study shows that posttranslational modification of β_2GPI via thiol-exchange reactions is a highly specific phenomenon in the setting of APS thrombosis. Quantification of posttranslational modifications of β_2GPI in conjunction with standard laboratory tests for APS may offer the potential to more accurately predict the risk of occurrence of a thrombotic event in the setting of APS.

The antiphospholipid syndrome (APS) is an autoimmune condition characterized by vascular thrombosis of the arterial and/or venous systems as well as recurrent miscarriages (1). Beta-2-glycoprotein I (β_2GPI) is the major autoantigen in APS (2). A number of studies have provided robust evidence that autoantibodies to β_2GPI are a significant risk factor for arterial thrombosis in young adults (3,4). In vivo and ex vivo studies by multiple groups have shown anti-β_2GPI autoantibodies to be directly thrombogenic (5).

At present it is not possible to stratify the risk for development of thrombosis in antiphospholipid antibody (aPL)–positive patients based on clinical features or use of currently available laboratory assays (6). The development of novel assays that could be used to stratify future thrombosis risk in patients with APS would hold immense clinical utility in informing the decision as to whether initiation of prophylactic therapy or intensification of therapy is warranted.

Beta-2-glycoprotein I is an evolutionarily conserved 50-kd protein circulating in the blood in relative abundance (~4 μM) (7). The physiologic role of β_2GPI is pleiotropic, with functional studies implicating a role in processes relating to coagulation (8), angiogenesis (9), and clearance of apoptotic cells (10). The crystal structure of β_2GPI, which has been ascertained based on the purified native protein, reveals that it does not possess free thiols (11,12). We have recently shown, however, that in vivo β_2GPI circulates in a free thiol form and that this free thiol form of β_2GPI is involved in the protection of endothelial cells against oxidative stress–induced cell injury (13). Beta-2-glycoprotein I can also participate in redox thiol-exchange reactions by acting as a substrate for oxidoreductase enzymes such as thioredoxin 1 (14). However, the proportion of β_2GPI circulating in the reduced state is unknown. Also unknown is whether the redox state of this autoantigen differs in patients with pathogenic anti-β_2GPI antibodies and a history of thrombosis.

In the present study we demonstrated that, in serum/plasma derived from healthy subjects, β_2GPI exists in a reduced biochemical state as the dominant molecular phenotype. Detailed in vitro quantitative assays to assess the levels of total and reduced β_2GPI were developed and used to screen >450 samples. Levels of both total and oxidized β_2GPI were found to be elevated in patients with APS as compared to disease and healthy control groups. These findings have implications with respect to understanding the antigenic drive for pathogenic aPL, as well as the potential for development of assays for purposes of thrombosis risk stratification.

PATIENTS AND METHODS

Patient samples. Samples were collected through an international collaborative multicenter effort involving 5 centers (University of New South Wales [Sydney, Australia], University of Athens [Athens, Greece], University College London [London, UK], Tianjin Medical University [Tianjin, China], and Hokkaido University School of Medicine [Sapporo, Japan]). An APS group, 2 disease control groups, and 1 healthy control group were studied. The disease control groups consisted of an autoimmune disease group (with or without aPL, but with no clinical features of APS) and a clinical event control group (clinical features of APS, but no aPL or autoimmune disease).

APS group. A total of 139 samples from patients with APS were collected and analyzed (24 from Sydney, 38 from Athens, 22 from London, and 55 from Sapporo). Every APS patient fulfilled the revised consensus classification criteria for vascular thrombosis–associated APS (1). All serologic tests for aPL were performed using standard commercially available kits and in accordance with the revised classification criteria. A venous thrombotic event was diagnosed based on a combination of clinical assessment and appropriate imaging with either Doppler ultrasonography or venography to confirm deep venous thrombosis, or isotope ventilation/perfusion scanning or computed tomography (CT) (with or without angiography) to confirm pulmonary embolism. An arterial event was diagnosed based on clinical findings along with one or more of the following: electrocardiographic evidence of myocardial ischemia or infarction, confirmation of infarction by brain CT or magnetic resonance imaging, or confirmation of peripheral vascular disease or arterial thrombosis by Doppler ultrasonography or angiography.

Autoimmune disease control group. Of the 189 autoimmune disease controls, samples from 188 were analyzed (42 from Sydney, 43 from Athens, 29 from London, and 74 from Sapporo). One sample (from a patient with systemic lupus erythematosus [SLE] and no aPL) was found to be deficient in β_2GPI and was withdrawn from the study. Among the autoimmune disease controls, 74 had persistently positive serologic findings for aPL satisfying the serologic component of the APS classification criteria (1), but did not have APS given the lack of a clinical event. All patients with SLE fulfilled the American College of Rheumatology revised classification criteria (15), and those with Sjögren's syndrome fulfilled the revised European classification criteria (16).

Clinical event control group. Thirty-eight samples from aPL-negative patients with a clinical event were collected and analyzed (26 from Sydney and 12 from Tianjin). Clinical events were diagnosed as described above for the APS group.

Table 1. Demographic and clinical characteristics of the groups studied*

		Control groups		
	APS	Autoimmune disease	Clinical event	Healthy
Patients	139	188	38	92†
Female	111 (79.9)	164 (87.2)	21 (55.3)	58 (63.0)
Age, median years	43	42	55.5	35
Race				
Caucasian	82	110	26	56
Asian	56	77	12	36
Afro-Caribbean	1	1	0	0
Autoimmune disease				
Total	75 (54.0)	188 (100)	1 (2.6)	0 (0)
SLE	58 (41.7)	106 (56.4)	1 (2.6)	–
SS	8 (5.8)	30 (16.0)	1 (2.6)	–
Other	10 (7.2)	58 (30.9)	–	–
Thrombosis				
Total	139 (100)	0 (0)	38 (100)	0 (0)
Arterial	80 (57.6)	–	21 (55.3)	–
Venous	72 (51.8)	–	20 (52.6)	–
aPL positive				
Total	139 (100)	74 (39.4)	0 (0)	0 (0)
aCL	93 (66.9)	43 (22.9)	0 (0)	–
Anti-β_2GPI	79 (56.8)	29 (15.4)	0 (0)	–
LAC	89 (64.0)	47 (25.0)	0 (0)	–
Antithrombotic therapy				
Total	103 (74.1)	54 (28.7)	29 (76.3)	0 (0)
Anticoagulant	58 (41.7)	52 (27.7)	6 (15.8)	–
Antiplatelet	63 (45.3)	3 (1.6)	23 (60.5)	–

* Except where indicated otherwise, values are the number (%). APS = antiphospholipid syndrome; SLE = systemic lupus erythematosus; SS = Sjögren's syndrome; aPL = antiphospholipid antibody; aCL = anticardiolipin antibody; LAC = lupus anticoagulant.
† One sample from this group was subsequently withdrawn from analysis because standard enzyme-linked immunosorbent assay revealed it to be deficient in β_2-glycoprotein I (β_2GPI).

Healthy control group. Samples from 93 healthy controls were collected, 92 of which were analyzed (28 from Sydney, 35 from Athens, and 29 from Sapporo). One healthy control sample was found to be deficient in β_2GPI by standard enzyme-linked immunosorbent assay (ELISA) and was withdrawn from the study.

Demographic and clinical details of the study groups are summarized in Table 1. Institutional ethics approval for patient sampling was attained from each center participating in the study, and informed consent was obtained from all subjects prior to venipuncture. Assays were performed under blinded conditions with regard to the underlying diagnosis.

Chemicals and reagents. HEPES and streptavidin beads were purchased from Sigma. *N*-(3-maleimidylpropionyl) biocytin (MPB) was purchased from Invitrogen. All other chemicals were of reagent grade.

Proteins. Bovine serum albumin (BSA), alkaline phosphatase (AP)–conjugated anti-mouse IgG, AP-conjugated anti-rabbit IgG, and AP-conjugated anti-human IgG were from Sigma. Purified native human β_2GPI was from Haematologic Technologies and also sourced as a kind gift from Dr. Inger Schousboe (University of Copenhagen, Denmark). Affinity-purified murine IgG2 anti-β_2GPI monoclonal antibody (mAb) 4B2E7 (previously designated "mAb number 16") and affinity-purified rabbit anti-β_2GPI polyclonal antibody were produced as previously described (17,18). Isotype control rabbit polyclonal IgG was purchased from BD PharMingen.

Assay for quantifying the absolute proportion of serum β_2GPI that can be labeled with MPB. With the demonstration that β_2GPI exists in vivo in a reduced state with free thiols (13), it was then pertinent to determine the absolute proportion of total β_2GPI that circulates in this reduced state. This was done in experiments with a sample of pooled serum derived from 10 healthy volunteers. The sex and age distribution of the pooled serum sample was chosen to match the APS disease group.

MPB-labeled and non–MPB-labeled serum samples were acetone precipitated to remove free MPB as described previously (13). The protein pellets were then dissolved in phosphate buffered saline (PBS)–0.1% Tween to a final dilution of 4,000-fold (total volume 1,400 μl), and streptavidin beads (50 μl) were added. After incubation with streptavidin beads (1 hour at 4°C), the beads were removed by centrifugation for 2 minutes at 3,000*g* and the supernatants assayed for β_2GPI. The proportion of β_2GPI that was labeled with MPB was calculated as (optical density at 405 nm [OD_{405}] of the biotin-depleted MPB-labeled sample/OD_{405} of the biotin-depleted non–MPB-labeled sample) × 100. Validation of this method is described in full in the supplementary information (available in the online version of this article at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

Assay for quantifying total human β_2GPI. A sandwich ELISA for quantifying total β_2GPI levels within serum/plasma samples was performed based on a previously published method (19), with modifications. Briefly, a high-binding 96-

well plate was coated overnight at 4°C with rabbit polyclonal anti-human β_2GPI (10 n*M*/well). Plates were washed 4 times with PBS–0.1% Tween and then blocked with 2% BSA/PBS–0.1% Tween for 1 hour at room temperature. Following washing, 100 μl of anti-human β_2GPI mouse mAb (clone 4B2E7) was added (10 n*M*/well, diluted in 0.25% BSA/PBS–0.1% Tween) and then 100 μl of the patient sample diluted 4,000-fold in PBS–0.1% Tween was coincubated for 1 hour at room temperature. After washing 4 times with PBS–0.1% Tween, AP-conjugated goat anti-mouse IgG was added (1:1,500 dilution) and incubated for 1 hour at room temperature, and samples read at OD_{405} after addition of chromogenic substrate. An in-house standard, consisting of pooled serum from 10 healthy controls, was used to construct a standard curve for every ELISA. The level of β_2GPI in the pooled-serum in-house standard was determined initially using a β_2GPI in-house standard curve and then validated with a calibrator from a commercially available β_2GPI quantification kit (Hyphen BioMed). Each new batch of the pooled-serum in-house standard was recalibrated against the commercial calibrator. Samples were assayed in duplicate.

Within-plate coefficients of variation (CVs) for this ELISA were calculated by running 10 duplicates of the same patient sample on a single plate. Between-plate CVs were calculated by taking 10 independent assays performed consecutively on separate days and calculating the CV based on the variation of the number obtained by dividing the OD of the standard at 4,000-fold dilution by the OD of the standard at 8,000-fold dilution for each plate.

Assay for measuring the relative amount of β_2GPI with free thiols within patient samples as compared to a pooled-serum in-house standard sample. The amount of β_2GPI with free thiols in patient samples relative to the standard sample was assayed as previously described (13), with minor modifications. Measurement of the amount of β_2GPI that is reduced is based on labeling of free thiols of β_2GPI with the biotin-conjugated selective free thiol binding reagent MPB, capturing biotin-labeled proteins on a streptavidin plate, and detecting the presence of MPB-labeled β_2GPI with a specific anti-β_2GPI mAb. The mean ± SD within-plate CV for this ELISA is 5.08 ± 3.09%, and the between-plate CV is 6.25% (13).

MPB (4 m*M*) was added to 50 μl of patient plasma or serum and incubated for 30 minutes at room temperature in the dark with agitation, diluted 50-fold in 20 m*M* HEPES buffer (pH 7.4), and incubated for a further 10 minutes at room temperature in the dark. Unbound MPB was then removed by acetone precipitation. The protein pellet was resuspended in PBS–0.05% Tween (final dilution 100-fold). The samples were then diluted a further 40-fold (4,000 times final), added in duplicate to a streptavidin-coated 96-well plate (100 μl/well; Nunc), and incubated for 90 minutes at room temperature. Prior to addition of MPB-labeled serum samples, streptavidin-coated plates were washed 3 times with PBS–0.1% Tween and blocked with 2% BSA/PBS–0.1% Tween. After washing 3 times with PBS–0.1% Tween, the murine anti-β_2GPI mAb (clone 4B2E7) was added (25 n*M*) and incubated for 1 hour at room temperature. After 3 further washings with PBS–0.1% Tween, AP-conjugated goat anti-mouse IgG (1:1,500 dilution) was added for 1 hour at room temperature and samples read at 405 nm after addition of chromogenic substrate. For each experiment, the pooled in-house standard used for the above-described β_2GPI quantification ELISA was MPB labeled, acetone precipitated, and used as an internal control and standard. The degree of MPB labeling in each patient sample was expressed as a percentage of that observed with the pooled in-house standard, after correction for the total amount of β_2GPI. The proportion of non–MPB-labeled β_2GPI represents the oxidized form of the molecule.

Statistical analysis. Box plots were created to depict the distributions of β_2GPI across groups. Medians and interquartile ranges (IQRs) were calculated. For comparisons between individual samples, the Mann-Whitney U test was used. Odds-ratios (ORs) and 95% confidence intervals (95% CIs) of exposure or disease incidence were computed using logistic regression. Adjustment for age and sex was carried out to remove potential confounders linked to these predictors.

RESULTS

A significant proportion of β_2GPI in vivo in healthy volunteers circulates in the reduced form. We have recently demonstrated that β_2GPI circulates in vivo in a reduced form (13), and we therefore wished to determine the absolute proportion of β_2GPI that is in this biochemically reduced state. This was investigated using a sample of human serum pooled from 10 healthy volunteers. Figure 1 shows that a mean of 45.6% of β_2GPI in pooled serum from healthy subjects was labeled with the biotin-conjugated free thiol binding reagent MPB. Validation of this method is demonstrated in detail in Supplementary Figure 1, available in the online version of this article at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

Total β_2GPI levels are elevated in APS and are associated with thrombogenic pathogenicity in aPL-positive patients. Given that biochemically reduced β_2GPI was found to represent a large proportion of circulating β_2GPI in healthy subjects, it was then relevant to ascertain whether this level was altered in patients with APS as compared to both disease control and healthy control groups. Serum or plasma levels of total β_2GPI were quantified in each individual patient sample so that a relative proportion of reduced and oxidized β_2GPI could be calculated for each sample.

The assay used for detecting total levels of β_2GPI in patient serum and plasma was optimized for use with in-house anti-β_2GPI antibodies, as shown in Supplementary Figure 2 (http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131). The within-plate CV for this assay was 5.8% and the between-plate CV was 3.3%, indicating good reproducibility.

The median level of total β_2GPI in the healthy control group was 178.4 μg/ml (IQR 149.4–227.5) (n = 91). In addition to healthy controls, an autoimmune

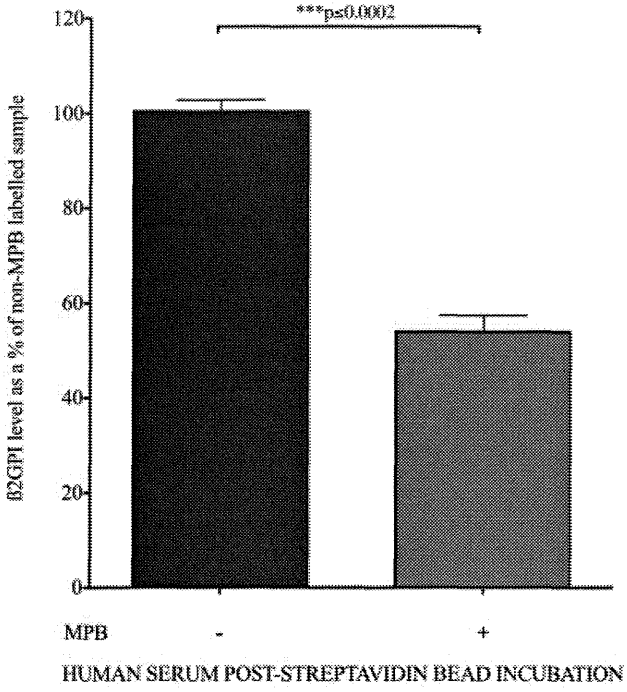

Figure 1. Beta-2-glycoprotein I (β_2GPI) with free thiols represents a large proportion of total circulating β_2GPI in vivo. Pooled serum from 10 healthy volunteers was labeled with *N*-(3-maleimidylpropionyl) biocytin (MPB) (4 m*M*) or treated with control buffer alone, after which the MPB-labeled proteins were depleted by incubation with streptavidin beads. Both samples were then centrifuged at 3,000*g* for 10 minutes to remove the beads, and an enzyme-linked immunosorbent assay for total β_2GPI was performed on the supernatant of both MPB-labeled and non–MPB-labeled samples post–streptavidin incubation. The relative reduction (in optical density) of the MPB-labeled sample as compared to the non–MPB-labeled sample indicates the relative amount of β_2GPI with free thiols labeled with MPB. Values are the mean ± SD.

disease control group (autoimmune disease with or without aPL but without APS) and a clinical event control group (thrombosis without aPL) were included, as described above. As shown in Figure 2A, the concentration of total β_2GPI was significantly higher in the APS group (median 216.2 μg/ml [IQR 173.3–263.8]) (n = 139) as compared to the healthy control group ($P < 0.0002$), the autoimmune disease control group ($P < 0.0001$), and the clinical event control group ($P < 0.0001$). Compared to healthy controls, cases were twice as likely to have an elevated β_2GPI level (defined as plasma levels ≥200 μg/ml). The effect remained after adjustment for age and sex (OR 2.2 [95% CI 1.2–3.9]). Given that the odds ratios of disease and of exposure can be considered the same, this translates to a 2-fold increase in thrombosis for patients with elevated β_2GPI

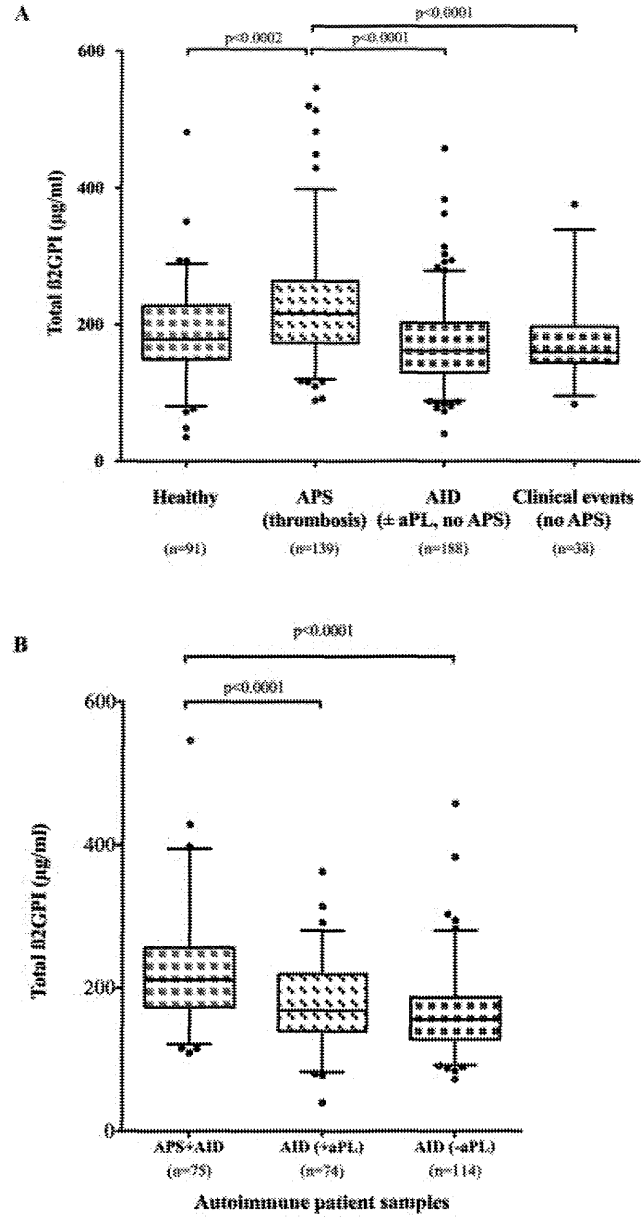

Figure 2. Elevated levels of β_2-glycoprotein I (β_2GPI) in patients with the antiphospholipid syndrome (APS). **A,** Total β_2GPI in the serum of patients with thrombosis-associated APS and in the serum of patients in the 3 control groups, i.e., healthy controls, patients with autoimmune disease (AID) with or without antiphospholipid antibodies (aPL) but without APS, and patients with clinical thrombotic events without APS. **B,** Total β_2GPI in the serum of patients in the APS group who had an autoimmune disease compared to patients in the autoimmune disease control group who were positive for aPL and patients in the autoimmune disease control group who were negative for aPL. Elevated levels of β_2GPI were demonstrated only when aPL positivity was combined with a thrombotic clinical event. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles. Circles indicate outliers.

levels, in the absence of further confounding effects. The association was stronger when the comparison was with the control group consisting of patients with autoimmune disease with or without aPL (OR 4.6 [95% CI 2.9–7.5]). It is also possible to treat total β_2GPI as a continuous variable in the model. When this was done, the results were consistent with the other findings (i.e., there was a strong positive association between total β_2GPI level and thrombosis risk).

Figure 2B shows that elevated β_2GPI levels were observed only when persistent aPL positivity was combined with a thrombotic event, thus fulfilling classification criteria for APS. Levels of β_2GPI in the autoimmune disease controls (without thrombotic events) with persistent aPL did not differ from levels in autoimmune disease controls without aPL, and also were not different from levels in healthy controls.

Subgroup analysis of the total level of β_2GPI within the APS group revealed no differences between those with and those without an additional autoimmune disease. Furthermore, there was no difference between those with arterial thrombosis and those with venous thrombosis (Supplementary Figure 3, http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

APS is associated with a greater proportion of β_2GPI being in an oxidized state. Each patient sample was labeled with MPB, and the amount of β_2GPI in the reduced form was compared and expressed as a percentage of that observed in a pooled standard (derived from 10 healthy volunteers who were matched for age and sex with the APS group), after correction for the total amount of β_2GPI. The same in-house pooled standard was used for every MPB labeling experiment and assay. The sensitivity for detecting reduced β_2GPI with this assay extends to a dilution of >128,000-fold, indicating marked sensitivity (Figure 3). The linear range was found to be between dilutions of 400- and 128,000-fold. The dilution found to yield ~50% of maximum OD was found to be 1:4,000, and hence this dilution was used to screen all patient samples for reduced β_2GPI. This assay has previously been shown to yield identical results when serum and plasma sampled from the same patient are tested in parallel (13).

Figure 4 shows that the relative proportion of β_2GPI in the reduced form, expressed as a percentage of that observed with the in-house standard, was significantly less in APS patients presenting with vascular thrombosis as compared to healthy controls, autoimmune disease controls, and clinical event controls (all

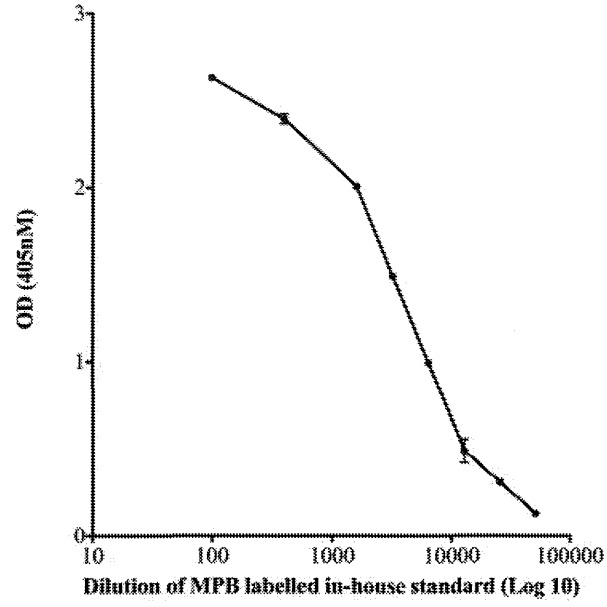

Figure 3. High level of sensitivity of the assay for quantifying relative amounts of reduced β_2GPI. Pooled human serum from healthy volunteers (n = 10) was labeled with MPB, and a streptavidin-coated plate–based enzyme-linked immunosorbent assay for reduced β_2GPI was performed on varying dilutions of this labeled sample, as described in Patients and Methods. The linear range for this assay was at dilutions between 1:400 and 1:128,000. OD = optical density (see Figure 1 for other definitions).

$P < 0.0001$). Thus, β_2GPI in APS patients presenting with thrombosis is in an oxidized state relative to each of the other 3 control groups. Similar to the findings in the analysis of total β_2GPI, a lower level of the reduced β_2GPI (proportion ≤50%) was associated with a greater risk of thrombosis. An OR of 4.1 (95% CI 1.9–8.8) in relation to healthy subjects was observed after adjustment for age and sex. A similar but somewhat smaller effect (OR 2.0 [95% CI 1.2–3.4]) was also obtained when the reference group was patients with autoimmune disease with or without aPL but without thrombosis.

Patient positivity for lupus anticoagulant (LAC) activity has been reported to be a strong predictor of thrombosis compared to anti-β_2GPI or anticardiolipin antibodies without LAC activity, particularly with regard to arterial thrombosis and the development of stroke (4,20). Subgroup analysis of the various aPL subtypes within the APS group revealed that the proportion of β_2GPI circulating in the reduced state was significantly lower in the APS patients who were positive for both anti-β_2GPI and LAC as compared to those positive for anti-β_2GPI but not LAC (median 53.58% [IQR 39.18–73.56] [n = 45] versus 74.80% [IQR 60.69–84.51] [n =

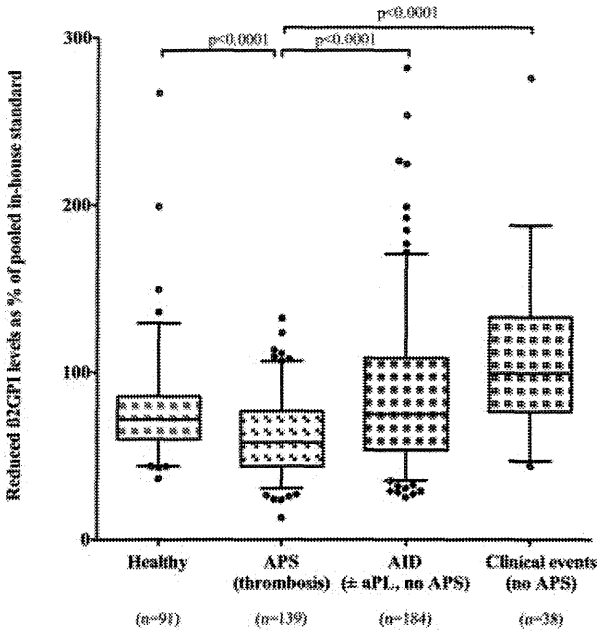

Figure 4. Circulation of β_2GPI in an oxidized form in patients with APS. Levels of β_2GPI in the reduced form were assayed and expressed as a percentage of that observed in an in-house standard (pooled serum from 10 healthy volunteers) after correction for the total amount of β_2GPI. The same pooled standard was used throughout. APS patients presenting with thrombosis had significantly lower amounts of β_2GPI in the reduced form as compared to each of the 3 control groups. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles. Circles indicate outliers. See Figure 2 for definitions.

29]; $P \leq 0.001$) (Figure 5). Interestingly, levels of β_2GPI were also lower in APS patients presenting with arterial thrombosis only (median 53.81% [IQR 39.38–74.62] [n = 67]) versus those presenting with venous thrombosis only (62.09% [IQR 49.64–83.11] [n = 59]) ($P < 0.045$), as shown in Supplementary Figure 4, http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

DISCUSSION

This is, to our knowledge, the first reported demonstration that the redox state of the autoantigen β_2GPI, in conjunction with plasma concentration levels, is different in APS patients compared to healthy or disease control subjects. Our study is the first to definitively confirm that β_2GPI levels are elevated in APS patients—both those with and those without an additional autoimmune disease—as compared to healthy and disease control groups. The finding of elevated levels of β_2GPI was observed by our group previously, albeit utilizing far lower numbers of patients (19). In addition, it is reported herein that levels of oxidized β_2GPI are elevated in APS patients compared to healthy and disease controls. A novel assay to measure relative amounts of reduced β_2GPI, as well as the ELISA for total β_2GPI, had good reproducibility and demonstrated strong associations with the APS disease phenotype. The robust nature of these findings is highlighted by the large numbers of well-characterized patients (>450) screened through this large international collaborative multicenter effort coupled with the use of both healthy and 2 distinct disease control groups. Such assays that precisely quantify the amount of posttranslationally modified autoantigen are unique in the field of APS, and even autoimmunity.

An extensive number of in vitro and in vivo studies suggest that anti-β_2GPI autoantibodies in complex with β_2GPI directly contribute to the APS clinical phenotype of thrombosis (5). In the present study, we

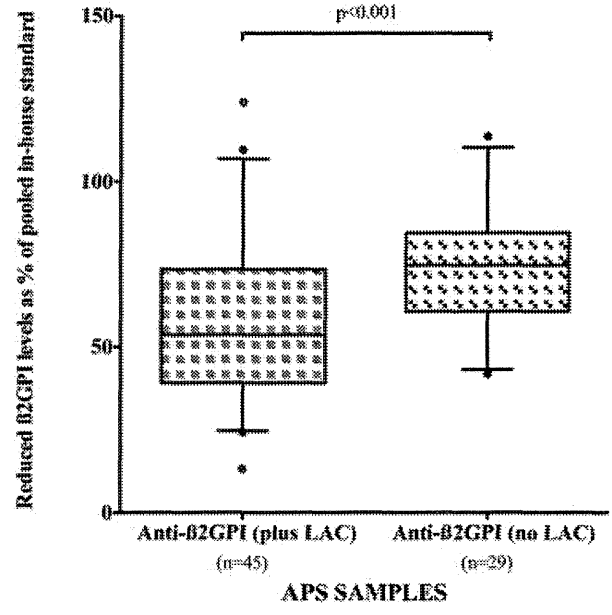

Figure 5. Association of positivity for anti-β_2GPI combined with lupus anticoagulant (LAC) with an elevated proportion of β_2GPI circulating in an oxidized state. Samples from APS patients presenting with vascular thrombosis who were positive for both anti-β_2GPI and LAC had significantly lower amounts of β_2GPI in the reduced form as compared to those from patients who were positive for anti-β_2GPI but not for LAC. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles. Circles indicate outliers. See Figure 2 for other definitions.

have demonstrated that patients who are persistently positive for aPL and have the clinical features of APS have higher levels of total and oxidized β_2GPI compared to controls. It is reasonable to hypothesize that clinical states associated with an increased oxidative stress load, such as pregnancy and infection (21), may lead to further increases in the levels of oxidized β_2GPI in the plasma, potentially elevating the risk of pathologic thrombosis in patients who are positive for anti-β_2GPI antibodies. This is based on the premise that an increased plasma load of oxidized β_2GPI may lower the threshold for provoking an anti-β_2GPI autoantibody–mediated dysregulated prothrombotic response. A recent study demonstrated that oxidative stress may drive β_2GPI production in vivo through activator protein 1 and NF-κB–mediated up-regulation of β_2GPI gene promoter activity (22). Hence, an enhanced oxidative stress load may increase antigenic load, potentially driving anti-β_2GPI production in autoimmunity-prone subjects and lowering the threshold for a clinical event. This hypothesis supports a rationale as to why SLE in particular is associated with anti-β_2GPI antibodies, given that this condition is characterized by a propensity toward autoreactivity, B cell hyperactivity, and oxidative stress (23,24).

It was recently shown that β_2GPI with free thiols protects endothelial cells against oxidative stress–induced cell injury, whereas oxidized β_2GPI (which lacks free thiols) has no such protective effect (13). Given the present finding that a significant proportion of circulating β_2GPI is in this protective reduced form in healthy individuals, it may be reasonable to hypothesize that the relative abundance of oxidized β_2GPI in APS lowers the threshold for development of vascular thrombosis. If this hypothesis is correct, then one would expect elevated levels of oxidized β_2GPI to represent an independent risk factor for thrombosis. Analysis of posttranslational modifications of β_2GPI on patient samples collected prospectively and subsequent determination of the presence or absence of a thrombotic event would allow for predictive calculations that could be used to test such a hypothesis.

With the development of novel assays to detect and quantify plasma β_2GPI–related redox changes, it is expected that stratification of anti-β_2GPI antibody–positive individuals for thrombotic risk according to the levels of total, reduced, and oxidized β_2GPI may be possible, with the attendant potential opportunity for implementing medical prophylactic measures during these periods of elevated risk. Prospective longitudinal studies aimed at validating the predictive and diagnostic role of such an approach are needed.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Krilis had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Ioannou, Zhang, Lau, Vlachoyiannopoulos, Moutsopoulos, Atsumi, Giannakopoulos, Krilis.

Acquisition of data. Ioannou, Zhang, M. Qi, Gao, J. C. Qi, Lau, Sturgess, Vlachoyiannopoulos, Moutsopoulos, Rahman, Pericleous, Atsumi, Giannakopoulos, Krilis.

Analysis and interpretation of data. Ioannou, Zhang, M. Qi, Gao, J. C. Qi, Yu, Lau, Vlachoyiannopoulos, Moutsopoulos, Rahman, Atsumi, Koike, Heritier, Giannakopoulos, Krilis.

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Pathophysiology of Thrombosis and Potential Targeted Therapies in Antiphospholipid Syndrome

Olga Amengual, Tatsuya Atsumi* and Takao Koike

Department of Medicine II, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Abstract: The antiphospholipid syndrome (APS) is an autoimmune disease in which recurrent vascular thrombosis, pregnancy morbidity or a combination of these events is associated with the persistent presence of circulating antiphospholipid antibodies (aPL). Evidence shows that the dominant antigenic targets for aPL in APS are phospholipid-binding plasma proteins such as β2glycoprotein I and prothrombin.

The pathogenic role of aPL in thrombosis is widely accepted but the mechanisms by which these antibodies mediate disease are only partially understood. aPL may affect the normal procoagulant and anticoagulant reactions occurring on cell surface, and also may interact with certain cells, altering the expression and secretion of procoagulant substances.

The intracellular signal transduction triggered by aPL has been a focus of intensive research and the p38 mitogen activated protein kinase (MAPK) pathway has been revealed as a major player in the aPL-mediated cell activation. In addition, some candidates as cell-receptor for phospholipid-binding plasma proteins have been identified. The recognition of the intracellular signaling triggered by aPL is a step forward in the design of new modalities of targeted therapies for thrombosis in APS including specific inhibitors of MAPK pathway or antagonists of the putative receptors. Furthermore, novel findings regarding the role of aPL in T-cells responses mark new advances in the understanding of the immunological reactions in APS and open new insights into possible therapeutic approaches to APS.

In this article, we review the pathophysiological mechanisms of thrombosis and the specific new targeted therapies for the treatment in APS.

Keywords: Antiphospholipid antibodies, p38MAPK, β2GPI, prothrombin, tissue factor.

INTRODUCTION

The antiphospholipid syndrome (APS), also known as Hughes' syndrome, is an autoimmune and multisystem disorder characterized by vascular thrombosis and pregnancy morbidity in association with the persistent laboratory evidence for antiphospholipid antibodies (aPL) [1, 2].

The APS was initially characterized in patients with systemic lupus erythematosus (SLE), but it can also occur in the absence of autoimmune diseases [3]. The most common clinical manifestations of the APS are venous thrombosis, particularly deep vein thrombosis in the lower extremities, followed by cerebral infarction or transient ischemic attacks [4]. Antiphospholipid syndrome represents one of the major risk factors for ischaemic cerebral events in young people without congenital atherosclerotic diseases [5].

Recurrent fetal losses in APS may happen at any stage of pregnancy, but are strikingly frequent during the second or third trimester. APS patients are susceptible to early onset of pregnancy complications such as severe pre-eclampsia and HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count) [2, 4, 6]. Moreover, aPL may be found in up to 20% of woman with recurrent pregnancy losses [7]. Other aPL-related abnormalities include non-thrombotic neurological syndromes, psychiatric manifestations, skin ulcers, livedo reticularis, haemolytic anemia, thrombocytopenia, nephropathy, pulmonary hypertension and heart valve abnormalities [4].

A minority of patients with APS develops an accelerated form of this syndrome with life-threatening multiple organ thromboses, severe thrombocytopenia and adult respiratory distress syndrome recognized as catastrophic APS [8].

The original concept of aPL considers that those antibodies were directed against anionic phospholipids. However, it is now well established that aPL include a heterogeneous group of circulating immunoglobulins and that proteins that bind to anionic phospholipids, such as beta2 Glycoprotein I (β2GPI) and prothrombin, are the main antigenic targets recognized by aPL in patients with APS [9-12].

aPL can be broadly categorized into those antibodies that bind to immobilized anionic phospholipid in solid phase enzyme linked immunosorbent assay (ELISA), known as anticardiolipin antibodies (aCL) [13], or those that prolong phospholipid-dependent coagulation assays, called lupus anticoagulant (LA) [14]. New assays have been developed for the detection of antibodies targeting phospholipid-binding protein complexes, comprising antiβ2GPI antibodies [15-17] and antiprothrombin antibodies [18-21].

*Address correspondence to this author at the Department of Medicine II, Hokkaido University Graduate School of Medicine, N15 W7, Kita-ku, Sapporo 060-8638, Japan; Tel: + 81-11-706-5915; Fax: + 81-11-706-7710; E-mail: at3tat@med.hokudai.ac.jp

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In 1998, an international consensus on classification criteria for definite APS was met in Sapporo; the criteria were thus called Sapporo criteria [2], and they were revised in 2006 [22]. The diagnosis of APS is made when at least 1 of the 2 clinical criteria (vascular thrombosis or pregnancy morbidity) occurs in a patient whose laboratory tests for aPL are positive (Table 1).

Table 1. Revised Classification Criteria for the Antiphospholipid Syndrome [2]

Clinical Criteria
1. Vascular thrombosis
≥ 1 clinical episodes of arterial, venous, or small vessel thrombosis, in any tissue or organ confirmed by objective validated criteria by imaging studies or histopathology in the absence of significant evidence of inflammation in the vessel wall.
2. Pregnancy morbidity
≥ 1 unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation, or,
≥ 1 premature births of a morphologically normal neonate before the 34th week of gestation due to eclampsia, severe pre-eclampsia or placental insufficiency, or
≥ 3 unexplained consecutive spontaneous abortions before the 10th week of gestation (maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded).
Laboratory Criteria
a) LA present in plasma, on ≥ 2 occasions at least 12 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Haemostasis [14, 23]
b) IgG and/or IgM aCL present in medium or high titer in serum or plasma, on ≥ 2 occasions at least 12 weeks apart, measured by a standardized ELISA [24]
c) IgG and/or IgM antiβ2glycoprotein I antibodies present in titer >99th percentile, in serum or plasma, on ≥ 2 occasions at least 12 weeks apart, measured by a standardized ELISA [25]

Antiphospholipid syndrome is present if at least 1 of the clinical criteria and 1 of the laboratory criteria are met. ELISA: enzyme-linked immunosorbent assay.

Numerous mechanisms have been proposed to explain the pathogenicity of aPL in APS as shown in Table 2.

Table 2. Proposed Mechanisms of Antiphospholipid Antibody-Mediated Thrombosis

1. Interference with the coagulation pathway:
a) Protein C pathway:
b) Contact activation pathway
c) β2GPI-thrombin interaction
d) Protein Z pathway
2. Disruption of fibrinolysis
3. Cell interaction:
a) Induction of pro-coagulant activity on endothelial cell and monocytes
b) Pro-coagulant effects on platelets
c) Release of membrane-bound microparticles
4. Complement activation

Genetic and acquired factors may trigger to develop thrombosis in susceptible individuals. However, it is yet not possible to assert whether a dominant mechanism is responsible for some specific clinical manifestations of APS or whether different antibodies acting together predispose to thrombosis [26].

Primary and secondary thrombosis prevention is crucial in APS, but treatment is conditioned by the lack of appropriate studies due to the poor laboratory standardization.

This article summarizes some of the major pathophysiological mechanisms that may contribute to the APS manifestations. In addition, the current modalities of treatment and the potentially specific new targeted therapies for APS are reviewed.

PATHOPHYSIOLOGY OF APS

Multifactorial thrombotic mechanisms, such as the inhibition of the natural anticoagulant systems, the impairment of fibrinolysis and the direct effect of aPL on cell functions, are involved in the development of thrombosis in APS. Evidence suggests that complement activation is also required for aPL-mediated tissue injury [27].

1. Interference with the Coagulation Pathway

The coagulation system is an amplification cascade of enzymatic reactions resulting in thrombin formation. Thrombin has several prothrombotic properties and also activates protein C. Protein C is a major constituent of the anticoagulant system and its impairment may lead to blood clot. Thrombin triggers the protein C system by binding to thrombomodulin and initiating rapid protein C activation. Activated protein C complexes with protein S on the surface of either platelets or endothelial cells. These complexes proteolytically catalyze the inactivation of activated factors V and VIII. Because both protein C and protein S are phospholipid-binding plasma proteins, this system could be one of the most likely to be involved in development of thrombosis in the APS.

aPL may interfere with the protein C pathway in different ways. aPL have been reported to inhibit both the activation of protein C by the thrombin-thrombomodulin complex [28] and the activated protein C-catalysed inactivation of activated factor V [29-33]. The inhibitory effect of IgG purified from patients with aPL on activated factor V inactivation mediated by activated protein C was subsequently confirmed [30, 34]. Rabbit polyclonal [35] and human monoclonal antiβ2GPI antibodies inhibit activated protein C function [32]. Moreover, aCL bound to protein C in the presence of both phospholipids and β2GPI, and binding activities strongly correlated with antiβ2GPI antibody titers, indicating that protein C might be a target of aCL resulting in the protein C dysfunction [36]. Izumi *et al.* [37] confirmed the inhibitory effect of antiβ2GPI antibodies purified from APS patients on the anticoagulant activity of activated protein C. Those authors demonstrated that binding of β2GPI to the phospholipid membrane surface is necessary to express this inhibitory activity

Most prothrombin-antiprothrombin immune complexes may predispose to thrombosis by interfering with the inactivation of activated factor V by the activated protein C in the presence of protein S [38]. This inhibitory effect of anti-

prothrombin antibodies on activity of the activated protein C was also demonstrated in the absence of protein S [39].

Finally, aPL may alter the effect of protein S in the protein C pathway. Decreased levels of protein S have been detected in plasma from APS patients [40, 41]. Some of the IgG that inhibit activated factor V degradation were directed not only to phospholipid-bound protein C but also to phospholipid-bound protein S [42].

There are some conflicting data between studies regarding the involvement of protein C pathway in the pathophysiology of APS, probably related to the heterogeneity of aPL found in patients with APS. The thrombotic predisposition for individual patient will vary depending on antibody-dependent and independent variables.

Other possible mechanism of thrombosis in APS is the interference of aPL with the contact pathway of coagulation. This coagulation pathway is initiated by the activation of factor XII by negative charged surfaces, then activated factor XII cleaves factor XI to activated factor XI in the presence of high-molecular kininogen and prekalikrein. β2GPI inhibited the phospholipid-mediated autoactivation of factor XII and the contact activation pathway of coagulation [43]. Further, β2GPI directly binds to factor XI and inhibits activation of factor XI by thrombin and activated factor XII; this inhibition attenuates thrombin generation [44]. Monoclonal antiβ2GPI antibodies enhanced the inhibition of factor XI activation by β2GPI and thrombin complex [45].

Thrombin, the final enzyme in the coagulation cascade, is generated from its inactive precursor prothrombin by activated factor X as part of the prothrombinase complex, on the surface of activated cells. Apart of the prothrombotic properties, thrombin is also involved in the regulation of many biological functions *in vivo*. β2GPI participates in thrombin generation as demonstrated by the significant reduction of *in vitro* ability to generate thrombin observed in plasma from β2GPI–null mice [46]. β2GPI directly binds to thrombin [45], indicating that β2GPI-thrombin interaction may interfere not only with the coagulation system but also with many of the biologic functions in which thrombin participates.

The inhibition of protein Z, has been proposed as an additional thrombotic mechanism in APS. Protein Z is a vitamin K dependent protein that functions as a natural anticoagulant. Protein Z serves as cofactor for the inactivation of activated factor X by the plasma protein Z-dependent protease inhibitor [47]. Reduced plasma levels of protein Z were detected in patients with aPL [48, 49] and were associated with thrombosis [50]. In the presence of β2GPI, aPL greatly impair the inhibition of activated factor X by protein Z-dependent protease inhibitor [51].

2. Disruption of Fibrinolysis

The fibrinolytic system involves the formation of plasmin from plasminogen by the tissue-type plasminogen activator (tPA); this, in turn, degrades fibrin into fibrin degradation products. The regulation of plasmin generation and activity is highly important to maintain the homeostatic balance *in vivo*. Inhibition of the fibrinolytic system may occur at the level of plasminogen activators by specific plasminogen activator inhibitors (PAI-1 and PAI-2) or at the levels of plasmin by α2-antiplasmin. Endothelial cells when activated, secrete the PAI-1 to regulate fibrinolysis by blocking tPA activity.

The effect of aPL in the fibrinolytic system has been investigated with controversial results probably due to the heterogeneity of the cohorts. Several other reports pointed toward a hypofibrinolytic state in APS characterized by elevated PAI-1 indicating a perturbation of endothelial cells with consequent fibrinolytic impairment [52-54]. Patients with connective tissue diseases, including APS, might have a hypofibrinolytic condition related to high PAI-1 levels [55]. Ames *et al.* [40] showed up-regulation of PAI-I in females with primary APS. They further showed a reduction in tPA release by endothelial cell stimulation, suggesting that tPA/PAI-1 balance was decisive to develop thrombosis in some APS patients. Monoclonal aCL appear to inhibit fibrinolysis by a β2GPI-dependent increase in PAI-1 activity [56]. Monoclonal antiβ2GPI antibodies significantly suppressed the intrinsic fibrinolytic activity *in vitro*. The inhibition was attributed to a reduced contact activation reaction started by activated factor XII [57]. Impaired activated factor XII-dependent activation of fibrinolysis was observed in pregnant woman with APS which developed late-pregnancy complications [58]. Antibodies against the catalytic domain of tPA were found in patients with APS and might represent a cause of hypofibrinolysis [59].

On the other hand, aPL might directly inhibit plasmin activity. High affinity antiplasmin antibodies that inhibit degradation of fibrin have been detected in patients with APS [60]. Moreover, IgG from APS patients significantly retard fibrin dissolution by plasmin [61].

Finally, the influence of lipoprotein a (Lp(a)) in the fibrinolytic system has been evaluated. Lp(a) inhibits fibrinolysis by acting as an uncompetitive inhibitor of tPA, but also by increasing PAI-1 expression in endothelial cells [62-65]. This behaviour confers a prothrombotic potential to Lp(a). Plasma levels of Lp(a) were found to be significantly increased in patients with APS [64, 65]. Further, patients with maximal elevation of Lp(a) showed a reduced fibrinolytic activity, estimated by low D-Dimer and high PAI-1 levels [64].

3. aPL and Cell Interaction

Damaged and/or activated endothelial cells or monocytes are predominant targets of aPL. Cultured endothelial cells incubated with aPL expressed high levels of adhesion molecules [66, 67], tissue factor (TF) [68-71] and endothelin-1 [72]. This effect is mediated by β2GPI and cell surface receptors and may promote inflammation and thrombosis [73, 74]. Prothrombin also binds to endothelial cells, and this binding was enhanced by a human monoclonal IgG antiprothrombin antibody, IS6. IS6 up-regulates expression of TF and E-selectin on endothelial cells [75].

The production of microparticles is a hallmark of cell activation, and aPL stimulated the release of microparticles from endothelial cells [76]. Finally, some aPL that recognize annexin-V are able to induce apoptosis on endothelial cells [77].

Platelets are prone to agglutinate and aggregate after exposure to aPL [78], and circulating activated platelets have been found in patients with APS [79, 80]. β2GPI binds to surface membranes of activated platelets and inhibits the generation of activated factor X [81-83] Antiβ2GPI antibodies interfered with this inhibition [82].

The cell activation mediated by aPL requires the interaction between phospholipid- binding plasma protein complexes and probably a specific cell receptors. A number of potential receptors for the binding of β2GPI to cellular membranes have been identified including annexin A2, apolipoprotein E receptor 2 (ApoER2'), low-density-lipoprotein receptor-related protein, megalin, toll like receptor (TLR) 2, TLR 4, the very-low-density-lipoprotein receptor and P-selectin glycoprotein ligand-1. β2GPI directly binds to glycoprotein Ibα subunit of the platelet adhesion receptor glycoprotein (GP)Ib/IX/V and to the platelet factor 4 [84-92]. It seems very unlikely that so many different receptors will be substantial involved in the pathophysiology of thrombosis in APS. Further studies are necessary to clarify the biological and pathological roles of those receptors in the aPL-mediated cell activation in patients with APS.

The signal transduction mechanisms involved in aPL mediated cell activation have been the centre of interest for many researchers. The adapter molecule myeloid differentiation protein (MyD88)-dependent signaling pathway and the nuclear factor kappa B (NFkB) have been involved in endothelial cell activation by aPL [86, 93-95]. The p38 mitogen-activated protein kinase (MAPK) pathway is an important component of intracellular signaling cascades that initiate various inflammatory responses. The p38MAPK pathway has a crucial role in mediating the effects of aPL on cell activation. Monoclonal antiβ2GPI antibodies from APS patients induce phosphorylation of p38MAPK, a locational shift of NFkB into the nucleus and up-regulation of TF on monocytes [96]. The importance of the p38 MAPK pathway in cell activation was also reported in platelets [97], and endothelial cells [98]. Activation of p38 MAPK pathway increases activities of cytokines such as tumor necrosis (TNF)α and interleukin (IL)-1β and macrophage inflammatory cytokine 3β [96, 99, 100].

4. Complement Activation and aPL

Complement activation was determined to be relevant to the pathophysiology of APS, especially with regard to pregnancy morbidity [101]. Placenta trophoblast cells are targeted by phospholipid-binding plasma protein-aPL complexes, leading to the activation of the complement cascade through the classical pathway. Then generated component complement C5a, through the alternative pathway, recruits and activates monocytes and polymorphonuclear leukocytes, stimulating the release of mediators of inflammation, which ultimately results in fetal injury [102]. In patients with unexplained pregnancy loss, elevated levels of complement components C3 and C4 predicted subsequent miscarriages [103]. Moreover, patients with cerebral ischemic events had higher levels of complement activation products compared to patients with non-APS-related cerebral ischemia [104].

We [105] analyzed the profile of complement activation in patients with primary APS and found that hypocomplementemia related to complement over-activation is common in those patients. The serum complement levels correlated with LA activity and plasma levels of TNFα, implying that complement activation induced by aPL may be one of the responsible mechanisms of the thrombotic state in APS

The IgG isotype of aPL is the most frequently found in patients with APS, and the IgG2 subclass is the most prevalent [106, 107]. IgG2 and IgG4 subclasses have a relatively weak ability to fix the complement *via* the classical pathways: thus, other additional mechanisms may be involved in the enhancement of complement activation in patients with aPL.

TREATMENT OF APS

Strategies to prevent thrombosis should be part of the management of patients with APS. Smoking cessation, the use of protective medications and stratification of the risk of thrombosis, are important aspects of the care of those patients. The estrogen component of oral contraceptive increases blood coagulability and the risk of thrombosis in the general population. There are case reports of patients with APS developing thrombosis on contraception, thus oral contraceptives are generally contraindicated in aPL-positive patients. Contraceptives containing only progesterone do not increase the risk of venous thromboembolism [108] and they can be considered in patients with aPL. However, there are no controlled studies supporting this approach [109].

The current treatment of APS involves antithrombotic agents to control and prevent recurrent thrombosis. Therapy to inhibit the immunological or inflammatory mediators of the disease has been used in some life-threatening cases of APS without promising outcomes.

The great advances in the understanding of the molecular basis of aPL-mediated pathogenicity over the last half decay have lead to the development of targeted biological therapies. However, the optimum therapy for APS has not been yet reached. Current and potential therapeutic options for APS are summarized in Table 3.

THERAPIES CURRENTLY USED FOR APS

Anticoagulation with therapeutic heparin followed by life-long secondary thromboprophylaxis with oral vitamin K antagonist is the current advise treatment of thrombosis in APS [110]. However, there is not yet consensus on the optimal intensity of anticoagulation with oral vitamin K antagonists. Low dose of aspirin (LDA) is often added to the anticoagulation therapy in patients with arterial events [111].

Prevention of fetal loss and maternal thrombotic complications during pregnancy include the combination of LDA and heparin. Both heparin and low molecular weight heparin (LMWH) do not cross the placental barrier but the latter is preferable during pregnancy because of the lower risk for heparin-induced thrombocytopenia and osteoporosis. If this regimen is not effective, the addition of intravenous immunoglobulins (IVIG) may effective to improve fetal outcome in some cases [112, 113]. IVIG would act by favoring the clearance of pathological immunoglobulins and blocking the pathological autoantibodies due to the effect of anti-idiotype antibodies.

Table 3. Therapeutic Options in the Treatment of APS

I. Current treatment
• Anticoagulants: Heparin, LMWH and warfarin
• Low dose aspirin
• IVIG in pregnancy morbidity refractory to aspirin and heparin combination
• Antiplatelet agents rather than aspirin
• Corticosteroids*
• Plasma exchange*
• Vasodilators*
II. Drugs currently used for other diseases with potential to be effective in APS
• Hydroxychloroquine
• Statins
• Angiotensin-converting enzyme inhibitors
• Recombinant human activated protein C
• Recombinant human thrombomodulin
• New anticoagulants
• Tumor necrosis alpha antagonists
• Anti CD20$^+$ B cells antibody (Rituximab)

LMWH: low molecular weight heparin, IVIG: intravenous immunoglobulins.
*Those drugs are currently used in case of catastrophic APS or refractory APS.

Aspirin showed a prophylactic role for primary prevention of thrombosis in aPL-positive patients [114], but those results are no supported by data from a prospective controlled study [115]. In addition, long-term oral anticoagulation at a relative high intensity is associated with an increase risk of bleeding and under-anticoagulation with a risk of recurrences [110]. Given these concerns, new, safer and more-efficient modalities for prevention and treatment of thrombosis are needed.

Antiplatelet agents, other than aspirin, such as dipyridamole, ticlopidine, clopidogrel bisulfate and cilostazol had been empirically used in selected patients with APS and may represent an alternative to warfarin to prevent further aPL-related arterial thrombosis. Those drugs could offer benefit in short-term secondary prevention after non-cardioembolic strokes or transitory ischemic attacks [116]. Dilazep and dipyridamole block the monocyte-TF expression induced by purified IgG from APS patients [117], but there are no clinical trials evaluating their efficacy on primary or secondary thrombosis prevention in patients with aPL.

In case of a life-threatening condition such as catastrophic APS, the combination of intravenous anticoagulation, corticosteroids and IVIG or plasma exchange with fresh frozen plasma showed the highest survival rates [118]. Plasma exchange can remove, not only pathologic aPL, but also activated complement and other inflammation mediators. The fresh frozen plasma provides natural intact anticoagulants, mainly antithrombin and protein C.

Rituximab, a monoclonal antibody that selectively depletes CD20$^+$ B cells, has been successfully employed in a small number of patients with resistant APS including catastrophic APS. This biological reagent reduces the aPL titers, but its effect on the prevention of thrombotic recurrence has not been proven [119-121].

Prostacyclin and prostaglandin E1 treatment result in vasodilatation and inhibition of platelet aggregation. The use of those drugs in patients with aPL is anecdotal and further studies are necessary to evaluate the effectiveness and the risk of re-thrombosis [122].

POTENTIAL THERAPIES FOR APS

In this section, some drugs with anti-thrombotic and immunomodulatory effects currently used to treat other disease are discussed. Those agents potentially can be used as additional drugs for the treatment and the prevention of aPL-related complications.

1. Hydroxychloroquine

Hydroxychloroquine is considered an integral component of the treatment of APS in patients with concomitant SLE, and has been associated with a decreased thrombotic risk in aPL-positive SLE patients [123]. In addition to anti-inflammatory and immunomodulatory effects, hydroxychloroquine has anticoagulant potential which may be due to the ability to inhibit platelet aggregation and the release of arachidonic acid from stimulated platelets. Hydroxychloroquine repressed aPL- induced platelet activation *in vitro* [124], and reduced both thrombus size and time of thrombus persistence in an APS mouse model [125]. This drug may be of benefit in the management of patients with aPL, but its effectiveness for thromboprophylaxis should be determined by controlled studies [126].

2. Statins

Statins are cholesterol-lowering drugs that act as competitive inhibitors of 3-hydroxy-3-ymethylglutaryl-coenzyme A (HMG-CoA) which catalyzes the cholesterol synthesis in the mevalonate pathway. Beside cholesterol lowering activity, statins have anti-inflammatory effects modifying the function of endothelial cells, platelets and monocytes/macrophages. Statins down-regulated the production of cytokines in endothelial cells, interfered with leukocyte-endothelial interaction, inhibited TF expression by mononuclear cells, increased fibrinolytic activity and hampered platelet function [127]. Fluvastatin interfered with the expression of adhesion molecules and IL-6, and reversed the up-regulation of TF mediated by aPL in endothelial cells [93, 128, 129]. Moreover, in aPL-treated mice, fluvastatin diminished thrombus size [130].

Statins are widely used drugs known to be effective for the prevention and treatment of atherosclerosis and cardiovascular diseases [131], but still there are not data regarding its potential on thrombosis prevention in aPL-positive patients. In an animal model of mice treated with aPL, simvastatin and pravastatin decreased the expression of TF and protease-activator receptor 2 on neutrophils and prevented fetal loss, suggesting that statins may be a favorable treatment in woman with aPL-induced pregnancy complications [132].

3. Angiotensin-Converting Enzyme Inhibitors

Angiotensin-converting enzyme (ACE) inhibitors have beneficial effects in vascular diseases and significantly improved survival in patients with atherosclerosis. ACE inhibitors reduced TF levels and TF expression on monocytes in patients with myocardial infarction [133, 134]. Experimental studies demonstrated the inhibitory effect of ACE inhibitors on NFkB with consecutive down-regulation of TF [135-137]. Therefore, ACE inhibitors have the potential to reduce TF expression. Recently, angiotensin receptor blockers (ARB) are taking the place of ACE inhibitors as antihypertensive drugs. Clinical trials for ACE inhibitors or ARB in aPL-positive patients are warranted.

4. Recombinant Human Activated Protein C and Recombinant Human Thrombomodulin

Activated protein C is one of the major regulators of thrombin generation, and also possesses anti-thrombotic and anti-inflammatory properties reducing cytokine production and expression of adhesion molecules on endothelial cells [138]. Recombinant human activated protein C (Drotecrogin alpha) has been demonstrated beneficial in patients with severe sepsis where microvascular thrombi are the major feature. Administration of recombinant human activated protein C may restore the dysfunctional anticoagulant mechanisms, prevent amplification and propagation of thrombin generation and formation of microvascular thrombosis, and may simultaneously modulate the systemic pro-inflammatory response [139].

Thrombomodulin, a receptor of thrombin and thrombin-thrombomodulin complex, is a natural activator of protein C. Thrombomodulin works as anticoagulant only when thrombin is excessive. Therefore, thrombomodulin is the ideal anticoagulant with minimal risk of bleeding complications. The use of recombinant human soluble thrombomodulin has been approved for the treatment of disseminated intravascular coagulation [140].

Impairment of protein system C by aPL have an important role in the pathogenesis of APS. Those drugs are promising for APS when infection events trigger the development of thrombosis.

5. New Anticoagulants

Anticoagulants such as warfarin and heparin act on a number of targets, whereas the newer anticoagulants have been designed to selective inhibit one specific target in the coagulation system. Therefore, those new agents might be more effective and safer than current anticoagulants with a promising role in the management of APS. Some of these emerging anticoagulants agents are already approved and used in clinical practice but others are still in development phase.

New anticoagulants that selectively inhibit thrombin either directly through binding to thrombin and inhibiting its interaction, or indirectly through antithrombin, have been developed. Direct thrombin inhibitors include lepirudin and argatroban, approved for patients with heparin-induced thrombocytopenia [141]. Bivalirudin was approved for percutaneous coronary interventions in patients with acute coronary syndrome [142]. Ximelagatran, an oral direct thrombin inhibitor, showed very promising data and entered in late phase clinical development. However, there were many concerns in safety, especially with liver toxicity. In 2004, the Food and Drug Administration rejected the drug license in the United States, and in 2006 it was finally withdrawn from the world market [143].

Fondaparinux is a synthetic pentasaccharide, which binds to antithrombin thereby indirectly inhibiting activated factor X and thrombin generation. This drug has little effect on platelet aggregation. Fondaparinux is subcutaneously administrated and has been approved for the prevention and treatment of venous thromboembolism [144].

Rivaroxaban a selective direct inhibitor of activated factor X with high oral bioavailability, and dabigatran, an oral direct thrombin inhibitor, are approved for the prevention of venous thromboembolism following orthopaedic surgery [145, 146]

Other emerging anticoagulant is recombinant nematode anticoagulant protein c2 (rNAPc2), an 85-amino acid protein that is a potent inhibitor of the activated factor VII/ TF complex, the key physiological initiator of blood coagulation. Recombinant NAPc2 might be effective in thrombosis prophylaxis by attenuating the initiation and propagation of thrombin generation. This drug was effective in reducing the postoperative venous thromboembolism in patients undergoing total knee replacement [143], and suppressed thrombin generation in patients undergoing coronary angioplasty [147]. Because of its unique mechanism of inhibition of activated factor VII/TF, rNAPc2 should be considered as future therapeutic options for APS

6. TNFα Antagonists

In an animal model of APS, TNFα was a critical mediator of aPL-induced fetal loss and was released in response to complement activation. TNFα DNA vaccination prevented clinical manifestations of experimental APS [148], and TNFα deficiency provided fetal protection in aPL-treated mice. In addition, TNFα was released from monocytes treated with antiβ2GPI antibodies [100]. Those observations identify TNFα as a potential target for the therapy of the pregnancy complications of APS. However, TNFα blockade does not completely protect pregnancy problems, thus it is likely that other effector pathways contribute to the fetal death [149].

On the other hand, TNFα blockers therapy has been associated with increase in the frequency of aCL or antiβ2GPI antibody positivity [150-152]. No consensus exists on the merit of TNF blockade in patients with APS.

NEW TARGETED TREATMENTS FOR APS

New targeted therapies with potential to be effective for the management of APS are reviewed and summarized in Table 4.

1. Inhibitors of p38MAPK and NFkB

The p38MAPK pathway is essential in mediating the effect of aPL. Both p38MAPK phosphorylation and NFkB

translocation are required for aPL-mediated TF up-regulation on endothelial cells and monocytes. Treatment with an specific p38MAPK inhibitor, SB203580, inhibits platelet aggregation and thromboxane A2 production induced by aPL [97]. Moreover, SB203580 reversed the effect of aPL on TF expression and IL-6 and IL-8 up-regulation [98].

Table 4. New Therapeutic Strategies for APS Treatment

I. Specific targeted therapies
• p38MAPK pathway and NFkB inhibitors
• Tissue Factor inhibitors
• Platelet activation inhibitors: - Receptor specific antagonists - Thromboxane A2 inhibitors
• Specific complement inhibitors
II. Immunomodulation
• Bone marrow transplantation
• T cell blockage
• Specific B cell molecular targeting

p38 MAPK: p38 mitogen activated protein kinase, NFkB: nuclear factor kappa B.

The specific NFkB-inhibitor MG132 significantly reduced up-regulation of TF and enhancement of thrombosis mediated by aPL *in vivo* [153]. The activation of NFkB by aPL was also inhibited by SN50, a specific inhibitor of NFkB translocation [154]. Those findings represent innovative modalities of targeted therapies for the treatment of thrombosis in APS, but there is still many questions which need to be answered such a long-term effect and safety.

2. Inhibitors of Tissue Factor expression

Tissue factor is a transmembrane protein normally found in sub-endothelial structures of blood vessels and expressed upon activation on the surface of monocytes, endothelial cells and smooth muscle cells. Following its exposure to blood, TF binds specifically to activated factor VII. The formation of the activated factor VII/TF complex is critical for the initiation of the proteolytic reactions leading to the generation of thrombin, and eventually to clot formation. [155]. Increased TF expression is widely recognized as one the aPL-mediated mechanism of hypercoagulability [69, 70, 156, 157].

TF is one the major potential target for pharmacological interventions and blockage of TF activity is one of the promising therapies in APS. Therapeutic approaches targeting TF include dilazep dipyridamole, pentoxifyllines, defibrotide and ACE inhibitors such as captopril [117, 133, 158]. Those agents suppressed the monocytes TF expression induced by aPL or lipopolysaccharide. However, there are no clinical studies on patients with APS

3. Inhibitors of Platelet Activation

a) Receptor Specific Antagonists

Antiphospholipid antibodies can cause platelet activation and aggregation in the presence of low concentration of platelet agonists [159]. Also, aPL can enhance the expression of platelet membrane GP particularly GPIIb/IIIa, a receptor involved in platelet aggregation [124]. Infusions of a GPIIb/IIIa antagonistic monoclonal antibody (1B5) abrogated the aPL-induced thrombosis *in vivo*. Further, aPL-mediated thrombosis is not observed in GPIIb/IIIa deficient mouse [127]. Double heterozygosity polymorphisms for platelet GPIa/IIa and GPIIb/IIIa increase the risk of arterial thrombosis in patients with APS [160].

Platelet GPIIb/IIIa antagonists abciximab, tirofiban and eptifibatide, have been used in the treatment of myocardial infarction and acute ischemic stroke, showing its efficacy in acute coronary syndrome in combination with other therapies. However, results have been less consistent in ischemic stroke. While phase I and II trials of abciximab as the sole agent were promising, the phase II trial was abandoned because of unfavorable benefit to risk ratio. Presumably, the benefit of these drugs for patients with APS is limited [161].

The effects of aPL in GPIIb/IIIa expression are significantly reduced by hydroxychloroquine and this inhibitory effect may be one of the mechanisms by which this drug prevent thrombosis [124].

The blockage of the platelet receptor ApoER2' using a receptor-associated protein (RAP) abrogated the increased adhesion of platelet to collagen induce by β2GPI-antiβ2GPI antibody complex [162]. Thus, inhibition of ApoER2' might contribute to the prevention of thrombosis in APS patients.

Recently, the binding of β2GPI to platelet factor 4 has been reported [163]. β2GPI forms stable complexes with platelet factor 4, leading to the stabilization of β2GPI dimeric structure that facilitates the antibody recognition. The β2GPI-platelet factor 4 complex is strongly recognized by serum of patients with APS. Moreover, platelets may be activated by β2GPI-antiβ2GPI antibody-platelet factor 4 or β2GPI-platelet factor 4 complexes. Almost every cell type can be a source of platelet factor 4 especially under some stimulation. Both, β2GPI I and platelet factor 4 are abundant in plasma, thus the preformed β2GPI-platelet factor 4 complexes may prime several pro-coagulants cells culminating in coagulation. The blockage of the interaction between β2GPI-platelet factor 4 might be a novel approach in the targeted therapies in APS.

The other putative receptor proteins described before may be potential target therapies to reduce the aPL-mediated effects, but there are not yet data available to suppose this therapeutic strategy.

b) Thromboxane A2 Inhibitors

Thromboxane A_2 is one of the most powerful agonist for platelet activation and exerts a vasoconstrictor effect by serving as agonist of the thromboxane receptor. Clinical and experimental data suggest that inhibition of thromboxane production may be effective to prevent thrombotic complication in patients with aPL. Patients with APS had elevated urinary excretion of thromboxane A_2 metabolites [80]. Preincubation with β2GP-aCL complexes results in production by platelets of higher levels of thromboxane B_2 which is a stable metabolite of thromboxane A_2 [164]. Indomethacin, a thromboxane A_2 inhibitor, and theophylline, a phosphodi-

esterase inhibitor, abrogated aPL-mediated thromboxane A_2 production [165, 166]. On experimental APS, a long-term actin thromboxane receptor antagonist, BMS180,201, was effective to reduce the fetal resorption rate [167]. No data exist in patients who have APS regarding thromboxane receptor antagonists.

4. Specific Complement Inhibition

Inhibition of the complement cascade *in vivo*, using the C3 convertase inhibitor complement receptor 1-related gene/protein y (Crry)-Ig, blocks aPL-mediated fetal loss [168]. In addition, C3 and C5 knock-out mice less frequently developed aPL-related complications and antiC5-antibody reverse the thrombogenic properties of aPL [169]. Finally, treatment with heparin prevented aPL-induced complement activation *in vivo and in vitro*, and protected mice from aPL-related pregnancy complications [101]. Specific complement inhibitors are attractive therapies for APS. Potential targets for the therapies are drawn in Fig. (1).

IMMUNOMODULATION

Bone marrow transplantation (BMT) is currently used for the treatment of some autoimmune diseases based on the fact that autoimmunity can be either transferred or eliminated by BMT. In APS, both transfer of APS and induction of tolerance to disease has been reported [170, 171], thus BMT may have some future application for APS. On the other hand autologous stem-cell transplanted patients with scleroderma developed APS [172].

Antigen uptake, processing and presentation are the first steps following the exposure of antigen to the immune system. If we could artificially control this procedure to reduce β2GPI-reactive $CD4^+$ T cell response, the subsequent reactions, including antiβ2GPI antibody production, would not occur, thus curing the disease. Therapeutic strategies should target interrupting the continuous autoimmune loop carried out by macrophages and β2GPI-reactive $CD4^+$ T cells and B cells [173, 174].

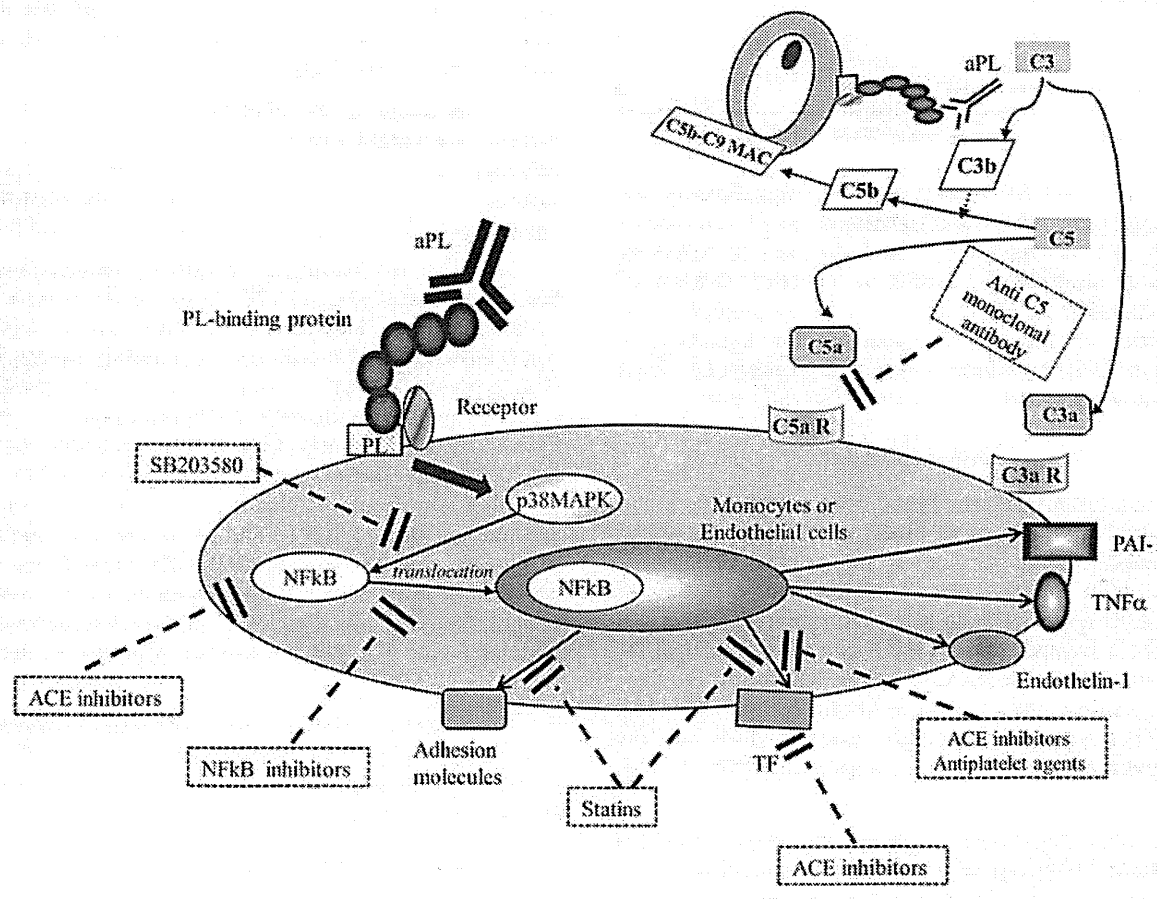

Fig. (1). Targets for novel therapies in APS.

Stimulation of procoagulant cells (monocytes or endothelial cells) by antibodies against phospholipid-binding proteins induce phosphorylation of p38MAPK which leads to the nuclear translocation of NFkB, and to the up-regulation of procoagulant substances. Specific inhibitors of p38MAPK (SB203580) or NFkB may block those processes. ACE inhibitors exert inhibitory effect on NFkB with consecutive down-regulation of TF. TF expression mediated by aPL can be abrogated by statins, and by some antiplatelet agents. aPL induce the production of cellular adhesion molecules by endothelial cells which may be reduced by statins. Finally, aPL may activate complement through the classical pathway. C3a, C5a and C5b-9 (MAC) may bind to specific receptors on endothelial cells and enhance the effects of aPL on cells. AntiC5-monoclonal antibody can reduce this binding.

aPL: antiphospholipid antibodies, PL: phospholipid, p38MAPK: p38 mitogen activated protein kinase; NFkB: nuclear factor Kappa B, ACE: angiotensin-converting enzyme, TF: tissue factor, TNFα: tumor necrosis factor alpha. PAI-1: plasminogen activator inhibitor-1, C5a R: C5a receptor, C5b R: C5b receptor, MAC: membrane attack complex.

The induction of immune tolerance at B-cell level is another future therapeutic approach for the management of APS. β2GPI-induced oral tolerance showed immunomodulatory effect in experimental APS [175]. A β2GPI-specific B cell toleragen, LJP 1082, was developed. This drug is a tetravalent conjugate of recombinant human domain I of β2GPI that has been shown to reduce domain I specific antibodies and levels of antigen-specific antibodies producing B cells [176]. Results from a phase I/II clinical trial in patients with antibody-mediated thrombosis showed that the drug was well tolerated and no differences on safety were found between patients receiving LJP1082 or placebo [177]. Further development is needed to assess the effect of this drug in the reduction of thrombotic events in APS.

CONCLUSIONS

Ongoing research focused on the thrombotic mechanisms mediated by aPL has significantly advanced the understanding of the pathophysiology of the APS. Those novel discoveries opened new insights into the management of APS leading to the investigation of specific target therapy.

Data from animal models suggested attractive and novel therapeutic approaches for the prevention and treatment of aPL-related complications. However, there is still not enough information to warrant the use of those agents in APS patients. In the coming years, studies are required to validate the data in humans and to evaluate the efficacy and security of new targeted therapies in APS.

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LIST OF ABBREVIATIONS

ACE	=	Angiotensin-converting enzyme
aCL	=	Anticardiolipin antibodies
aPL	=	Antiphospholipid antibodies
ApoER2'	=	Apolipoprotein E receptor 2
APS	=	Antiphospholipid syndrome
ARB	=	Angiotensin receptor blockers
β2GPI:	=	Beta 2Glycoprotein I
BMT	=	Bone marrow transplantation
ELISA	=	Enzyme-linked immunosorbent assay
GP	=	Glycoprotein
IL	=	Interleukin
IVIG	=	Intravenous immunoglobulins
LA	=	Lupus anticoagulant
LDA	=	Low dose of aspirin
LMWH	=	Low molecular weight heparin
Lp(a)	=	Lipoprotein a
MAPK	=	Mitogen activated protein kinase
NFkB	=	The nuclear factor kappa B
PAI	=	Plasminogen activator inhibitor
SLE	=	Systemic lupus erythematosus
TF	=	Tissue factor
TLR	=	Toll like receptor
TNFα	=	Tumor necrosis-α
tPA	=	Tissue-type plasminogen activator

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