aPLs and the lack of standardization of these assays makes the laboratory diagnosis of APS challenging.

Heterogeneity of aPLs

The antiCL assay detects different types of antibodies: antibodies directed against cardiolipin itself, directed against a complex of cardiolipin and β_2GPI and directed against other cardiolipin-binding proteins [6]. AntiCL antibodies directed against cardiolipin itself are thought to be infection-related and transient [7]. Therefore, antiβ_2GPI assays using immobilized β_2GPI have a better specificity for the laboratory diagnosis of APS. Studies have shown that aPLs that recognize the cryptic epitope G40-R43 on domain I (DI) of β_2GPI have been proven to be pathogenic, while antibodies against other domains of β_2GPI appear to be unrelated to the clinical symptoms of APS.

Detection of antibodies against thrombosis-related DI epitope

Various assays for detecting antiDI antibodies have been developed and are expected to improve the laboratory diagnosis of APS. However, no consensus is reached on whether detecting antiDI antibodies is of added value for the classification of APS (**chapter 2**). Chemiluminescence (CLIA) is currently the most widely used method to detect antiDI IgG antibodies, whose presence is a strong indicator for clinical manifestations of APS. We found that the antiDI CLIA was less sensitive but more specific compared to the laboratory criteria aPLs tests. However, it hardly improved the Odds Ratio for the occurrence of thrombosis or pregnancy morbidity. Therefore, our study demonstrated that measuring antiDI IgG by CLIA was not of added value on top of the current criteria (**Chapter 3**). A possible explanation could be the high variability in exposure of the G40-R43 epitope on DI. This epitope has proven to be cryptic and only exposed when β_2GPI is in its open conformation.

Interestingly, our in-house antiDI ELISA that ensures enough exposure of the cryptic epitope, was able to detect more samples with clinical manifestations of APS, which resulted in a higher sensitivity compared to the antiDI CLIA assay (**Chapter 4**). Taken together, our results suggest that this antiDI ELISA is able of detecting a specific population of pathogenic antiDI antibodies, thereby preventing that the patients are misdiagnosed as false negative.

Standardization of assays for detecting aPLs

Standardization of the assays used to detect aPLs antibodies in APS is of utmost importance. This standardization includes the confirmation of enough exposure of the pathogenic epitope G40-R43 on DI of β_2GPI to ascertain that at least the specific pathogenic antibody population is detected. The charge of the solid phase surface used to immobilize antigen (β_2GPI or DI) can affect the amount of exposure of this epitope. Therefore, verifying the correct exposure of the cryptic epitope in all available aPLs assays will help to improve standardization of aPLs assays and thereby patient classification.

Relevance for patients

The current APS diagnostic procedure may result in misdiagnosis of the syndrome, with major implications regarding the treatment of patients. Current treatments for APS are aimed at attenuating the procoagulant state of the patient and take into account the risk of recurrence of thrombotic events and/or pregnancy morbidities. The current treatment methods are mainly

based on oral anticoagulant therapy. Given the fact that also non-pathogenic antiβ_2GPI antibodies exist, quantitative assays measuring reactivity against the full protein, will result in false positive results. Patients with thrombosis and aPLs antibodies may be given indefinite oral anticoagulant treatment. Falsely diagnosed patients may thus be exposed to a high risk of bleeding, without having any benefit of such treatment. AntiDI assays measuring the reactivity of anitbodies against DI can improve the specificity of APS laboratory diagnosis, thereby reducing the false positive rate.

Current laboratory tests included in the criteria fail to exploit potential pathophysiological processes associated with aPLs antibodies, thereby resulting in false negative results. False-negative results also have serious consequences for patients suspected having APS because they need long-term anticoagulation to prevent recurrence. Our study firstly determined the impact of the variable exposure of the pathogenic DI epitope of β_2GPI in the commercial antiDI assays on the patient classification. Subsequently, we provided preliminary results of our in-house anti-DI hydrophobic ELISA. We demonstrated that our in-house anti-DI ELISA is able to detect the specific anti-DI antibody population against the pathogenic G40-R43 epitope, thereby improving patient diagnosis sensitivity and reducing the false negative rate. In addition, our study proved that at least this specific thrombosis-associated antibody population will not be missed when there is enough exposure of the G40-R43 epitope, thereby improving the standardization of existing aPLs assays based on the binding of aPLs to β_2GPI.

Improving risk stratification of APS patients

The current laboratory criteria include LAC, antiCL IgG and IgM and antiβ_2GPI IgG and IgM. It is sufficient for the diagnosis of APS to have one positive test when the patient is also positive for one of the clinical criteria [1]. However, not every test has the same predictive value and positivity. Therefore, risk stratification can be done by categorizing patients according to the number of positive tests and by the analysis of the aPLs profile. Each aPLs profile confers a characteristic thrombotic risk. When LAC is positive together with antiCL and antiβ_2GPI antibodies (triple positivity), it carries a significant risk for a first thrombotic event [8] and for recurrence of thrombosis [9]. Triple positivity is also an independent risk factor for pregnancy failure [10]. Our study observed that anti-DI IgG antibodies are highly correlated with triple positivity, indicating that anti-DI IgG positivity confirms the patients at higher risk for clinical events related to APS. Moreover, combined DI and triple positivity confirms an even higher risk for both thrombosis and pregnancy morbidity compared to only triple positivity (**Chapter 3**).

The LAC assay measures the functional effect of a heterogeneous group of aPLs antibodies. LAC positivity could be based on antiβ_2GPI antibodies or anti-phosphatidylserine (PS) or prothrombin (PT) antibodies, or possibly even other inhibitors. A positive LAC is considered to be a strong risk factor for thrombosis in APS. We demonstrated that an isolated LAC (in the absence of antiCL and antiβ_2GPI IgG and IgM) was also strongly correlated with a history of thrombosis with an even higher predictive value for thrombosis than triple positivity. The presence of anti-PS/PT antibodies could not explain LAC positivity in isolated LAC. As samples were negative for

antibodies against β_2GPI and PT, further studies are needed to identify other target antigen responsible for isolated LAC activity (**Chapter 7**).

Relevance for patients

Identifying the presence of factors associated with a high risk for thrombotic and/or obstetric events is critical for patient management. Our study demonstrated that a major risk factor is: the presence of LAC, the presence of triple (all three subtypes) aPLs positivity or antiDI IgG positivity. Improving the risk stratification of patients can better define high-risk and low-risk aPLs profiles and better describe the risks associated with different aPLs profiles to improve patient management.

A high-risk aPLs profile not only indicates the first clinical event[8], but also suggests recurrence [9]. Clinical decision may be modified if APS patients have a high-risk aPLs profile. For patients with APS and a first unprovoked venous thrombosis, it is recommended to receive long-term treatment with vitamin K antagonists (VKA) [11]. While for patients with a provoked first venous thrombosis, when patients have a high-risk aPLs profile, longer anticoagulation could be considered to avoid recurrence. Based on the current evidence, treatment with direct oral anticoagulants (DOACs, such as Rivaroxaban) is not recommend in triple aPLs-positive patients with obstetric APS and APS with arterial events, due to the high risk of recurrent events[12,13] . Furthermore, for women with prior obstetric APS, if an individual has a high-risk profile, it is recommended to combine treatment with low dose aspirin heparin during pregnancy.

Conclusion

The results of our study provide evidence for an update of the diagnostic criteria including risk stratification for the diagnosis and management of APS. Hereby is assay standardization of utmost importance. Subsequently, the improved diagnosis and risk stratification should improve the current treatment procedures and reduce APS-related morbidity and mortality. In addition, our findings open the way for investigating new potentially important antigen targets in the pathogenesis of APS.

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). *Journal of thrombosis and haemostasis* 2006; **4**(2): 295-306.
2. Duarte-García A, Pham MM, Crowson CS, et al. The epidemiology of antiphospholipid syndrome: a population-based study. *Arthritis & Rheumatology* 2019; **71**(9): 1545-52.
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 & Rheumatology* 2002; **46**(4): 1019-27.
4. Andreoli L, Chighizola CB, Banzato A, et al. Estimated frequency of antiphospholipid antibodies in patients with pregnancy morbidity, stroke, myocardial infarction, and deep vein thrombosis: a critical review of the literature. *Arthritis care & research* 2013; **65**(11): 1869-73.
5. Nayer A, Ortega LM. Catastrophic antiphospholipid syndrome: a clinical review. *Journal of nephropathology* 2014; **3**(1): 9.
6. Pengo V, Bison E, Denas G, Jose SP, Zoppellaro G, Banzato A. Laboratory Diagnostics of Antiphospholipid Syndrome. *Seminars in thrombosis and hemostasis* 2018; **44**(5): 439-44.
7. Levy RA, de Meis E, Pierangeli S. An adapted ELISA method for differentiating pathogenic from nonpathogenic aPL by a beta 2 glycoprotein I dependency anticardiolipin assay. *Thrombosis research* 2004; **114**(5-6): 573-7.
8. Pengo V, Ruffatti A, Legnani C, et al. Incidence of a first thromboembolic event in asymptomatic carriers of high-risk antiphospholipid antibody profile: a multicenter prospective study. *Blood* 2011; **118**(17): 4714-8.
9. Pengo V, Ruffatti A, Legnani C, et al. Clinical course of high-risk patients diagnosed with antiphospholipid syndrome. *Journal of thrombosis and haemostasis* 2010; **8**(2): 237-42.
10. Ruffatti A, Tonello M, Visentin MS, et al. Risk factors for pregnancy failure in patients with anti-phospholipid syndrome treated with conventional therapies: a multicentre, case-control study. *Rheumatology (Oxford)* 2011; **50**(9): 1684-9.
11. Tektonidou MG, Andreoli L, Limper M, et al. EULAR recommendations for the management of antiphospholipid syndrome in adults. *Annals of the Rheumatic Diseases* 2019; **78**(10): 1296-304.
12. Pengo V, Denas G, Zoppellaro G, et al. Rivaroxaban vs warfarin in high-risk patients with antiphospholipid syndrome. *Blood* 2018; **132**(13): 1365-71.
13. Cohen H, Hunt BJ, Efthymiou M, et al. Rivaroxaban versus warfarin to treat patients with thrombotic antiphospholipid syndrome, with or without systemic lupus erythematosus (RAPS): a randomised, controlled, open-label, phase 2/3, non-inferiority trial. *The Lancet Haematology* 2016; **3**(9): e426-e36.

Appendix III

Scientific output

Publications

1. **Yin D**, Chayoua W, Kelchtermans H, de Groot PG, Moore GW, Gris JC, Zuily S, Musial J, de Laat B, Devreese KMJ. Detection of anti-domain I antibodies by chemiluminescence enables the identification of high-risk antiphospholipid syndrome patients: A multicenter multiplatform study. *J Thromb Haemost. 2020 Feb;18(2):463-478.*

2. **Yin D**, de Laat B, Devreese KMJ, Kelchtermans H. The clinical value of assays detecting antibodies against domain I of β2-glycoprotein I in the antiphospholipid syndrome. *Autoimmun Rev. 2018 Dec;17(12):1210-1218.*

3. Chayouâ W*, **Yin D***, de Groot PG, Zuily S, Wahl D, de Laat B, Kelchtermans H. Brief Report on the 11th Meeting of the European Forum on Antiphospholipid Antibodies. *Curr Rheumatol Rep. 2019 Feb 11;21(2):6.* *Both authors contributed equally.

4. **Yin D**, de Laat B. Commentary on: Circulating plasmablasts contribute to antiphospholipid antibody production, associated with type I interferon upregulation. *J Thromb Haemost. 2019 Jul;17(7):1030-1032.*

5. Walid Chayoua, **Dong mei Yin**, Hilde Kelchtermans, Gary W. Moore, Jean-Christophe Gris, Jacek Musia, Stéphane Zuily, Hugo ten Cate, Bas de Laat, Katrien M. J. Devreese. Is there an additional value in detecting anti-cardiolipin and aβ2glycoprotein I IgA antibodies in the Antiphospholipid Syndrome? *Thrombosis and Haemostasis. 2020 Nov;120(11):1557-1568.*

6. **Dongmei Yin**, Philip G. de Groot, Bas de Laat, Katrien M. J. Devreese. The clinical relevance of isolated lupus coagulant positivity in antiphospholipid syndrome. *Thrombosis and Haemostasis. 2020 Dec 31. doi: 10.1055/a-1344-4271.*

7. **Dongmei Yin**, Hilde Kelchtermans, Philip G. de Groot, Bas de Laat, Katrien M. J. Devreese. Detection of anti-domain I antibodies by an in-house developed ELISA improve the classification of antiphospholipid syndrome. *Manuscript in preparation.*

8. de Laat-Kremers R, Di Castelnuovo A, van der Vorm L, Costanzo S, Ninivaggi M, Cerletti C, Huskens D De Curtis A, Gialluisi A, Bai C, de Gaetano G, **Yin D**, Donati MB, de Laat B, Iacoviello L. Increased BMI and Blood Lipids are Associated with Procoagulant Thrombin Generation Profile in the Moli-sani Cohort. *Submitted*. 2020.

9. Joke Konings, **Dongmei Yin**, Dana Huskens, Romy Kremers, Viola Strijbis, Saartje Bloemen, Mark Roest, Bas de Laat, Hilde Kelchtermans. Stability of Thrombin Generation, Thrombin Dynamics and Platelet Function in Healthy Individuals over a One-year Period. *To be submitted.*

Oral Presentations

1. **Dongmei Yin**, Walid Chayoua, Bas de Laat, Philip de Groot, Gary Moore, Jean-Christophe Gris, Stephane Zuily, Jacek Musial, Katrien Devreese, Hilde Kelchtermans. Detection of anti-domain I antibodies enables the identification of high-risk antiphospholipid syndrome patients: results of a multicenter study. ISLH 2019, XXXII International Symposium on Technical Innovations in Laboratory Hematology, Vancouver, Canada, May 9-11, 2019.

2. **Dongmei Yin**, Bas de Laat, Philip de Groot, Gary Moore, Jean-Christophe Gris, Stephane Zuily, Jacek Musial, Katrien Devreese, Hilde Kelchtermans. Role of anti-domain I β2-glycoprotein I antibodies measured by different methods, in the diagnosis and risk stratification of antiphospholipid syndrome. 65th Annual Scientific and Standardization Committee (SSC) of the ISTH, Melbourne Australia, July 6-10 2019.

3. **Dongmei Yin**, Walid Chayoua, Bas de Laat, Philip de Groot, Gary Moore, Jean-Christophe Gris, Stephane Zuily, Jacek Musial, Katrien Devreese, Hilde Kelchtermans. Detection of anti-domain I antibodies enables the identification of high-risk antiphospholipid syndrome patients: results of a multicenter study. 27th Congress of the International Society on Thrombosis and Haemostasis (ISTH), Melbourne Australia, July 6-10 2019.

4. **Dongmei Yin**, Hilde Kelchtermans, Philip G. de Groot, Bas de Laat, Katrien M. J. Devreese. Multicenter study on solid phase assays: detection of anti-domain I β2 glycoprotein I antibodies by an in-house assay and a commercial chemiluminescent assay. SSC of the ISTH 2020 Virtual Congress, June 23th 2020.

Poster

1. D. Yin, W. Chayouâ , B. de Laat , P.G. de Groot , S. Zuily , G.W. Moore , J. Musial , J-C. Gris , K. M. J. Devreese , H.Kelchtermans .The role of antibodies against domain I of β2-glycoprotein I in the antiphospholipid syndrome: an international multicenter study. De Nederlandse Vereniging voor Trombose en Hemostase (NVTH), Koudekerke, The Netherlands, April 2019.

2. **D. Yin**, W. Chayouâ , B. de Laat , P.G. de Groot , S. Zuily , G.W. Moore , J. Musial , J-C. Gris , K. M. J. Devreese , H. Kelchtermans. The role of antibodies against domain I of β2-glycoprotein I in the antiphospholipid syndrome : an international multicenter study. 11th Meeting of the European Forum on Antiphospholipid Antibodies, Maastricht, The Netherlands, September 25-26 2018.

Awards

Young investigator award. ISLH, Vancouver, Canada (2019).

Appendix IV

Curriculum Vitae

About the author

Dongmei Yin was born on the 24th of December 1982 in Suizhou City, China. After completing her pre-university education in her hometown, Dongmei Yin was admitted to Tianjin University of Traditional Chinese Medicine (Tianjin, China) in 2001. In the last year of five years of study (2005), she completed her clinical internship in the First Affiliated Hospital of Tianjin University of Traditional Chinese Medicine. After receiving her bachelor's degree, she continued to study for a master's degree in 2006. During her master (2006-2009), she studied Critical Care Medicine at Burn Institute, First Hospital Affiliated to Chinese PLA General Hospital, where her research focused on levels of microparticles in peripheral blood and coagulation system in the septic rat. During her master, she passed the Doctor's License in 2007. After two years of clinical work (2009-2011), she assisted in the establishment of the "Practical Organ Transplant Electronic Journal" and worked as an editor in Tianjin First Central Hospital for four years. Subsequently, she got the opportunity to go to Magdeburg University (Germany) as a Visiting Scholar to learn about stem cell transplantation. In 2016, she was awarded a scholarship under the State Scholarship Fund from China Scholarship Council. With the support of this scholarship, she came to the Netherlands and started as a Ph.D. candidate at the Department of Biochemistry of Maastricht University and Synapse Research institute, under the supervision of prof. dr. H. ten Cate, prof. dr. Katrien M. J. Devreese and dr. B. de Laat. In 2021, she went back to China and would start her residency training in Internal medicine at the Second Affiliated Hospital of Fujian Medical University.

Appendix V

Acknowledgements

Acknowledgements

I am extremely grateful to my supervision team, colleagues, friends and family. If it was not because of their selfless help, I would not have attained what I could achieve until this moment in my life.

First of all, I would like to thank my primary promotor, **Prof. Dr. Hugo ten Cate**, for giving me this opportunity to complete my PhD with you. When I need to extend my residence permit to complete my project, thank you for your quick response and help me contact KCIS. Thank you also for your insightful comments and suggestions on my thesis. Your valuable suggestion makes the thesis more professional and accurate. Your insightful comments and suggestions have tremendously improved my thesis, and I have learned great deal from you.

I would like to thank my secondary promotor, **Prof. Dr. Katrien M. J. Devreese.** Dear Katrien, thank you for your guidance at every stage of my project process. With your support and leadership, the anti-DI antibody research project has applied for the ISTH-SSC fund. Although this project has only achieved preliminary results due to the covid-19 pandemic, my interest in antibody research will continue in the future. If there is a chance, I will continue this research. Thank you for traveling back and forth between Gent and Maastricht and bringing research samples. Thank you for coming to Synapse to participate in the APS group meeting and to give detailed answers to the questions I encountered in my research. Thank you for your encouragement in my oral presentation at international conferences. During my presentations, it was very important for me to see you. Thank you for your detailed comments on my manuscripts and thesis and for helping me to revise them. Your elegant demeanor, rigorous academic attitude, and efficient time management will be a role model for me in my future work and life.

I would like to show my sincere gratitude to my co-promotor, **Dr. Bas de Laat.** Dear Bas, thank you for providing me with the opportunity to study for a PhD at Synapse. Thank you for taking care of me and switching to English instead of Dutch at the APS group meetings. Thank you for supporting me in participating in various courses and participating in the (inter)national conferences**.** In the first year of my PhD**,** you supported me in the provision of English courses in order to improve my spoken English. You helped me in my practices of academic presentation, especially the on-site questioning session, which eased my nervousness and made me more confident. You helped me organize my thoughts and inspired me to start the isolated LAC project**.** Also, I would like to thank you for your valuable suggestions on my manuscripts and thesis, as well as your quick and positive response to my questions. In addition to academics, as the CEO of synapse, you have also given Chinese PhD students a lot of care and help. In the first year of our PhD life, you invited us to the typical Dutch dinner you prepared, which made us feel at home. You also prepared special Christmas gifts for us. You provided us with the opportunity to go to Paris to participate in the Ecorun charity event. It was a wonderful and impressive experience. Your love for sports, academia, flexible business acumen, and attention to work-family life balance left a deep impression on me.

I am grateful to my Assessment Committee: **Prof. Dr. Y.M.C. Henskens**, **Prof. Dr. J.W.M. Heemskerk**, **Prof. Dr. J.C.M. Meijers**, **Dr. K. Winckers** and **Dr. R.T. Urbanus**, for taking time in reading and assessing my thesis critically.

The next one, who I would like to show my great gratitude to, is **Dr. Hilde Kelchtermans**, my daily supervisor. Dear Hilde, my research would have been impossible without your aid and support. During my application for the PhD position at Maastricht University, your efficient and timely response made my transition to the Netherlands a smooth process. No matter what information I need, you will prepare it in time and email it to me. After arriving in Maastricht, you and **Iris** helped me to apply for a bank card and find a house. Without your help, my trip to the Netherlands and life in Maastricht would not be so pleasant. In my research, as my daily supervisor, you have provided me with unparalleled help. For my first paper, you took the trouble of revising it over and over again. When I presented a presentation in English for the first time, you helped me practice and gave me critical suggestions. You gave me great support throughout the whole process and said "I am proud of you" after the speech. When I was not confident in myself, you always encouraged me and helped me. When I encountered a problem in the experiment, you guided me step by step to find the problem. The formulation of my scientific research thinking, the way to solve problems, and the cultivation of academic writing ability are all inseparable from your guidance and support. In my third year, you paid attention to my research work and helped me even when you have left Synapse due to an opportunity. Thank you very much, Dear Hilde! I am very fortunate to meet you and have started my PhD career under your guidance. Your noble personality charm, rigorous academic attitude, and problem-solving ability are all examples I have been learning from.

My sincere thanks to **Dr. Marisa Ninivaggi**. Dear Marisa, thank you for your invaluable help after Hilde left Synapse. You spent a lot of time and energy to help me although you have other PhD students in need of guidance. Thank you for helping me practice my oral presentation for the international conference. When I was confused, you gave me many valuable suggestions and helped me find my way again. Thank you, Marisa, also thank you for helping me revise the thesis and rewrite the English summary into the Dutch version. When I returned to China during the Covid-19 pandemic period, thank you for scheduling my PhD thesis defense.

I would like to show my greatest appreciation to **all the volunteers and patients** for their contribution to the multicenter study, I also warmly thank the technician **Michael Luypaert** of the Coagulation Lab of Ghent University Hospital for performing all commercial solid phase assays. In addition, I would like to thank HemosIL AcuStar®, BioPlex®2200, Phadia®, QUANTA Lite ELISA® and QUANTA Flash® for providing the assay kits and/or the corresponding devices.

I also want to warmly thank all the **co-authors**. Thank you for providing patient samples, your comments and suggestions, which have greatly improved the quality of my works.

I would also like to thank the **China Scholarship Council (CSC)** for providing funds to support my study in the Netherlands.

Then it comes to the big group, all present and former colleagues from Synapse Research Institute and Maastricht University, thank you for their assistance at every stage of the research project. It is nice to work with you during my PhD career. First of all, I would like to offer my special thanks to **Prof. Dr. Philip G. de Groot**. Dear Philip, you used your deep knowledge to give me inspiration and help me lift the fog of thought. Thank you for your constructive suggestions for my paper, and thank you for helping me in revising the paper. Other colleagues at synapse, even though we are not participating in the same project, your experience and knowledge have helped me a lot. The communications with you inspired me and helped me avoiding many detours in my experiments. Dear **Professor Hemker**, you are approachable and we can get your detailed answers whenever we have any questions. Dear **Mark**, thank you for your suggestions in my ELISA experiments, and gave detailed answers to my questions. Dear **Adam**, thank you for giving me detailed answers when I have questions to ask you. Dear **Joke**, thank you for letting me join your TG stability project and for giving me detailed guidance on the project. Dear **Dana**，
I remember that you taught me to do PACT experiments, and discussed with me when I had questions about my project, and gave me detailed instructions. Thank you, Dana. Because of your guidance, I have learned a lot about platelet testing, and know how to think and find out the reasons when the experimental results were not in line with the expected results, so as to decide how to proceed. Dear **Romy**, thank you for your help to familiarize me with how to conduct web conferences, and thank you for helping me to get familiar with the laboratory environment when I first arrived. Dear **Eric and Rob (Wagenvoord)** Thank you for producing the proteins and other products we need for our research, so that our research can proceed smoothly. Lively and enthusiastic **Tessa**, thank you for providing the protein and other products we need every time, and thank you for your warm and bright smile, which lights up our life. Dear **Rob (van der Zander),** thank you for helping me to apply for an extension of my residence permit. Dear **Caroline**, thank you for helping me to order reagents for experiments, for sending emails to others, for the reimbursement and all the other daily affairs. Dear **Eartha**, thank you for the nice communication. Dear **Tea** and dear **Monica**, thank you for your nice presence. Dear **Iris**, thank you very much for helping me arrange everything at the beginning in my study and work. You guided me in my application for a bank card, helped me purchase insurance, and helped me find a super cute and cost-effective house so that we could settle down. Every time there is something at work that requires your coordination and help, you can complete it efficiently. You are such a considerate and helpful person. Dear **Saartje**, I remember that when I first started learning the thrombin generation, you taught me step by step how to do it. Thank you for your patience and guidance.

Dear talented PhD students **Walid Chayoua, Lisa, Martijn**, **Susanne**, **Cecil**, and **Viola**. I am so lucky to be able to work with you. Dear **Walid,** thank you for your help during my experiments and answered my questions. Thank you for sharing samples and reagents when I need them. Thank you for your help without hesitation when I have any questions or new ideas to discuss with you. Dear **Lisa**, thank you very much for always answering my questions patiently. Dear **Martijn**, you love sports, you are always helpful and positive. Dear **Susanne**, you are always so friendly. Thank you for creating a joyous atmosphere at work or for leisure. Dear **Cecil**, thank you very much for your blood draw and cheerful presence. Dear **Viola**, thank you very much for your company in the first year of my Ph.D. career.

All the previous and current interns, **Joyce**, **Benji**, **Oriona**, **Guy**, **Daphne**, **Youssra**, **Dion**, **Yves**, **Nikita**, **Maikel**, **Lucas**, **Maike**, **Damien, Melanie, Hilde and many others.** Thank you so much for donating blood and creating joy and energy in the lab.

Thanks to **all the people** who helped me during my life in the Netherlands and Europe. Your friendly attitude and enthusiastic help make me feel warm. Your enthusiasm left a deep impression on me, and let me return to you with the same enthusiasm.

I am extremely grateful to all of my Chinese colleagues at Synapse. **Cui**，记得我刚来马城首先就是与你联系，你带我熟悉 Synapse 的环境，帮我翻译，初到一个陌生的地方，语言交流没有那么顺畅时，有一个同胞给予帮助是多么的让人安心。您性格平和，乐于助人。你和 **Armando** 一起招待我们，带我们熟悉荷兰的生活, 然后可爱的**小馒头**到来了，天使般的安琪儿又给我们的生活带了许多乐趣，尤其在 pandemic 期间，看看**小馒头**的视频，生活都被点亮了。**万俊，亚秋**，我们是同一届的难兄（姐）难弟（妹），在第一年我们开展研究的艰难时刻，有你们在身边真好。无论是学业还是生活，我们互相支持，一起吐槽，太多的细节了, 都历历在目，珍藏在我的记忆里……**亚秋**，你超级有行动力，性格又大方热情，多才多艺。祝你在美国事业顺利，生活丰富多彩（有你心爱的尤克里里陪伴，还有画儿般的幽静森林和灵动小鹿陪着你）。**万俊，**祝你和**珊珊**生活幸福美满。事业就不用说了，你聪明又有想法，肯定没有问题啦，等着你功成名就，报效祖国。亲爱的**珊珊，**祝你越来越美，工作也越来越好。**大黄，秋婷，李莉**，你们的加入，让我们 Synapse 中国小分队的队伍壮大了，难忘大家相聚的时光……**大黄**，谢谢您帮我搬家，邀请我们聚餐，难忘你美味的清蒸鱼，祝你顺利毕业回国，早日和**张舒**团聚！温柔的**秋婷**，谢谢你和我一起分享的时光：散步，做实验和一起吐槽。加油！祝你按时毕业，找到满意的工作和美丽的爱情！**李莉，**谢谢你分享的 Western Blot 的经验，虽然我最终没有做蛋白组学分析，谢谢你的乐于分享。相信自己，你超级优秀的，祝您顺利毕业！**思雨，今幂，**你们加入 Synapse 的时候我快要回国了，跟你们接触的时间不多。**思雨**，走之前还见识了一下你的厨艺，那么多好吃的。你上进勤奋，生活不会薄待努力的姑娘，祝你收获甜蜜的爱情，事业顺顺利利。**今幂**，你淡定平和，谢谢我回国的时候你送我到火车站，谢谢你和**辛真**帮我存放行李，祝你和**辛真**学业顺利，生活甜蜜蜜！

I also want to thank all friends I met in Maastricht! **金涵**，谢谢你在 R 语言上对我的帮助，分享给我的资料，谢谢你积极为我们实验室献血。祝你课题顺利早日毕业。**乔奇**，谢谢你热情的分享答辩流程以及回国的经验。谢谢你带我买自行车，质量超级好，用了四年都没有坏。**刘红**，谢谢您在我刚来马城时带我熟悉超市，谢谢您的无私分享。**李雯婕**，谢谢您热心分享的统计学知识。**宗盛华师兄**，谢谢你和**徐青师姐**聚餐时的各种信息交流，谢谢**小玉米**带给我们的快乐。还有**丽佳**，**宗亚楠**，**刘晓松，张明，陈琳，杨丹，龚英**等等所有中国留学生们，我多么想写上你们所有的名字，谢谢你们对我的帮助，无论在学业上，还是在生活上，你们的存在，让我知道自己不是孤独的。想到我们一起参加中秋节，春节活动的时候，想到我们参加 Brightlands 中国区活动的时候，想到我们一起开办数据分析学习小组的时候，想到我们在马大课堂上，会议中，在健身房，超市, 在公园，在马斯河边，在路上，在火车上，在公交上偶遇的时候，想到你们在身边，想到大家在一起在努力着，就感到莫大的安慰和支持。

我要感谢我在荷兰的朋友**乔姐和她的丈夫 Jan，圆圆，赵平安，刘峰**以及其他所有人。我们一起去旅游，一起自由地交谈，使我能够更多地了解当地信息，能更好的融入当地的生活。

最后，我要感谢我的丈夫**周军**，谢谢你陪我到荷兰，无论我遇到什么困难，你都一直陪在我的身边，给我支持和安慰。不管是深夜去实验室接我，还是在我心情低落的时候陪我散心，给我做各种美食，有你在我身边，我才能坚持到现在。我要感谢我的**爸妈，**亲爱的爸妈，中国有句古言，父母在，不远游，可是你们却放手让我飞。你们的支持，让你们为我骄傲也是我坚持下来的动力。我还要感谢我亲爱的**妹妹们**还有**孟帅**，亲爱的妹妹，有你们我真的很幸福，我们不仅是姐妹，还是朋友，每次回国，能跟你们相聚，都让我重新充满了能量；每次我回国，谢谢**孟帅**的接送。还要感谢亲爱的**周涛，刘姐，昌俣**及我的**公公婆婆**，感谢每次回国你们安排的相聚，感谢你们的支持和鼓励。我还要感谢我的朋友们**秦庆福，胡帆，吴遥，肖红，成观星等**，我的同学**余洁英，张红艳，刘西贤，武子霞，郑贵军，焦丽娜，朱海云，归永刚等，**每次回国跟你们聚会，都能让我更加明确我的方向。还要谢谢我的朋友**潘山**，你的鼓励和答疑解惑缓解了我的焦虑，谢谢你帮助我练习。谢谢所有的亲人朋友们~ 谢谢你们，你们的爱支撑着我一直走下去，爱你们~~