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Laboratory Methodology Important in the Diagnosis and Prognosis of Antiphospholipid Syndrome

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Abstract

Antiphospholipid syndrome (APS) is an autoimmune disease, characterized by thrombosis and pregnancy complications with persistently elevated levels of antiphospholipid antibodies (aPL). Recently, a unique mathematical calculation has been presented to assess the risk of thrombosis in patients with APS called antiphospholipid score or global antiphospholipid syndrome score (GAPSS). This new approach in the diagnosis of APS leads to the assessment of the risk of thrombosis considering the results of different aPL (lupus anticoagulants (LA), anticardiolipin antibodies (aCL), antibodies against β 2GPI (anti- β 2GPI), and phosphatidylserine-dependent antiprothrombin antibodies (aPS/PT) (isotypes IgG and IgM). This chapter provides an overview of the algorithm strategy for APS diagnosis with the aims of characterizing in detail the laboratory methodology of criteria aPL (LA, aCL, and anti- β 2GPI) and noncriteria aPL, such as IgA aCL and IgA anti- β 2GPI, anti-domain I β 2GPI, and antiprothrombin antibodies. In order to improve APS diagnosis, several new approaches in aPL detection have recently been suggested, such as multiline immunodot assay, detection of aPL by flow cytometry using beads with particular surface properties, and the newly developed automated BioPlex system technology for parallel detection of aCL and anti- β 2GPI antibodies of IgG, IgA, and IgM isotypes. A completely different and promising approach in future research lies in the potential of microRNAs as biomarkers for risk of thrombosis and/or obstetric complication.

Keywords: Anticardiolipin antibodies, lupus anticoagulants, anti-beta2-glycoprotein I antibodies, antiprothrombin antibodies, ELISA, multiplex assays, microRNA studies

1. Introduction

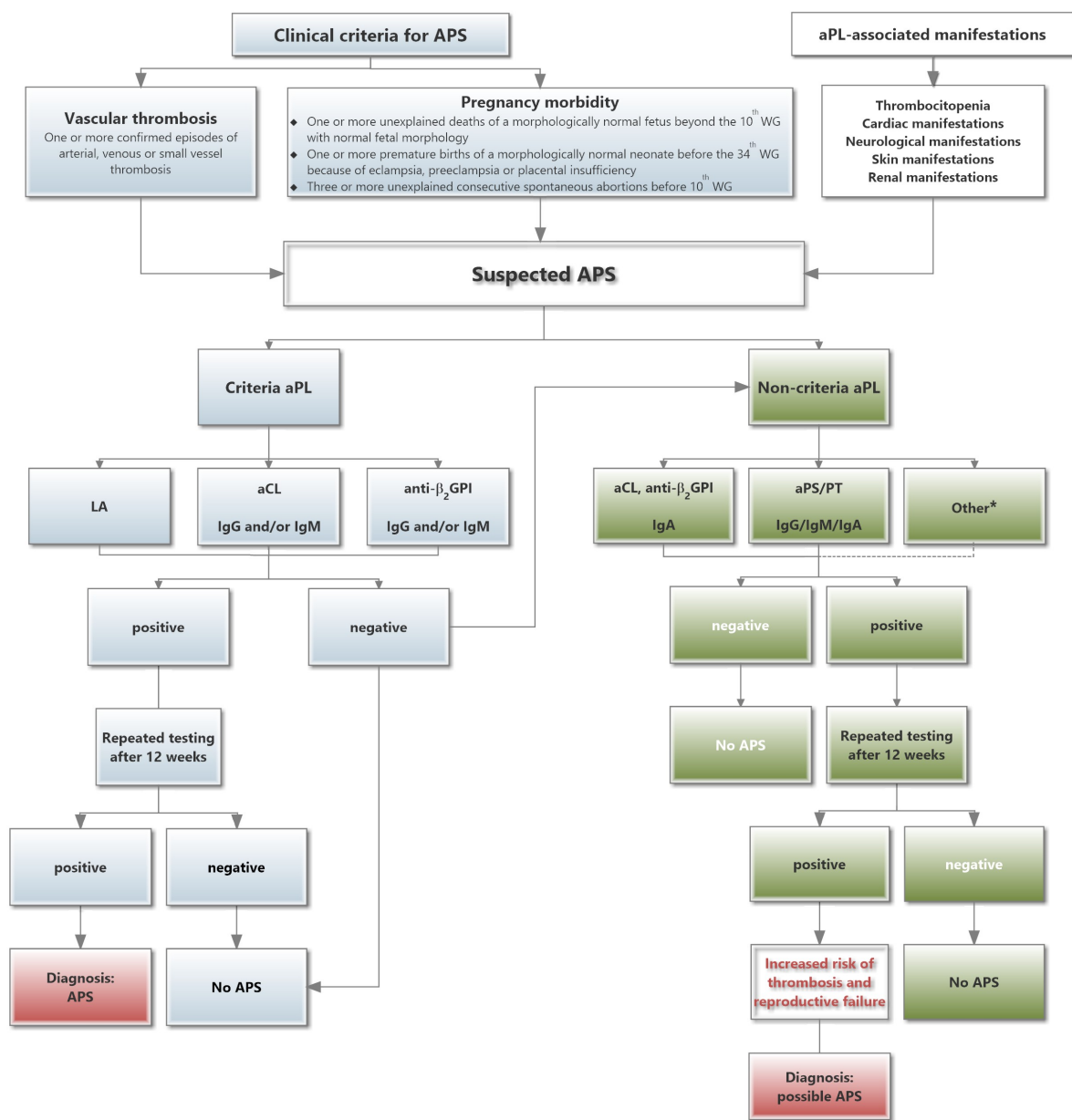
The antiphospholipid syndrome (APS) or Hughes syndrome was first described three decades ago in patients with systemic lupus erythematosus and positive pathogenic autoantibodies named antiphospholipid antibodies (aPL), who experienced arterial or venous thrombosis [1]. APS is now recognized as an autoimmune disease, characterized by thrombosis and pregnancy complications with persistently elevated levels of aPL. While deep veins of the lower limbs and the cerebral arterial circulation are the most common sites of venous and arterial thrombosis, any tissue or organ can be affected [2]. The risk of recurrent thrombosis in APS over 5 years is 16.6% despite the use of anticoagulants and/or salicylates [3, 4]. APS is also associated with a 5-year mortality of 5.3% with most deaths occurring within the first year of diagnosis due prevalently to bacterial infections, myocardial infarction, stroke, and cerebral hemorrhage [5]. The catastrophic APS (CAPS) is a rare life-threatening form of APS in which widespread intravascular thrombosis results in multiorgan ischemia and failure. Intravascular thrombosis affecting predominantly microcirculation characterizes CAPS. In addition, thrombosis of arteries, veins, or both can occur. CAPS affects about 1% of cases with APS and is the initial presentation of APS in nearly half of patients, while the remaining half has a history of APS [6–9]. Despite aggressive treatment, mortality rates still range between 44.0% and 55.6% [5, 10]. The other major clinical manifestations of APS are obstetrical. They include the unexplained death of one or more morphologically normal fetuses at or beyond the 10th week of gestation, the premature birth of one or more morphologically normal neonates before the 34th week of gestation due to eclampsia, severe preeclampsia, or recognized features of placental insufficiency, and three or more unexplained consecutive spontaneous abortions before the 10th week of gestation.

According to the international consensus statement on classification criteria established in 1999 in Sapporo and updated in 2006 in Sydney, APS is classified when persistently elevated levels of specific aPL are confirmed in addition to clinical manifestations [11, 12]. These consensus classification criteria for definite APS enabled substantial improvement in APS recognition (Figure 1); however, the diagnosis of APS still remains difficult. In the general population, the incidence of clinical manifestations which can be included in APS is high and could often be triggered by other underlying factors. Consequently, the diagnosis of APS relies predominantly on the laboratory results. The laboratory criteria comprise persistently elevated levels of either lupus anticoagulant (LA) or IgG/IgM antibodies to cardiolipin (aCL) or antibodies to β_2 GPI (anti- β_2 GPI). Methods for their determination differ and have not yet been standardized [13]. The common weaknesses of aPL determination are high interassay and interlaboratory variations, problems with the interpretation and clinical evaluation of the test results, as well as their low diagnostic specificity. Elevated aPL can be associated with many other conditions such as infections, malignancy, and also exposure to certain drugs. The absence of reliable, robust diagnostic markers for APS thus limits patient identification and management which challenges researchers to find better diagnostic marker(s). Among many autoantibodies that have been found to be associated with APS but not included in the current laboratory criteria,

antiprothrombin antibodies have shown the highest diagnostic applicability. Moreover, antiprothrombin antibodies are often present in APS patients and are sometimes the only aPL elevated [14, 15]. A recently published review concluded that an immunoassay using phosphatidylserine/prothrombin (aPS/PT ELISA) as an antigen on solid phase, leads to higher diagnostic accuracy as compared to the method using prothrombin alone (aPT ELISA) [14]. It seems that in the future there might be a change in the perception of the role of aPL from the criterion for APS classification to the role of risk factors estimating the probability for developing APS clinical manifestation. Modern trends for the diagnostic evaluation of APS patients therefore propose a determination of multiple classes of aPL, among them also antiprothrombin antibodies, to gain a common score which estimates the risk for arterial/venous thrombosis in APS patients [16–18]. Risk stratification is a major challenge in treating patients with APS and a potential role of aPL as a risk or even as a prognostic factor for arterial/venous thrombosis and miscarriages has been intensively debated [16, 19]. Single, double, and triple aPL positivity is not rare in patients with APS and such multiple positivity is mostly associated with higher risk for appearance or recurrence of thrombotic events and miscarriages [20, 21]. Recently, two research groups presented a unique mathematical calculation to assess the risk of thrombosis in patients with APS (called antiphospholipid score or global antiphospholipid syndrome score-GAPSS) [16, 17]. This new approach in the diagnosis of APS leads to the assessment of the risk of thrombosis considering the results of different aPL (LA, aCL, anti- β_2 GPI, and aPS/PT—isotypes IgG and IgM). The common calculation can help clinicians decide about introducing, eliminating or changing the appropriate therapy.

In order to improve APS diagnosis, several new approaches in aPL detection have recently been suggested. One is to detect aPL antibodies by multiline immunodot assay which may provide an interesting alternative to ELISA. Multiline immunodot assay could be a candidate for an effective multiparameter test system and simultaneous, semi-quantitative detection of several aPL antibodies in one sample; however, the technique has not yet been widely studied [22]. Apart from the immunodot technique, the detection of aPL by flow cytometry using beads with particular surface properties could represent another promising approach in aPL antibody multiplex testing [23]. Very recently, an automated technology for the parallel detection of aCL and anti- β_2 GPI antibodies of IgG, IgA, and IgM isotypes was developed in the BioPlex system [24]. The instrument combines the multianalyte profiling technology with antigen-coated fluoromagnetic beads in an automated platform where sampling, processing, and data reduction are performed automatically.

A completely different and promising approach in future research lies in the potential added value of microRNAs (miRNAs) as biomarkers for risk of thrombosis and/or obstetric complication. These are small noncoding RNAs that regulate gene expression at the posttranscriptional level by degrading or blocking translation of messenger RNAs. Many miRNAs have been found significantly altered in sera or plasma of patients with thrombosis [25] or specific pregnancy complications [26, 27].



*Anti-annexin V antibodies, anti-phosphatidylethanolamine, antibodies to domains of β_2 GPI, antibodies against anionic phospholipids other than cardiolipin [28, 29].

Figure 1. Algorithm strategy for APS diagnosis. APS: antiphospholipid syndrome; aCL: anticardiolipin antibodies, anti- β_2 GPI: antibodies against β_2 glycoprotein I; aPS/PT: antiphosphatidylserine prothrombin antibodies; aPL: antiphospholipid antibodies, LA: lupus anticoagulant.

2. Criteria aPL

Despite several attempts to standardize the LA, aCL, and anti- β_2 GPI tests, a considerable degree of interlaboratory variation still exists [13, 30]. While many laboratories worldwide

have managed to obtain consistently reproducible LA, aCL, and anti- β_2 GPI results, there are others that still report variations between different runs, which may potentially affect the consistency of the diagnosis of APS. This is mainly due to laboratories performing in-house aPL assays or using commercial kits not conforming to proposed guidelines for these tests [11]. Such apparent inconsistencies limit clinical usefulness and effectiveness as well as interlaboratory comparability of LA, aCL, and anti- β_2 GPI tests results. In general, aPL assays are very heterogeneous, poorly standardized, poorly harmonized, and consequently diversely sensitive and specific for APS. The standardization of autoantibodies assay is still a challenging task, and particularly limited progress has been made in the detection of aPL. Therefore, comparison studies are needed to further analyze the agreement between the different technologies.

It is well known that different assays for the detection of aPL show different sensitivities. By one plausible speculation LA assay is relatively “undersensitive” to aPL, so that only higher titre and more clinically relevant forms of aPL are detected by the clot-based tests [31]. On the other hand, immunoassays, such as aCL, may be “oversensitive,” as they detect both clinically relevant and clinically irrelevant (e.g., infection-associated) forms of aPL. Furthermore, aCL test more often detect “low levels” of aPL that are less likely associated with APS than LA assay. The hypothesis is also supported by the observation that a positive result in the anti- β_2 GPI assay (which is theoretically more “specific” but less “sensitive” than aCL for APS), is more strongly associated with clinical features of APS such as thrombosis than a positive result in the standard aCL assay [30]. For the physician, it is important to have in mind there is no gold standard aPL assay when defining the presence or absence of APS. However, data are emerging that the combination of multiple aPL findings is a superior risk stratification tool for clinical events in APS such as thrombosis [32, 33]. According to the revised classification criteria, APS patients should be divided into four categories: category I includes patients with more than one positive test in any combination, while patients with a single positive test should be classified in category II (IIa if LA positive, IIb if positive for antibodies against CL (aCLs), and IIc if positive for anti- β_2 GPI antibodies) [11]. Triple positivity, defined by the presence of LA and medium/high titers of aCL and anti- β_2 GPI antibodies (above the 99th percentile), is the most predictive profile for clinical manifestations and recurrences despite conventional treatment [34–36]. Patients in category II, expressing single aPL positivity, have a lesser risk to develop APS manifestations.

2.1. Lupus Anticoagulants (LA)

LA denotes a subgroup of aPL antibodies that prolong phospholipid-dependant clotting reactions. The clotting-based nature of the LA assay is fraught with preanalytical limitations, the need for multiple reagents, diversity in platforms, as well as challenges with interpreting results. It was shown that the presence of LA correlates better with thromboembolic complications than the presence of aCL [4]. The superiority of LA over aCL was explained by the nature of the assay; LA measures the activity of aPL, while in an ELISA, both the antibodies that influence functional activity and the antibodies that have no aPL activity are measured [37]. However, LA testing has several drawbacks, such as the absence of a reference method,

low degree of standardization, and difficult test result interpretation. Consensus guidelines for the detection of LA were first published in the 1990s [38, 39]. Significant improvement in standardization has been achieved with the updated guidelines of the Scientific Standardization Subcommittee (SSC) of the International Society Thrombosis and Haemostasis in 2009 [40] and with the Clinical and Laboratory Standards Institute (CLSI) document in 2014 [41] that has resolved several issues of the preanalytical, analytical, and post-analytical phase.

The preanalytical phase plasma preparation is the crucial step. Plasma for LA analysis must be rendered platelet poor (platelet count less than $10 \times 10^9/L$), as residual platelets contain phospholipids that can shorten clotting times and generate false-negative results. Double centrifugation of blood is preferred, although a single centrifugation can also be suitable if the recommended platelet count is achieved. Ultracentrifugation as the second step is discouraged due to possible generation of phospholipid-containing microparticles [42]. Filtration through $0.22 \mu m$ cellulose acetate filters is also discouraged because of the binding of coagulation factors V, VIII, IX, XII, and von Willebrand factor to the filter [43]. Whether assays, such as dilute Russell's viper venom time (dRVVT), that bypass these coagulation factors are affected by filtration has not yet been studied.

Testing may be performed on fresh or properly frozen/thawed samples [41]. According to our experience, however, all the samples should be treated in the same manner as were plasma samples of healthy donors from which the reference values were obtained. For example, we found significantly longer activated partial thromboplastin times (APTTs) measured with the Staclot LA (Diagnostica Stago, France) on Behring Coagulation Timer (Dade Behring, Germany) in fresh (73.0 ± 17.7 s screening and 59.6 ± 12.1 s confirming test) compared to frozen/thawed plasma (59.8 ± 9.3 s screening and 53.7 ± 4.2 s confirming test, both paired t-test $p < 0.05$), while no differences were found for the dRVVT assay.

Several plasma-based (clotting) assays were traditionally used for LA detection that include intrinsic, extrinsic, or common coagulation pathway. On one hand, no single assay is 100% sensitive and specific for LA; therefore, the use of more than one assay was recommended. On the other hand, by increasing the number of different assays, the probability of false-positive results increases [44]. Guidelines therefore suggest using (no more than) two assays that are known to be responsive to LA, preferably dRVVT and APTT [41, 44].

Several strategies that include screening and confirming tests (with or without the mixing test) can be used for LA detection (Figure 2). In the screening test, low phospholipid levels are used, while confirmation test, preferably based on the same principle as the screening test, utilizes high phospholipid levels.

Each laboratory should establish its own cutoff value for a screening test above which confirming (and mixing) test needs to be performed. The SSC guidelines recommend using the 99th percentile [44], while the CLSI guidelines recommend average + 2 standard deviations (equal to 97.5th percentile) as the appropriate cutoff [41]. It should be noted that a higher cutoff insures less false-positive results, but a false-positive screening test from a statistical outlier in case of the lower cutoff will not generate false-positive composite interpretation. Yet a false-negative result in case of the higher cutoff could lead to inappropriate diagnosis and treatment [42].

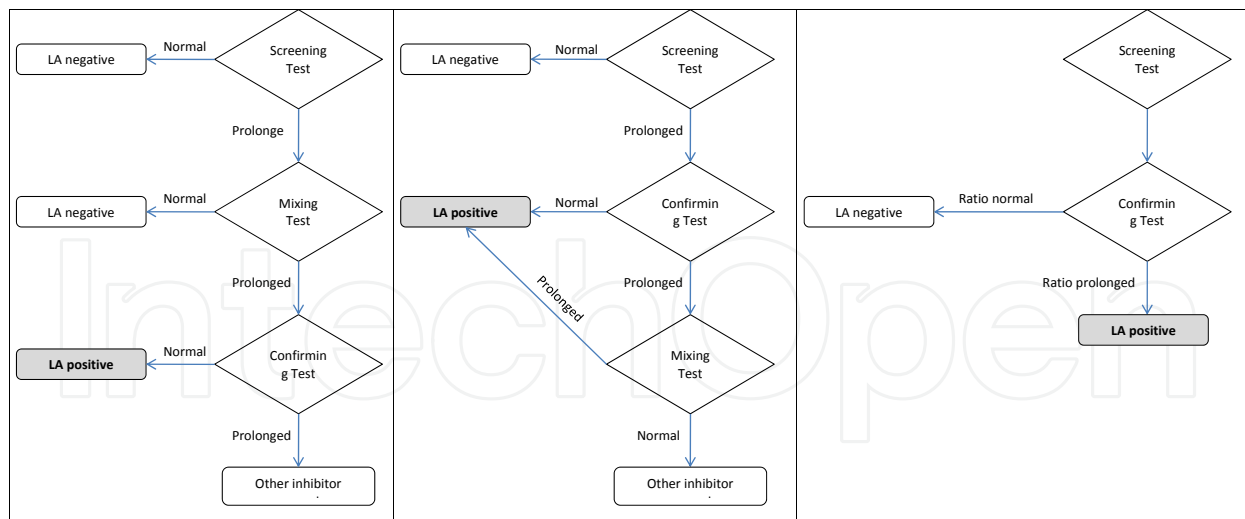


Figure 2. Strategies for LA detection. LA: lupus anticoagulant.

The need for a mixing test has been highly debated. When using an integrated test system (Figure 1, right panel), a mixing step is not necessary, and many laboratories avoid this step because of the additional work, cost, testing time, and the need for normal pool plasma. In addition, it is not clear how the mixing test should be performed (patient to normal pool plasma volume ratio, incubation time). Finally, there is some evidence that the mixing step produces false-negative results where a weak but clinically relevant LA is present [45]. On the other hand, integrated test systems without the mixing step may produce false-positive [46] as well as false-negative results [34]; therefore, further studies are needed.

LA testing results should be expressed as the normalized screen to confirm ratio [41, 44] as this practice reduces the variability of the analyzer and reagent performance. When the result is positive, additional assays, such as prothrombin time (to exclude vitamin K antagonists), thrombin time (to exclude heparin or direct thrombin inhibitor), and anti-Xa (to exclude low-molecular-weight heparin or direct factor Xa inhibitor), may be performed to make sure that the patient is not on anticoagulant therapy. The test panel should be repeated at or beyond 12 weeks to determine persistence of the LA [41, 44]. Laboratories should provide the final interpretation of LA testing results as positive (LA present), negative (LA not detected), or inconclusive (to be retested).

LA testing remains much more labor intensive and complicated to perform than ELISA but must be performed in parallel with aCL and anti- β_2 GPI. Significant improvement has been achieved through SSC guidelines [44], but some issues are still unresolved and further reports of the SSC are awaited.

2.2. Anticardiolipin antibodies

The first molecularly defined aCL test was a radioimmunoassay established in 1983, using cardiolipin as an antigen, with a mixture of gelatin/PBS to dilute patient serum and radiola-

beled anti-human IgG or IgM secondary antibodies. In 1990, three groups independently reported that aCL do not bind to cardiolipin in the absence of serum proteins, as summarized by Roubey [47]. The designation is somewhat misleading because aCL are an undefined group of antibodies that bind directly (1) to the cardiolipin (CL) and (2) to various (usually calf or bovine) serum proteins (most likely β_2 GPI) linked to CL. The first group are β_2 GPI-independent aCL (binding to CL does not require this cofactor), which are often associated with infectious diseases. Infectious aCL do not pose a risk for the occurrence of clinical signs associated with APS and are therefore indicated as nonthrombogenic autoantibodies. Infectious diseases can therefore determine the transient aCL positivity. Patients with syphilis, HIV, Lyme disease, and other infections triggered by cytomegalovirus, hepatitis C, and Epstein–Barr viruses may be mistakenly diagnosed as APS based on high levels of aCL especially, when concomitant arterial thrombosis or cerebrovascular accident is present [48]. In the second group, antibodies against β_2 GPI prevail. These are the β_2 GPI-dependent aCL antibodies (aCL/ β_2 GPI), which require the presence of cofactor β_2 GPI for their binding to CL. They are characteristic of autoimmune diseases, as high titers of these antibodies detected in plasma represent a risk for thromboembolic complications. Therefore, for the detection of aCL (IgG and IgM), an ELISA method is used [49, 50], which measures the immune reactivity to CL as a phospholipid or to β_2 GPI as a protein bound to phospholipids. The affinity of β_2 GPI for CL is 30 to 40 times higher than affinity to phosphatidylserine [51]. Briefly, the ELISA test involves medium binding microtitre plates coated with highly purified negatively charged CL incubated overnight at 4°C and diluted patients sample in bovine serum (FBS), which acts as a cofactor for the recognition of CL as the antigen. After 2.5 h incubation at room temperature and two washes, goat antihuman IgG or IgM conjugated with alkaline phosphatase diluted in FBS/PBS was applied to the plates. Following washing, para-nitrophenylphosphate in diethanolamine buffer (pH 9.8) was applied, and optical density at 405 nm (OD405) was kinetically measured by a spectrometer (Figure 3) [50, 52]. The essential weakness of this assay is that patients with aCL antibodies that bind human but not bovine β_2 GPI can be missed. Therefore, some laboratories use human β_2 GPI instead of bovine or calf serum as a source of β_2 GPI in the aCL ELISA.

Several international workshops and forums have been conducted in an attempt to standardize the aCL test [53–58]. Despite these efforts, a considerable degree of interlaboratory variation still exists [59]. There is also a high degree of variability between different commercial kits for the detection of aCL even when assessed within the same laboratory, with lower variability seen with different commercial anti- β_2 GPI kits [60]. This keeps raising the question of the standardization of commercial and/or in-house laboratory methods and results [61]. Although laboratory results represent an essential contribution to the diagnosis of APS, many questions regarding methodology and reference values of various in-house and commercially available kits that are not comparable still remain unanswered. In an effort to improve the diagnostic value of aPL antibody assays, aCL determination is still necessary and should be complemented by anti- β_2 GPI determination in accordance with revised Sydney laboratory classification criteria for the diagnosis of APS [11].

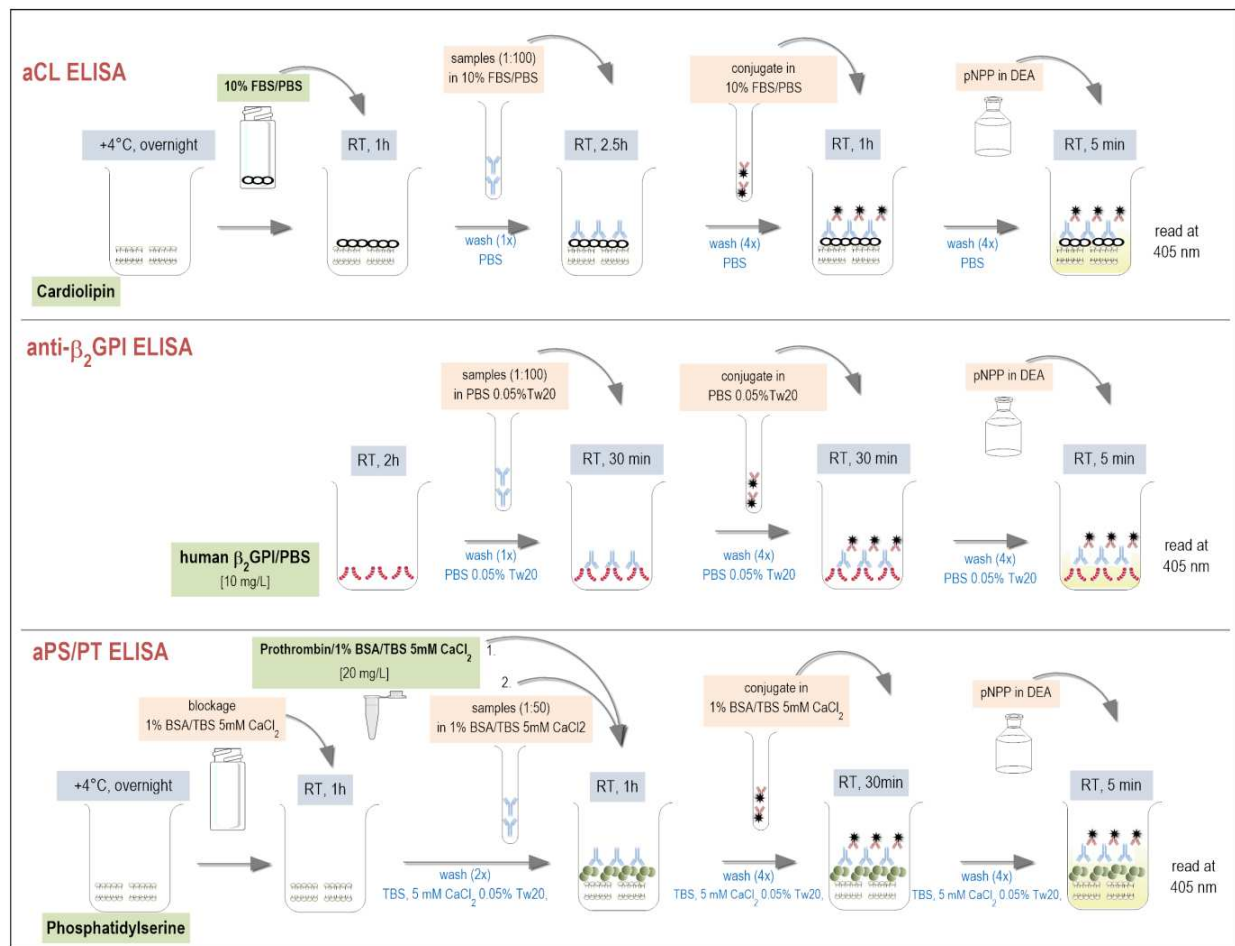


Figure 3. Laboratory protocols for aCL, anti- β_2 GPI, and aPS/PT ELISA. BSA: bovine serum albumin; conjugate: goat antihuman IgG/M/A conjugated with alkaline phosphatase; DEA: diethanolamine buffer; FBS: fetal bovine serum; PBS: phosphate-buffered saline; pNPP: para-nitrophenylphosphate; RT: room temperature; TBS: tris buffer saline; Tw20: Tween-20.

2.3. Antibodies against β_2 GPI

A large part of autoantibodies in patients with APS are directed against β_2 GPI and anti- β_2 GPI therefore represent one of the main subgroups of antiphospholipid antibodies [62]. β_2 GPI, also known as apolipoprotein H, is a multifunctional and evolutionary conserved single-chain glycosylated protein present in plasma at concentrations ~ 180 mg/L (range 20–300 mg/L) [63–65]. The major site of β_2 GPI synthesis is the liver, although its mRNA has also been found in endothelial cells, neurons, astrocytes, and in the extra villous cytotrophoblast and syncytiotrophoblast of placenta [66]. The ~ 50 -kDa protein consists of 326 amino acids, which are folded into five short consensus repeat domains, termed also “sushi” domains. Domains I–IV are composed of ~ 60 amino acids, and each contains two disulfide bridges, while the domain V consists of 82 amino acids. Domain V also has specific characteristics, such as a positively charged, lysine-rich region, a hydrophobic loop, and a 19-residue C-terminal extension, which is cross-linked by an additional disulfide bond allowing β_2 GPI to bind to negatively charged

phospholipids [67–70]. Due to specific interactions between the domains I and V of β_2 GPI, the protein exists in three configurations, i.e., circular plasma protein, as observed by electronic microscopy [71], open fishhook conformation, as revealed by its crystal structure [69, 72], and an intermediate S-shape, which has been recently observed using small-angle X-ray scattering [73]. After binding to suitable anionic surface, for example, to phospholipids, β_2 GPI changes its conformation from circular to open (“J”), exposing a cryptic epitope, which is recognized by anti- β_2 GPI-directed against domain I [74].

Although routine laboratories detect and interpret anti- β_2 GPI as one set of autoantibodies, they actually represent a very heterogeneous group directed against the same antigen. Due to their polyclonal nature subpopulations of anti- β_2 GPI vary in epitope recognition [63, 75–78], avidity [79–82], and pathological mechanisms of their action [63, 83, 84], which results in their different pathogenicity and clinical relevance [63, 77, 78, 80, 85, 86]. A subset of IgG anti- β_2 GPI directed against domain I on β_2 GPI seems to have a prominent role in the pathology of thrombotic complications in patients with APS [74]. According to de Laat *et al.* [87], pathologic anti- β_2 GPI can be divided into those targeting epitope on domain I (directed against the G40-R43 residues), which highly correlate with thrombosis, and those targeting nondomain I epitopes. According to Giannakopoulos *et al.* [88], these differences are due to anti- β_2 GPI avidity. Therefore, avidity must also be considered when describing the diversity of anti- β_2 GPI subpopulations and their clinical relevance. Several research groups/clinical studies indicated that difference in strength of binding to the same antigen/same epitope could be an important marker of the pathogenicity of the specific anti- β_2 GPI subgroups [80, 85, 89]. An association between high-avidity IgG anti- β_2 GPI and thrombotic or obstetric complications in patients with APS was reported [85, 90]. This was in contrast to low-avidity IgG anti- β_2 GPI, which was more prevalent in autoimmune patients without APS. The involvement of high avidity IgG anti- β_2 GPI in the pathogenesis of APS was also confirmed in an *in vitro* study on human coronary artery endothelial cells [91]. In this study, high-avidity IgG anti- β_2 GPI was shown to increase the expression of inflammatory and chemotactic cytokines leading to higher migration of monocytes. These effects were further intensified in presence of the major acute phase protein serum amyloid A (SAA) [91], denoting that along with lipopolysaccharide [83], SAA as a marker of inflammation, also represents a trigger or a second hit uncovering the full pathological potential of anti- β_2 GPI [91]. The differences among antigen binding sites on anti- β_2 GPI of differently avidity were further confirmed by the location of the recognized epitopes on β_2 GPI. Therefore, using heptamer phage display libraries two binding epitopes of high-avidity IgG anti- β_2 GPI have been characterized: FNPYWYV and QGOAHSK [92]. These sequences mimicked specific amino acid clusters on domains II and III of β_2 GPI and were accessible to high-avidity IgG anti- β_2 GPI in the fishhook and circular conformations. In contrast, the sequence KMDGNHP has been characterized as a surface binding epitope for low-avidity IgG anti- β_2 GPI located between domains III and IV of β_2 GPI. It was accessible for the binding of low-avidity IgG anti- β_2 GPI only in the open/fishhook conformation [92]. Epitopes of low-avidity IgG anti- β_2 GPI are cryptic and not reachable for the antibodies when β_2 GPI molecules occupy the closed plasma conformation, which explains a negligible binding of the respective anti- β_2 GPI to β_2 GPI in the solution. Epitopes of high-avidity IgG anti- β_2 GPI are native and reachable regardless of conformation of the β_2 GPI, which is in accordance with

the proven binding of the respective anti- β_2 GPI to antigen in the solution. High-avidity monoclonal anti- β_2 GPI recognizes β_2 GPI in the solution by binding to the epitope, which is crucial for the restoration of the closed conformation of the β_2 GPI [81]. Since enzyme-linked immunosorbent assays currently used in routine diagnostics detect anti- β_2 GPI antibodies of unknown avidity, these observations are potentially useful for the development of improved diagnostic tests capable of detecting clinically relevant antibodies. The avidity of IgG anti- β_2 GPI was determined by chaotropic anti- β_2 GPI ELISA [79]. The procedure was in principle the same as in the anti- β_2 GPI ELISA, using chaotropic conditions during antibody binding. Samples were diluted in PBS–Tween containing increasing concentration of NaCl. Discrimination between anti- β_2 GPI with high or low avidity was made arbitrarily, comparing the initial binding at 0.15 M NaCl with bindings at higher salt concentrations [79, 85]. NaCl (0.5 M) was selected as the reference concentration for comparison with the initial binding. When the binding at 0.5 M NaCl remained higher than 70% of the initial one, the presence of high-avidity anti- β_2 GPI was declared. When the binding at 0.5 M NaCl decreased to or below 25% of the initial binding, low-avidity anti- β_2 GPI were designated. Samples that did not fulfill either of the above criteria were considered to be of heterogeneous avidity [79].

The addition of anti- β_2 GPI antibodies to the revised Sydney laboratory classification criteria for the diagnosis of APS was finalized in 2006 [11]. According to the consensus guidelines on anti- β_2 GPI testing and reporting [93], IgG anti- β_2 GPI should be performed on all requests for anti- β_2 GPI where isotype is not specified. If both IgG and IgM anti- β_2 GPI antibody testing is routinely performed on all requests (regardless of whether IgM anti- β_2 GPI testing has been specifically requested), then it has been suggested that a comment should be provided to the physician indicating that the association between isolated positive IgM anti- β_2 GPI results and thrombosis is uncertain but appears to be lower than for IgG anti- β_2 GPI [93].

ELISA that measures the immune reactivity directly to β_2 GPI is used for the detection of anti- β_2 GPI (IgG and IgM). The characteristics are similar to the detection of aCL with the major difference that in anti- β_2 GPI ELISA, the purified human native β_2 GPI [94] is coated directly onto the ELISA microtitre plates. Briefly, high binding polystyrene microtitre plates were coated with β_2 GPI in PBS and incubated with sera diluted in PBS containing 0.05% Tween-20. The detection system was the same as in aCL ELISA (Figure 3). Anti- β_2 GPI is directed against a cryptic epitope exposed when β_2 GPI is bound to anionic phospholipid or another suitable surface [95]. Namely, some brands of ELISA plates are unable to induce the conformational change due to difference in structure or charge and are unsuitable for use in anti- β_2 GPI ELISA [95]. There remains a lack of a formal, universally accepted method for performing anti- β_2 GPI ELISA, coupled with a lack of standardized WHO accepted calibrators. The in-house and commercial kits are calibrated by their own “calibrators” and expressed in arbitrary units. Consequently, there is a great variability in the results reported by the different laboratories. Meroni *et al.* suggested that the identification of common calibrators may be useful in obtaining more reproducible results [96]. Both polyclonal IgG/IgM anti- β_2 GPI antibodies affinity-purified from two APS patients with high IgG and IgM anti- β_2 GPI levels and chimeric monoclonal IgG antibody (INOVA, San Diego, CA, USA) reacting with human β_2 GPI have been selected as candidates for reference material for calibration [97]. This reference material may contribute significantly to better standardization of anti- β_2 GPI immunoassays.

3. Noncriteria aPL

The majority of APS patients have APS, as defined by the presence of clinical features and positive one or more laboratory criteria aPL. However, a small proportion of patients have significant clinical features of APS but lacking persistently elevated above-mentioned criteria aPL. These so-called “seronegative” APS patients (SNAPS) probably do exhibit certain aPL, which may not be identifiable given the limitations of currently available aPL assays. Even if such a patient does not fully satisfy the classification criteria, he/she may still have APS. Missing a diagnosis of APS in these individuals may lead to the absence of appropriate therapy and potentially significant adverse outcomes for such patients. For clinical studies, patients falling into any of these categories should be classified separately from those that fulfill the revised classification criteria for APS.

3.1. IgA aCL and IgA anti- β_2 GPI antibodies

Although standard serological tests included in the current laboratory classification criteria for APS [11] detect IgG and IgM isotypes for the aCL and anti- β_2 GPI but not the IgA isotype, clinicians will occasionally encounter patients with isolated IgA aPL who exhibit clinical manifestations of APS. Most studies to date have mainly focused on the IgG/IgM isotypes of aPL, with only rare reports pointing to the possible clinical significance of IgA aPL. The literature on IgA aPL is quite heterogeneous, reporting a high variability in their prevalence and clinical significance due to different study designs and populations studied and different laboratory methods (in-house as well as commercial kits) with different cutoffs and different ethnic populations (review in [98]). IgA aCL and anti- β_2 GPI antibodies have been reported in up to 70% of patients with systemic lupus erythematosus (SLE) and in those with primary APS [98]. The role of IgA isotypes of both antibodies in APS is however unclear. Lagos *et al.* [99] found that livedo reticularis, heart valve disease, thrombocytopenia, and epilepsy are more common among subjects with increased IgA anti- β_2 GPI antibodies [99]. A similar association was observed in SLE patients [100].

The reports on the prevalence of IgA aCL are extremely variable ranging from 0% to nearly 50% [28]. Altogether, twelve studies showed an association between IgA aCL and certain clinical features related to APS but fifteen studies failed to find any relationship between the presence of IgA aCL and the clinical signs of APS (review in [28]). Studies investigating diagnostic applicability and added value of IgA aCL determination show a general weakness, seeing as elevated value of IgA aCL are often accompanied with positive IgG and/or IgM aCL, making it difficult to understand the role of elevated IgA aCL alone. In addition, a great variability of results suggests that studies are barely comparable in the population included, regarding methods used and results obtained. Some differences among studies can also be due to different ethnicity. IgA seems to be the most prevalent isotype of aCL in African Americans [101], African Caribbean [102], and Japanese patients [103].

There is also a controversy in the literature regarding the meaning of elevated IgA anti- β_2 GPI. The majority of published papers have highlighted the value of IgA anti- β_2 GPI in the diagnosis of APS [28]. Thrombosis, particularly arterial thrombosis [86, 104, 105], is frequently found

associated with IgA anti- β_2 GPI, although the simultaneous presence of other isotypes makes it often difficult to interpret. Mehrani *et al.* [106] reported on a high prevalence of IgA aPL antibodies with 20% positive IgA anti- β_2 GPI associated with deep vein thrombosis. IgA anti- β_2 GPI seems to be more prevalent in SLE patients compared to IgA aCL (review in [98]). Therefore, IgA anti- β_2 GPI have gained clinical relevance and were recently included among aPL tests in the novel SLICC classification criteria for SLE [107]. Ruiz-García *et al.* [108] also suggested that the assessment of IgA anti- β_2 GPI in patients with suspected primary APS is important for the identification, treatment, and management of patients who in accordance with the current classification criteria are not diagnosed at this point of the disease since the serological profile of patients with primary APS (where IgA is the most prevalent isotype) is different from systemic autoimmune disorders-associated APS (where IgG is the most prevalent isotype). Therefore, in selected patients where the clinical suspicion of APS is high but all tests for other antiphospholipid antibodies are negative, testing for IgA anti- β_2 GPI might be clinically useful and therefore recommended [58, 93, 109]. Despierres *et al.* [105] also proposed that IgA anti- β_2 GPI should be searched for in all patients with unexplained thrombosis based on their finding that IgA anti- β_2 GPI was associated with thrombosis even in non-SLE patients. Most patients positive for IgA anti- β_2 GPI were negative for IgA aCL [108, 109].

In addition, a subpopulation of IgA anti- β_2 GPI directed against domain IV has been linked to atherosclerosis in a study using different β_2 GPI domain-deleted mutants and native β_2 GPI [86]. Akhter *et al.* also reported that in a large cohort of nearly 300 SLE patients, the only assay with a significant association with stroke was the IgA anti- β_2 GPI directed against domain IV/V [104].

3.2. Anti-domain I β_2 GPI antibodies

For better clinical diagnosis and management of APS, a lot of efforts have been implicated for better characterization of pathogenic function and clinical significance of anti- β_2 GPI. Several groups have studied the fine specificity of anti- β_2 GPI, and every domain of β_2 GPI has been described to bind antibodies [63]. From an immunologic point of view, it is hard to imagine a self-protein containing many immunodominant epitopes. Therefore, many studies have been initiated to identify the most immunodominant epitope. Most evidence points to the domain I (DI) of β_2 GPI. A specific epitope in DI is a positively charged discontinuous structure located in the N-terminal of β_2 GPI and has been identified as the most relevant antigenic target involved in the binding of anti- β_2 GPI to β_2 GPI [110]. The reactivity to DI was first described by Iverson *et al.* in 1998, who showed that most anti- β_2 GPI reacted with DI of β_2 GPI using domain-deletion mutants of the protein [111]. The binding of anti-DI anti- β_2 GPI (anti- β_2 GPI-DI) to β_2 GPI is conformation dependent [110]. In the circular conformation of β_2 GPI, DI interacts with domain V, and immunodominant epitope is therefore hidden. When β_2 GPI takes the intermediate S-shape, the epitope in the DI is covered by carbohydrate chains of domain III and domain IV, thus preventing the binding of antibodies to β_2 GPI [74]. The binding of β_2 GPI to the anionic surface may induce conformational change of β_2 GPI from circular to J-shaped conformation, which also results in the displacement of the domains III-IV carbohydrate side chains; the critical epitope arginine 39-glycine 43 is exposed and thus becomes accessible for domain-specific anti- β_2 GPI binding [74]. Despite a wide interest in the potential diagnostic

value of these DI-specific antibodies in the diagnosis of APS, specifically anti- β_2 GPI-DI, the number of studies is still limited, and their potential clinical utility has not been widely accepted [112]. Anti- β_2 GPI-DI antibodies have attracted particular interest as they are strongly associated with thrombosis [77, 113–115]. Ciesla *et al.* [116] found that increased IgG anti- β_2 GPI-DI antibodies were associated with livedo reticularis and heart valve disease in APS, which is also in concordance with the finding of de Laat and coworkers [63]. The latter have also reported of IgG anti- β_2 GPI-DI antibodies to be associated with heart valve disease presumably linked to thrombotic processes on a valve with autoimmune valvulitis, being a risk factor for stroke.

To date, there have been no commercial kits to detect anti- β_2 GPI-DI antibodies available on the market; however, several research methods have been developed [110]. As the binding of anti- β_2 GPI-DI antibodies might be directly affected with conformational changes of β_2 GPI, the preparation and coating β_2 GPI to ELISA trays is of major importance for the results of the assay. It has been consistently observed that anti- β_2 GPI-DI antibodies react with their target epitope only when DI is bound onto hydrophobic but not hydrophilic surface. The majority of studies used the two steps ELISA using β_2 GPI-DI coated on hydrophobic and hydrophilic microtitre plates [110, 113, 117]. This method is based on the assumption that the epitope of β_2 GPI-DI is exposed only to the hydrophilic surface of the microtitre plates and thus accessible for autoantibody binding [113]. Pozzi *et al.* showed that the capture ELISA with chemical synthesized N-terminally biotinylated β_2 GPI-DI on streptavidin plates was able to discriminate between APS patients and controls in contrast to nonbiotinylated β_2 GPI-DI directly immobilized onto ELISA plates [118]. A liquid phase inhibition assay using a whole β_2 GPI immobilized on the solid phase and synthetic β_2 GPI-DI as inhibitor was also developed [119]. In addition, Iverson and coworkers developed an ELISA, which was based on recombinant β_2 GPI-DI expressed and purified from insect cells [120]. Recently, a novel β_2 GPI-DI chemiluminescence immunoassay (CIA, INOVA Diagnostics, San Diego, USA) based on the BIOFLASH system (Biokit, Barcelona, Spain) and using a recombinant β_2 GPI-DI coupled to paramagnetic beads has been developed [121]. Despite comparability between solid phase assays and a CIA immunoassay, results obtained by both approaches should be evaluated in a larger multicentre studies in order to confirm the reproducibility of different anti- β_2 GPI-DI antibody assays. It is also premature to replace the anti- β_2 GPI ELISA with the anti- β_2 GPI-DI assays, as there is still the possibility that other populations of thrombosis related antibodies are present.

3.3. Antiprothrombin antibodies

Prothrombin, a phospholipid binding protein, was first proposed as a possible co-factor for LA by Loeliger in 1959 [122]. In 1983, Bajaj and his colleagues were the first to ascertain the evidence of prothrombin binding antibodies in two patients with LA and severe hypoprothrombinemia [123]. These antibodies bound prothrombin without preventing its activation to thrombin. The authors assumed that the hypoprothrombinemia resulted in a rapid removal of prothrombin–antibody complexes from circulation. A year later, the existence of prothrombin–antiprothrombin immune complex was confirmed with counterimmunoelectrophoresis (CIE) in the plasma of patients with LA, but without hypoprothrombinemia [124]. Fleck *et al.*

confirmed these findings, showing that 74% of patients with LA had evidence of anti-prothrombin antibodies on CIE and that antiprothrombin antibodies exhibit LA activity [125]. Bevers *et al.* highlighted the role of antiprothrombin antibodies for the LA activity in patients with elevated aCL and LA [126]. In eleven out of sixteen patients' sera, the binding to phospholipid surface was detected only in the presence of prothrombin and calcium ions. Later, Oosting and his colleagues demonstrated that antibodies with LA activity inhibited prothrombinase complexes on the surface of endothelial cells and that IgG fraction with LA activity bound the phospholipid-prothrombin immune complexes [127]. A review elucidated that majority of antibodies with LA activity bind prothrombin and/or β_2 GPI [128]. Double diffusion and CIE were the first techniques used for the detection of antiprothrombin antibodies [123–125, 129]. Their main advantage was in the measurement of prothrombin–antiprothrombin immune complexes. These *in vitro* findings have indicated that such complexes might exist in plasma, but detection methods used did not enable quantitative measurements. In many cases, the titer and/or affinity of antiprothrombin antibodies were too low to obtain a clear precipitation line. Other techniques based their detection on the impairment of enzyme activity of prothrombin after binding with relevant antibodies [127, 130]. The need for isolation of antiprothrombin antibodies and purification of clotting factors makes these methods unsuitable for routine work.

In the last few years, several research groups developed various ELISAs, which are by far most widely used techniques for the determination of antiprothrombin antibodies, since they enable relatively quick determination of a titre and antibody isotype. Arvieux *et al.* first presented an ELISA, where prothrombin as the antigen was applied to high binding microtitre plates [131]. In this way, they determined antibodies directed against prothrombin alone (aPT-A or aPT). It is important to note that the manner of prothrombin presentation on a solid surface greatly affects its recognition by antiprothrombin antibodies [132]. Antiprothrombin antibodies only bind prothrombin when immobilized on an appropriate anionic surface such as γ -irradiated or high binding polyvinylchloride (PVC) microtitre plates, or to prothrombin exposed to the immobilized anionic phospholipids. Antiprothrombin antibodies cannot be detected when prothrombin is immobilized on nonirradiated plates [131]. In this respect, the reactivity of antiprothrombin antibodies in an ELISA strongly resembles the reactivity of low-avidity anti- β_2 GPI. The latter recognizes β_2 GPI only when bound to the anionic surface, while their binding to the antigen in liquid phase is minimal [133–135]. Matsuuda *et al.* [136] showed that antiprothrombin antibodies could be detected also by an ELISA where prothrombin is bound to immobilized phosphatidylserine (aPS/PT ELISA). Soon it was shown that aPS/PT ELISA was more efficient in determination of antiprothrombin antibodies in comparison to aPT ELISA [137]. The phenomenon can be explained in several ways: (1) prothrombin, bound to phosphatidylserine, is not limited in its lateral movement, in contrast with prothrombin bound to high-binding microtitre plates resulting in clustering and proper orientation of prothrombin offering better conditions for antibody binding; (2) ELISA with phosphatidylserine in solid phase may, through the calcium ions, capture the circulating prothrombin–antiprothrombin immune complexes present in certain samples; and (3) antiprothrombin antibodies may react with neoepitopes occurring on prothrombin molecules only when bound to phosphatidylserine through calcium ions. Altogether, aPS/PT ELISA better simulates the *in vivo* conditions, where prothrombin and antiprothrombin antibodies presumably bind to phospholipid surface of

endothelial cells. In addition, Atsumi *et al.* showed that the aPS/PT strongly correlated with LA activity [138]. This group showed there was no correlation between the levels of aPS/PT and aPT in patients with APS and therefore assumed that the aPS/PT ELISA determines, at least in part, different antibodies compared to aPT ELISA. The first published aPS/PT protocol was later modified in order to increase the analytical sensitivity of the test [139]. The modified procedure allowed the detection of the two allegedly different populations of antibodies and thus determination of all clinically relevant antiprothrombin antibodies with one method. The authors reported that their in-house aPS/PT ELISA was the most optimal method for the determination of all clinically relevant aPS/PT antibodies, exhibiting the highest percentage of LA activity, compared to aCL and anti- β_2 GPI [139]. The ELISA protocol is schematically presented in Figure 3. Briefly, medium binding plates were coated with phosphatidylserine in chloroform/methanol 1:4 and dried overnight at 4°C. Following blocking with Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) and 5 mM CaCl₂ (1% BSA/TBS-Ca) and two washes with 5 mM CaCl₂-TBS-0.05% Tween 20 (TBS-Tw), human prothrombin of final concentration 10 mg/L and patients' sera diluted 1:100 were applied to wells immediately one after the other and incubated for 1 h at room temperature. After 4 washes, alkaline-conjugated goat anti-human IgG or IgM were applied in 1% BSA/TBS-Ca and incubated for 30 min. Following 4 washes in TBS/Tw, 100 μ l/well of para-nitrophenylphosphate in diethanolamine buffer (pH 9.8) was applied and OD405 was kinetically measured by a spectrometer (Figure 3). Zigon *et al.* also showed that antiprothrombin antibodies are of different avidity, which greatly impacts their detection by different ELISAs [139, 140]. It was shown that low-avidity antiprothrombin antibodies were detectable only in the aPS/PT ELISA, while aPT ELISA detected only antibodies with predominantly heterogeneous avidity. Initially, only a few commercial kits for the detection of antiprothrombin antibodies were available, and they all measured aPT antibodies. Their overall diagnostic sensitivity and specificity was poor [141]. In 2010, the commercial QUANTA Lite™ aPS/PT IgG/IgM and LAC assays became available as an aid to the diagnosis of APS [15, 142]. Zigon *et al.* showed that results measured with QUANTA Lite™ aPS/PT highly correlated with their modified in-house procedure described above and confirmed that both ELISAs have high diagnostic specificity and sensitivity for APS [15].

Several retrospective and cross-sectional studies, which were first summarized in Galli *et al.* [143] and recently in Sciasca *et al.* [14], had examined the clinical significance of antibodies to prothrombin determined by ELISA. Different laboratories performed their variation of ELISA methods using commercially available assay kits for the determination of antibodies to prothrombin. International studies that have attempted to standardize and unify these methods were very rare [141]. The main reasons for significant differences in results among published studies are modified procedures, different reagents used, and the fact that international standards are not available. Comparative studies of two methods aPT or aPS/PT ELISA showed that the results only partially overlap, which means that some patients were positive in only one ELISA [141, 144–146]. Researchers then speculated that results from aPT ELISA and from aPS/PT ELISA allegedly do not belong to the same population of antibodies, although both are clinically associated with APS. Many have therefore estimated that in terms of clinical significance, it is necessary to consider the results of the two performances. A recent systematic review of studies on aPT (38 studies) and aPS/PT (10 studies) summarizes the results of more than 7000 patients and controls. Both types of antiprothrombin antibodies represent an increased risk for thrombosis, but meta-analysis in the article showed that elevated aPS/PT pose a greater risk for

arterial and/or venous thrombosis compared with aPT (OR = 5.1, 95% CI = 4.2 to 6.3 and OR = 1.8, 95% CI = 1.4 to 2.8). The authors concluded that routine measurements of aPS/PT, but not aPT, allow better insight to thrombotic risk in patients with systemic autoimmune diseases as well as confirm an undeniable need for further research toward the inclusion of these antibodies in laboratory criteria for APS.

Results of a modified aPS/PT ELISA [15] have shown that elevated aPS/PT represent an increased risk of venous thrombosis (OR = 3.5, 95% CI = 1.7 to 7.0) and less so for arterial thrombosis (OR = 1.9, 95% CI = 0.9 to 4.1) (Table 1). A very important finding was that antibodies to prothrombin compared to other aPL (LA, aCL, and anti- β_2 GPI) represented the highest risk for pregnancy complications (OR = 9.3, 95% CI = 3.5 to 24.6). Not many studies have investigated the relationship between antibodies to prothrombin and complications during pregnancy, and about half of these studies have not confirmed such correlations [145, 147–150]. In contrast, Akimoto [151] and Bertolaccini [152] and their colleagues showed strong and specific association between different types of antibodies to prothrombin and miscarriages. Clinical correlations between antibodies and late pregnancy complications were later confirmed by two other studies [153, 154], but none of the studies found any link between complications in early pregnancy before 12 weeks of pregnancy and the presence of antibodies to prothrombin. The most recent study clearly showed an overall prevalence of aPS/PT of 13.0%, aCL of 12.4%, LA, and anti- β_2 GPI less than 8.0% in a group of patients with obstetric complications characteristic for APS. Both aPS/PT and aCL were significantly more prevalent compared to healthy blood donors. However, aCL correlated only with late pregnancy morbidity and prematurity, while aPS/PT were the only antibodies associated with early recurrent pregnancy loss, as well as with late pregnancy morbidity and prematurity. Authors suggested that, aPS/PT measurement might improve the evaluation of patients with early recurrent pregnancy loss, undiscovered by other aPL tests [155].

		Arterial thrombosis (n=41)				Venous thrombosis (n=53)				Obstetric complications (n=28)			
		p-value	Odds ratio (95% CI)	Sensitivity %	Specificity %	p-value	Odds ratio (95% CI)	Sensitivity %	Specificity %	p-value	Odds ratio (95% CI)	Sensitivity %	Specificity %
LA		0.43	1.4 (0.6-2.9)	49	59	<0.001	5.6 (2.6-12.2)	70	70	<0.005	4.3 (1.6-11.9)	62	73
aCL	IgG	<0.001	5.5 (2.3-13.4)	83	53	<0.005	3.0 (1.5-6.2)	74	52	<0.001	5.8 (2.1-15.9)	79	61
	IgM	0.67	1.2 (0.4-3.5)	15	88	0.010	3.5 (1.3-9.2)	23	92	0.130	2.5 (0.8-7.8)	21	90
anti-β ₂ GPI	IgG	0.02	2.4 (1.2-5.1)	63	58	<0.001	3.2 (1.6-6.5)	66	63	0.002	4.1 (1.7-10.4)	68	66
	IgM	0.99	1.0 (0.3-2.9)	12	88	0.070	2.5 (0.9-6.5)	19	91	0.278	2.0 (0.6-6.6)	18	90
aPS/PT	IgG	0.08	1.9 (0.9-4.1)	44	71	<0.001	3.5 (1.7-7.0)	51	77	<0.001	9.3 (3.5-24.6)	64	84
	IgM	0.36	1.4 (0.6-2.9)	39	69	0.021	2.2 (1.1-4.5)	45	73	<0.005	4.0 (1.6-9.9)	54	78

Table 1. Antiphospholipid antibodies and LA in relationship to arterial thrombosis (AT), venous thrombosis (VT), and obstetric complications (OC). aCL: anticardiolipin; anti- β_2 GPI: anti- β_2 glycoprotein; aPS/PT anti-phosphatidylserine/prothrombin; LA: lupus anticoagulant; OR: odds ratio; CI: confidence interval.

4. Novel/future methodology

Given the poor standardization of single aPL testing, new technical approaches to aPL profiling have been proposed recently, which elucidated the possibility of aPL profiling for the diagnosis

of APS and outcome prediction [22, 24, 156]. In order to ensure the quality of routine laboratory measurements, along with time and cost-efficiency, multiline immunodot assays or bead-based multiplex techniques might represent a solution for these challenges. Apart from the immunodot technique, the detection of aPL by flow cytometry using beads with particular surface properties seems to represent another promising approach in aPL antibody multiplex testing [23]. In the future, we can also expect studies exploring miRNAs as biomarkers for risk of thrombosis and/or obstetric complication. Many miRNAs have been found significantly altered in sera or plasma of patients with thrombosis [25] or specific pregnancy complications [26, 27].

4.1. Multiplex assays

Recently, the parallel detection of aCL and anti- β_2 GPI antibodies of IgG, IgA, and IgM isotypes related to APS was developed in the BioPlex system (BioPlex™ 2200 multiplex immunoassay system using APLS reagents). A unique fully automated multiplex immunoassay platform that allows the qualitative and quantitative measurements of several antibodies was developed [24]. The instrument combines the multianalyte profiling technology with antigen-coated fluoromagnetic beads as the solid phase, in an automated platform where sampling, processing, and data reduction are performed automatically. Unlike other methods, beads are washed after incubation and labeling; magnetic beads are used to automate washing steps and support random access testing [157]. The BioPlex APLS system seems to have a good diagnostic accuracy for all tests. However, the simultaneous detection of aCL and anti- β_2 GPI of IgA isotype together with IgG and IgM isotypes did not increase the diagnostic sensitivity for APS. This was the first and until now the only study on the BioPlex APLS system; therefore, the applicability of this technology needs to be further explored and confirmed.

4.2. Multiline immunodot assays

A multiline immunodot assay (MLDA) uses a different solid phase and is an alternative to ELISA for simultaneous assessment of multiple aPL. Only one research group reported the use of hydrophobic membrane as the solid phase for immobilization of phospholipids in aPL antibody testing [22, 156, 158, 159]. The anionic phospholipids CL, phosphatidylinositol, and phosphatidylserine were immobilized in lines together with the co-factor protein β_2 GPI and a reaction control reactant on a polyvinylidene difluoride (PVDF) membrane strip to detect specific autoantibodies simultaneously in one sample. Membrane surfaces like the hydrophobic PVDF membrane seemed to induce surprisingly the same conformational changes in the β_2 GPI polypeptide required for disease-specific aPL. It may be assumed that the membrane immobilization mimics the *in vivo* presentation of anionic phospholipids in membranes more appropriately since the majority of aPL antibodies are supposed to be of medium or rather low affinity and bivalent binding is required for strong amplification of interaction. The formation of multiple interconnected immune complexes on an appropriate lipid surface might be of importance for antibody testing. Authors of the study explained that MLDA provided the appropriate reaction environment for the detection of three different aPL antibody reactivities. First, aPL antibodies to pure anionic phospholipids were determined; second, immobilized

phospholipids interacting with cofactor-aPL antibody complexes were detected; and third, antibodies against cofactor proteins alone could be detected. Therefore, this technique may assess a broader spectrum of reactivity in a multiplex format for aPL antibody profiling than the recommended ELISA technique does. However, the inability of quantification and the lack of confirmation by other researchers make this very novel methodology questionable.

4.3. miRNAs

MicroRNAs (miRNAs) are small noncoding RNAs with 21 to 25 nucleotides in length that posttranslationally regulate gene expression. miRNAs are fundamentally involved in the regulation of major biological processes in health and disease. The primary miRNA sequence repository—miRBase database (<http://www.mirbase.org/>), the latest miRBase release (v20, June 2013), contains 24521 miRNA loci from 206 species, processed to produce 30424 mature miRNA products [160].

Since the discovery that miRNAs are highly stable in circulation [161, 162], circulating serum/plasma miRNAs have gained an extensive research interest as highly promising noninvasive clinical biomarkers in diagnosis, prognosis, treatment response, and risk assessment in several diseases, including cancer [163, 164] autoimmune diseases [165, 166], myocardial infarction [167], heart failure [168], deep vein thrombosis [169], and pregnancy complications (reviewed in [170, 171]).

Williams *et al.* showed that the vast majority of circulating miRNAs in healthy human plasma originated from blood components and endothelial cells, and tissue-specific miRNAs from gut and liver were represented as well [172]. This suggested broad tissue contribution to the circulating pool of miRNAs in healthy human plasma. In contrast, skeletal muscle-, heart-, and brain-specific miRNAs were detected only at very low levels in healthy plasma, suggesting that such miRNAs, when detected in circulation at increased levels may specifically reflect source tissue damage or disease. The strong evidence for the existence of the circulating organ-specific miRNAs in tissue damage or disease comes, for example, from the study of circulating miRNAs in myocardial injury in patients with advanced heart failure [168]. In this study, cardiac-specific (miR-208b, miR-208a, and miR-449) and muscle-specific (miR-1-1 and miR-133b) circulating miRNAs (myomirs) increased up to 140-fold in patients with advanced heart failure as compared to subject without heart disease and highly correlated to the levels of cardiac troponin I, the established marker of heart injury.

The potential for shedding the tissue-specific miRNAs in the circulation during organ damage or disease, alongside their high stability in serum and plasma, equips circulating tissue-specific miRNAs with high potential for clinical biomarker applications. However, to reach significant levels in the circulation, tissue-specific miRNA biomarker has to be abundantly present in the damaged source tissue as, for example, in advanced heart failure [168]. In addition, the source tissue has to be well perfused and of considerable size to be able to contribute to the circulating miRNA pool. According to the experimental estimations of circulating placental miRNAs [172] and mathematical modeling [173], the tumors should reach a considerable size (0.3–0.6 g) before tumor miRNAs could reach the detection threshold in plasma. A lack of cancer-specific

miRNAs, small tumor biomass early in disease, and the dilution of tissue-specific miRNAs in circulation may hinder the early detection of tumor-derived circulating miRNAs. Given the promise of miRNAs as noninvasive clinical biomarkers, different strategies have been suggested to overcome these limitations early in disease, when the detection of tumors is most desirable [174]. Circulating miRNAs are primarily associated with Argonaute complexes, and a minor part of circulating miRNA is contained within extracellular vesicles (EVs) [175]. EVs are shed in a highly regulated manner from their cells of origin and are reported to have disease unique signatures of nucleic acids [176]. Therefore, the selective detection of EV-associated circulating miRNAs could prove valuable in enriching the low abundant tissue-specific circulating miRNAs [174].

Despite the exponential increase in the number of studies on circulating miRNAs as disease biomarkers over the last years, circulating miRNAs are in the infancy toward becoming highly specific clinical biomarkers. Many circulating miRNAs, identified as potential disease biomarkers, are associated with a variety of diseases. Furthermore, reported miRNA profiles for a given disease significantly differ between different studies. This can be in part attributed to the current methodological limitations for detecting low level circulating miRNAs, as well as to the preanalytical and the heterogeneity of studied cohorts.

Preanalytical variables during collection and processing of blood to obtain cell-free, circulating miRNAs may significantly affect the levels of circulating miRNAs as excellently reviewed by Nair *et al.* [177]. Therefore, all the study samples should be obtained in the same way, using standard operating procedure for sample collection and processing to minimize the preanalytical variability in circulating miRNA detection. An important source of the preanalytical variability, that considerably affect circulating miRNA levels, is the contamination of circulating cell-free miRNAs with cellular miRNAs, originating from epithelial cells at the site of skin puncture, red blood cell hemolysis releasing a large amount of contaminating miRNAs, as well as from contaminating monocytes and/or platelets. Such cellular contamination could be minimized by discarding first milliliters of withdrawn blood, using a larger-gauge needle, minimizing tourniquet use, using adequate centrifugation speed and preparing platelet-free plasma (reviewed in [177]). Several miRNAs have been identified, which can estimate the contribution of different cellular contaminants, such as platelets or red blood cell lysis, to the plasma miRNA profile [178, 179]. In addition, study subjects have to be matched for age, sex, smoking history, and end-stage organ dysfunction, or miRNA levels have to be adjusted for these confounders, known to significantly affect miRNA levels (reviewed in [177]).

To measure circulating miRNA levels, several methods could be used, including quantitative PCR, high-density microarray technology or next generation sequencing. When using plasma as a source biofluid for circulating miRNA detection heparin should be avoided as it may inhibit qPCR. Another critical step in the analysis of circulating miRNA is data normalization, which is rather challenging when compared to the analysis of cellular and tissue miRNA expression. Normalization strategies significantly vary across the published studies on circulating miRNAs, further contributing to analytical variability. In contrast to the measurement of cellular miRNAs, there is no optimal housekeeping gene for normalization of circulating RNA levels, and a global median normalization can be used as a normalization method.

The simplest method to normalize circulating miRNA levels is to use the defined plasma volume, from which RNA is isolated, combined with the “Spike In” exogenous miRNA controls. The use of “Spike In” exogenous miRNA controls, derived, e.g., from *Caenorhabditis elegans* (cel-miR-39), is highly recommended during the isolation of RNA from plasma or serum as quality control for the technical variation arising from variations in RNA isolation, and the differential efficiencies of the reverse transcription and real-time PCR [179, 180].

Up to date, no reports exist on investigating the circulating levels of miRNAs in APS patients. Nevertheless, the down-regulation of tissue factor-targeting miRNAs, miR-19b, and miR-20a in peripheral blood monocytes from SLE and APS patients as compared to healthy controls inversely correlated with the surface tissue factor expression on monocytes and may contribute to the increased thrombosis risk in APS and SLE patients [181]. Circulating miRNAs may prove valuable as biomarkers in APS, in particular in patients with obstetric APS and APS-related placental dysfunction. Human placenta is a relatively large, well-perfused organ, present only during pregnancy, which makes it an excellent solid tissue model, very likely to contribute to the pool of circulating miRNAs. During pregnancy, placenta-specific miRNAs are detected in maternal circulation. Many of these miRNAs exhibit strict placental specificity and cannot be detected in paternal circulation or plasma of nonpregnant control women [172]. Specifically, the characterization of circulating and placental miRNAs by small RNA deep sequencing showed strong 5240-fold and 28-fold enrichment of the miR-498 cluster in placentas and maternal plasma, respectively, as compared to paternal plasma. This indicated strong, tissue-specific contribution of placenta to the maternal circulating miRNA pool. Furthermore, placental miRNA fingerprints, including miR-498 cluster isomiRs, were sufficiently unique between the subjects to enable matching of the maternal plasma sample to its specific source placenta. This suggests that clinically relevant alterations of placental miRNAs may be present in maternal plasma of women with pregnancy complications, such as preeclampsia or obstetric APS. Such miRNAs could serve as noninvasive biomarkers of placental disease in obstetric APS, allowing for its better monitoring and on time intervention.

5. Conclusion

The classification of APS is based on three aPL laboratory tests that need to be positive in addition to its clinical manifestations. Both the clinical spectrum of APS and the serological markers are more polymorphic than it was thought in the past. Additional laboratory tests have been proposed in order to improve the diagnostic and predictive power, but promising results have been reported especially for anti- β_2 GPI-DI and anti-PS/PT antibodies. Discovering new markers did not ease the recognition of patients with APS, and therefore modern trends propose the determination of multiple classes of aPL to gain a common score, which could estimate the risk for arterial/venous thrombosis in APS patients. Risk stratification itself represents a major challenge. However, the potential roles of aPL (as a risk or even as a prognostic factor for arterial/venous thrombosis and miscarriages) will occupy future debates.

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