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## **B CELLS IN PRIMARY ANTIPHOSPHOLIPID SYNDROME: REVIEW AND REMAINING CHALLENGES**

**Section designation:** Review

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**Running Title:** B cells in primary anti-phospholipid syndrome

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## **ABSTRACT**

It is now widely accepted that antiphospholipid antibodies (aPL) have direct pathogenic effects and that B cells, notably through aPL production, play a key role in the development of antiphospholipid syndrome (APS). Recent findings strengthened the implication of B cells with the description of specific B cell phenotype abnormalities and inborn errors of immunity involving B cell signaling in APS patients. In addition, it has been shown in preclinical models that cross-reactivity between APS autoantigens and mimotopes expressed by human gut commensals can lead to B cell tolerance breakdown and are sufficient for APS development. However, B cell targeting therapies are surprisingly not as effective as expected in APS compared to other autoimmune diseases. Elucidation of the B cell tolerance breakdown mechanisms in APS patients may help to develop and guide the use of novel therapeutic agents that target B cells or specific immune pathway.

## **HIGHLIGHTS**

- Antiphospholipid syndrome (APS) is characterized by a hypercoagulable state related to pathogenic antiphospholipid antibodies (aPL).
- B cells, notably through aPL production, have a central role in the pathogenesis of APS.
- aPL development/persistence may result from mono/polygenic inborn errors of immunity and specific interactions with commensal hosts.
- Paradoxically, B cell targeting therapies are not as effective as expected in APS considering experiences in other autoimmune diseases.

## **KEY WORDS**

Autoantibody, antiphospholipid syndrome, B cell, tolerance, autoimmunity, thrombosis.

## 1. Introduction

1 Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by the presence of  
2 pathogenic autoantibodies directed against membrane phospholipids and/or their associated plasma  
3 proteins. Antiphospholipid antibodies (aPL) were first described by Hughes *et al.* in 1983 [1]. Annual  
4 incidence and prevalence are estimated at approximately 2 and 50 per 100 000 individuals [2]. Clinical  
5 features are related to a hypercoagulable state leading to obstetrical complications and/or venous or  
6 arterial thromboembolic events (TEs) affecting almost every organ system [3]. The criteria derived  
7 from the Sapporo classification revised in 2006 (Sidney) are quite consensual and require the  
8 association of one of these clinical manifestations and the persistence of “classical” aPL, namely  
9 lupus anticoagulant (LA), anticardiolipin antibodies (anti-CL) and anti- $\beta$ 2 glycoprotein I antibodies  
10 (anti- $\beta$ 2GPI) [4]. Patients may encompass non TEs manifestations or “non-classical” aPL (including  
11 phosphatidylserine/prothrombin complex, PS/PT, PE, or anti-annexin5A, Anx5A, antibodies) that are  
12 excluded from Sydney criteria due to a role that remain elusive [5].

13 Recently coronavirus disease 2019 (COVID-19) and the associated abnormalities in coagulation  
14 measures have put the spotlight back on aPL occurrence and their clinical relevance. There are strong  
15 evidences that B cells, notably through aPL production, have a central role in APS pathogenesis.  
16 Paradoxically, B cell targeting therapies are somehow not as effective as expected considering  
17 experiences in other autoimmune diseases. Herein we propose a review that highlights the major role  
18 of B cells and autoantibodies in APS, and how they provide a window into the pathogenesis, the  
19 clinical features and the risk assessment in APS.

20

## 21 **2. B cells: the leading role?**

### 22 *Pathogenesis of aPL*

23 B cells contribute to APS pathogenesis notably through antibody production. The pathogenic  
24 mechanisms mediated by aPL have been largely supplied by animal models, especially for  $\beta$ 2GPI-  
25 dependent aPL. In 1990 Mc Neil *et al.* found that the binding of aPL to cardiolipin requires the  
26 presence of  $\beta$ 2GPI as a cofactor [6]. Passive transfer of purified human anti- $\beta$ 2GPI IgG, but not anti-

27  $\beta$ 2GPI depleted IgG or normal human IgG, potentiates thrombosis formation in animal models after  
28 vessel injury or pre-treatment with lipopolysaccharide (LPS) [7,8]. Anti- $\beta$ 2GPI also induce fetal  
29 losses or growth retardation in pregnant mice without the need of additional factors [9], and the  
30 prominent role of the  $\beta$ 2GPI/anti- $\beta$ 2GPI complex is highlighted by the absence of thrombosis in  
31  $\beta$ 2GPI deficiencies [10]. The binding of anti- $\beta$ 2GPI antibodies to their antigen is dependent on  $\beta$ 2GPI  
32 conformation.  $\beta$ 2GPI comprised 5 domains, the fifth domain shielding the domain 1 epitope in the  
33 circular conformation form in plasma [11]. Autoantibodies do not bind this conformation form but  
34  $\beta$ 2GPI binding to negatively charged structures (such as anionic phospholipids) induce conformational  
35 changes [12,13]. As a result, cryptic epitopes in domain I are exposed and the autoantibodies are able  
36 to recognize  $\beta$ 2GPI [14]. The type of interaction is also critical since human IgG anti- $\beta$ 2GPI domain I  
37 (anti- $\beta$ 2GPI-DmI) with high avidity induced significantly larger thrombi *in vivo* compared to anti-  
38  $\beta$ 2GPI-DmI with poor avidity [15].

39 A part of APS pathophysiology is mediated by the disruption of AnxA5 shielding over phospholipid  
40 bilayers, making them available for coagulation reactions [16]. Interestingly, anti- $\beta$ 2GPI-DmI has  
41 been associated with significantly reduced annexin A5 anticoagulant activity in adults with aPL [17].  
42 Nevertheless, considering  $\beta$ 2GPI-dependent aPL as the only ones with pathological relevance would  
43 be too straightforward, since aPL targeting other phospholipid-binding proteins (such as prothrombin)  
44 [18,19] or cofactor-independent aPL [20] may also exert thrombogenic effects in mice, but it has  
45 become increasingly evident that the phospholipid binding protein  $\beta$ 2GPI is the major antigenic target  
46 for thrombogenic aPL antibodies.

47 The pathogenic effects of aPL do not depend solely on the immunogenicity of the phospholipid, but  
48 also on the downstream effect of the autoantibodies. aPL or the phospholipid/antibody complex can  
49 activate multiple cells and factors that finally drive clot formation, which is the key event  
50 underpinning vascular manifestations. A non exhaustive list of such mechanisms is listed in **Table 1**.

### 51 ***B cell phenotype in APS***

52 Despite the central role of aPL, little is known about B cell disturbance and characteristics of the aPL  
53 producing clones. Indeed, only few data on small cohorts are available concerning B cell phenotype in

54 APS. A work assessing B cell phenotypic abnormalities in obstetric APS women described reduced  
55 memory B cells and increased percentage and absolute count of CD27-IgM<sup>+</sup> naive B cells in patients  
56 with history of TEs. This higher number of naive B cells was associated with lower serum levels of  
57 C4, higher levels of anti-CL and anti-β2GPI IgG and with the development of thrombotic  
58 complications during follow-up [22]. The authors assumed that altered peripheral B cell differentiation  
59 and activation could bypass normal B cell differentiation leading to a bias toward plasma cell  
60 maturation. These results were confirmed on another cohort of 11 patients with thrombotic primary  
61 antiphospholipid syndrome (PAPS), which showed expansion of naive B cells as compared to  
62 controls, non-APS thrombotic patients, systemic lupus erythematosus (SLE) and rheumatoid arthritis  
63 (RA) patients [23]. An increased proportion of transitional B cells, a putative source of autoreactive B  
64 cells, was also noticed [23]. However, a recent work heightens the debate, showing a decrease of  
65 naive/immature B cell compartment only in obstetric PAPS patients [24]. Increase of CD19<sup>+</sup>CD5<sup>+</sup> B  
66 cells percentage was found in PAPS patients with TEs, which can represent up to 20% of total B cells  
67 in this population [25]. CD5, firstly described in T cells, is not a distinctive marker but is highly  
68 expressed by B1 cells in humans, a subset producing natural polyreactive IgM with a repertoire biased  
69 towards bacterial and self-antigens [26]. An increase of CD5<sup>+</sup> B cells have been described in several  
70 conditions, including SLE, RA, or chronic viral infections [27]. In APS, their number correlates with  
71 aPL IgM titers and most of them have reactivity against phospholipids [27]. Recently, Hisada *et al.*  
72 reported a decreased frequency of IL-10 producing regulator B cells (B-regs) and an increase of  
73 plasmablasts in 26 PAPS and 19 SLE/APS patients. This increase was associated with TLR7 SNP  
74 rs38553839 and type I IFN upregulation [28]. Another soluble mediator that could dysregulate B cell  
75 survival and development is B-cell activating factor (BAFF), known as a key cytokine in SLE [29]. In  
76 APS, soluble BAFF level is increased both in patients with secondary APS and PAPS and correlates  
77 with prognostic scores of thrombosis [24,30].

### 78 ***BCR repertoire in APS***

79 Features of the BCR repertoire have been investigated in several immune-mediated diseases and  
80 profound changes have been described in SLE, RA or vasculitides [31]. However, in depth BCR

81 repertoire analysis is lacking in APS. In a systematic analysis of 36 identified aPL sequences, there  
82 was no evidence of preferential gene or family usage in any group of aPL studied, but only two aPL  
83 were proven to be pathogenic and many of them were derived from asymptomatic patients [32]. Our  
84 group performed molecular and functional analysis of anti-CL autoantibodies in a patient with PAPS,  
85 by cloning the BCR of autoreactive B cells isolated as single cells [33]. The five different antibodies  
86 were highly heterogeneous in terms of V, D or J gene usage and mutations in variable regions.  
87 However, the only antibody with LA activity had the highest mutated rate [33]. In accordance, among  
88 five selected monoclonal aPL originating from another patient, the only one inducing fetal losses,  
89 when passively injected in mice, had the highest rate of mutation in the Vk region [34]. This  
90 pathogenic effect was reversible when this autoantibody was *in vitro* reverted to inferred naive  
91 germline configuration [34]. In this process of somatic hypermutation, the presence of newly inserted  
92 positively charge in CDR3 seems to play a particularly important role, and Pericleous *et al.* showed  
93 that new arginine residues enhance the binding to  $\beta$ 2GPI-DmI [35]. In conjunction with studies  
94 proposing that aPL belong to the natural antibodies [27], pathogenic aPL clones identified by our  
95 group were polyreactive, suggesting that they may be part of the natural repertoire [34]. However, in  
96 lupus-prone mice, B cells specific for phospholipid are largely found in germinal centers, whereas few  
97 are localized in the extrafollicular compartment, suggesting that follicular class-switch recombination  
98 of autoreactive clones is important to drive aPL pathogenicity [36].

### 99 **3. APS: a “hit(s)-parade” toward B cell maturation**

100 Although aPL are directly implicated in pathophysiology and correlate with the thrombotic risk, TEs  
101 occur occasionally, suggesting that other factors are needed to push the thrombotic/haemostatic  
102 balance in favor of thrombosis [37]. The “second hit” hypothesis is widely accepted and regarded as  
103 an additional procoagulant condition that is needed to precipitate clotting formation in aPL carriers  
104 [9]. This theory is strongly supported by several experiments in APS prone mice models. In this view,  
105 Pierangeli *et al.* described mouse model of injury-induced thrombosis with surgically induced  
106 thrombus formation and showed in this model the importance of complement activation in aPL’s  
107 pathogenicity [38]. Similarly, the same group showed that aPL mediated thrombogenic effects were

108 reduced in mice non-responsive to LPS [39]. Hence, a priming factor (e.g. vessel injury or pre-  
109 treatment with LPS) is needed for passively transferred human aPL to induce thrombosis [7,8].

110 Among various mechanisms, infection agents or oxidative stress may increase aPL exposure [40],  
111  $\beta$ 2GPI conformation modifications that expose cryptic epitopes [41], or complement activation.  
112 Whereas  $\beta$ 2GPI binding to endothelial cells requires priming with pro-inflammatory factors, uterine  
113 vessels and trophoblast physiologically express high level of  $\beta$ 2GPI [42]. Thereby, pregnancy  
114 increases specific epitope exposure and could act as a second hit *per se* [9]. Other clinical situations,  
115 such as traumatism, inflammatory state or surgery can trigger clot formation, leading to clinical events  
116 defining APS. However, and as discussed further, such additional hits may, in an alternative or  
117 complementary manner, step in earlier in the pathogenic process driving B cells from benign and  
118 transient aPL production toward persistent and pathological autoantibodies generation.

#### 119 ***aPL occurence***

120 It is widely accepted that autoimmune diseases, including APS, may arise via an inappropriate  
121 immune response towards infections. Apart from chronic infectious, such as the one induced by  
122 human immunodeficiency virus (HIV) or hepatitis C virus, in which link between aPL and APS-  
123 related features is controversial [43], most “infectious” aPL are transient, cofactor independent, bind  
124 neutral or negative PL with low affinity, and clinical APS-related complications remain an  
125 epiphenomenon [32,44]. While not frequently reported, cytomegalovirus (CMV), Epstein Barr Virus  
126 (EBV), Herpes simplex virus (HSV) and adenovirus infections could lead to anti-CL production and  
127 transient LA positivity [45]. Development of pathogenic aPL has been reported during Parvovirus B19  
128 infection, with aPL of IgG isotype and cofactor-dependent binding, similar to autoimmune disease  
129 [45–47]. Recently, high prevalence of LA have been described in patients with SARS-CoV2 [48].  
130 Among 216 patients, 44 (20%) were found to have a prolonged activated partial-thromboplastin time  
131 (aPTT), due to LA in 90% of them. Additionally, in a recent series, 15% of 122 infected patients were  
132 positive for anti- $\beta$ 2GPI IgG and about 5% for anti-CL IgG [49]. Similarly to patients with triple  
133 positive APS or CAPS, IgG isolated from COVID-19 patients increase thrombus extension in a mouse  
134 model of inferior vena cava thrombosis [50]. TEs are frequent during acute SARS-CoV2 infection

135 [48,51], and the severe form of COVID-19 share several clinical and laboratory features with  
136 hyperferritinemic syndromes [52,53]. However, no association between thrombosis and aPL is clearly  
137 established and, during COVID-19, anti- $\beta$ 2GPI displayed an epitope specificity different than in PAPS  
138 [49]. The role of aPL in this hypercoagulable state should be elucidated, as well as the question of  
139 their long-term persistence.

140 Regarding bacterial infections, aPL are most commonly associated with *Coxiella burnetii*,  
141 *Mycoplasma pneumoniae*, *streptococci*, and *Mycobacterium tuberculosis* infections. The latter three  
142 infections may be associated with persistent autoantibodies and authentic APS [54]. The relatively  
143 high prevalence of anti-CL antibodies found in syphilis is largely due to a cross-reactivity of syphilis  
144 antibodies against treponemal cardiolipins [43]. Multiple aPL positivity and  $\beta$ 2GPI-dependent binding  
145 are found in sera from patients with leprosy [55]. Mice immunized with proteins from *Haemophilus*  
146 *influenzae* or *Neisseria gonorrhoeae*, which share sequence homology with  $\beta$ 2GPI-DmIII, led to the  
147 production of anti-CL and anti- $\beta$ 2GPI IgG autoantibodies [56]. Parasite or yeast induced-aPL are very  
148 rare and mostly occur after *Plasmodium falciparum* or visceral leishmaniasis infection [57].

149 Finally, several other clinical conditions have been associated with the occurrence of aPL. Drug  
150 induced aPL, following antiarrhythmic or antipsychotic agents, are common, but they are usually  
151 benign and disappear after the drug withdrawal [44]. Reports documented the appearance of aPL after  
152 vaccine administration responses. Indeed, the frequency of aPL production after influenza  
153 immunization is higher than after infection [44]. In a prospective study, 8% (7/101) of healthy subjects  
154 developed anti-CL but not anti- $\beta$ 2GPI after seasonal influenza vaccination. Anti-CL positivity was no  
155 longer observed at week 12 post-vaccination [58]. Tetanus toxoid vaccination triggers antibodies  
156 which are cross-reactive with  $\beta$ 2GPI as shown in animal models [57]. The role of adjuvants as  
157 stimulators of innate actors in vaccine associated aPL is not elucidated.

### 158 ***From aPL to thrombosis***

159 Several potential mechanisms have been evoked to explain the production of aPL during infections,  
160 including epitope spreading, bystander activation, and molecular mimicry. However, little is known  
161 about mechanisms sustaining chronic autoimmunity in APS. The transient character of aPL after

162 infection might suggest that continuous antigen exposure is needed for the maintenance of chronic  
163 autoreactive B lymphocyte activation. The microbiome is a potential source of persistent self-antigens  
164 that either trigger or perpetuate an autoreactive B cell response [59]. Depletion of the gut microbiota  
165 with antibiotic regimen leads to lower anti- $\beta$ 2GPI titers and protection from thrombotic events in mice  
166 [60]. In a recent work, *Roseburia intestinalis* (*R. int*) was identified as a chronic driver of  $\beta$ 2GPI  
167 autoreactivity [61]. Indeed, Ruff *et al.* provided evidence for cross-reactivity between mimotopes of  
168 this common human gut commensal and the major T cell (p276-290 in DmV) and B cell (R39-R43 in  
169 DmI) autoepitopes of  $\beta$ 2GPI. APS-derived monoclonal antibody with LA activity binds to a mimotope  
170 of a *R. int* DNA methyltransferase (DNMT). *R. int.* immunization of BALB/c mice induces  
171 autoepitope-specific cross-reactivity to human  $\beta$ 2GPI, and oral gavage of the spontaneous APS mouse  
172 model (NZW x BXSB) F1 mice lead to a significantly elevated anti-human  $\beta$ 2GPI IgG autoantibody  
173 and TEs. In conjunction, elevated levels of anti-*R. int* DNMT IgG were found in 15 patients with APS,  
174 and positively correlated with anti- $\beta$ 2GPI IgG. The authors suggested that cross-reactivity with  
175 commensals is likely to impact mechanisms of tolerance of B cells but also T cells providing help to  
176 PL-specific B cells [62]. Could this hypothesis be sufficient to explain how aPL producing B cells  
177 bypass tolerance mechanisms? Indeed, autoreactive B cells are detectable in healthy individuals [63],  
178 but several checkpoints ensure their counterselection or their immunologic ignorance to thwart  
179 autoimmunity in physiological conditions [64]. Hence, pathogenic aPL producing clones may only  
180 arise in predisposed individuals.

181 In this way, primary abnormalities of tolerance mechanisms linked to genetical defects could favor the  
182 maintenance of aPL reactive B cell clones. *BLK* polymorphism (rs2736340) displayed a strong genetic  
183 association with PAPS [65]. Polymorphisms in *IRF5* or *BANK1*, classically reported in SLE, displayed  
184 low or no association in PAPS respectively [65]. In line with other autoimmune diseases, several  
185 candidate genes especially in HLA locus (eg. *HLA DR4*, *DRw53*) were identified in monozygotic  
186 twins and familial clusters of PAPS, and suggested to be involved in the pathogenesis of PAPS  
187 through genome wide association studies (GWAS) or next-generation sequencing (NGS) approach  
188 [66–69]. Monogenic inborn errors of immunity that affect B cells can be associated with aPL  
189 seropositivity or PAPS, and offer a prototypical condition to explore the tolerance breakdown against

190 aPL. We reported recently a family of patients with APS and an heterozygous variant of *IKZF1*  
191 causing loss of function for IKAROS, a key transcription factor involved in primary and secondary  
192 affinity maturation processes of B cells [70]. Long-term persistence of aPL may also depend on  
193 intrinsic characteristics of autoreactive B cells. Indeed, one could speculate that somatic mutations  
194 affecting aPL producing clones could provide selective advantage to escape negative selection, in line  
195 with findings in autoimmune lymphoproliferative (ALPS) syndrome, where somatic mutations appear  
196 relevant for the accumulation of Fas-deficient memory B cells and the development of autoimmunity  
197 [71].

198 These data strengthen a model whereby, on a predisposing genetic background, transient or benign  
199 phospholipid reactive clones could pass through B cell tolerance checkpoints, expand and mature,  
200 leading to aPL-secreting plasma cells accumulation. The development of such persistent and  
201 pathogenic clones may be in part sustained by an adaptive immune responses against cross-reactive  
202 microbiota, as opposed to acute infections due to pathogens that are cleared by the host [61]. Specific  
203 epitope exposure in pregnancy and/or other clinical situations, including vessel injury and/or  
204 inflammatory processes, could thereafter provide additional triggers leading to clinical events defining  
205 APS (**Figure 1**).

206

#### 207 **4. Autoantibodies and clinical implication**

##### 208 *aPL and diagnosis*

209 The main role of autoreactive B cells is emphasized by the central place of aPL in the APS diagnostic  
210 [72]. However, the various association between large panels of serological and clinical phenotypes  
211 remains elusive. According to Sydney classification, diagnosis laboratory criteria include LA, anti-CL  
212 and anti-β2GPI, of IgM or IgG isotype at medium-to-high titres (generally tested by ELISA), that  
213 should be tested repeatedly positive at least 12 weeks apart. Usually, anti-CL and anti-β2GPI from the  
214 same isotype are detected together [73]. The positivity for LA reflects the ability of aPL to prolong  
215 phospholipid-dependent coagulation assays. The LA activity is often displayed by anti-β2GPI,

216 especially those directed against domain I of  $\beta$ 2GPI. Seropositivity of all three-classification criteria-  
217 aPL is linked with a more aggressive APS phenotype, named "triple-positive".

218 Alternative aPL, named "non-classical", have been excluded from Sidney criteria due to an initial lack  
219 of clinical implication. However, patients with clinical manifestations compatible with APS, positive  
220 for such extra criteria aPL, and negative for classical aPL exist and were named "seronegative APS  
221 patients" (SN-APS). This entity introduced in 2003 by Hughes and Khamashta [74] remains  
222 controversial and three possible explanations have originally been considered to explain the existence  
223 of such SN-APS patients: (i) a wrong diagnosis, (ii) previously positive aPL tests that became  
224 negative, or more likely (iii) aPL not detected by the current consensual tests. A retrospective study  
225 found no significant differences in clinical manifestations between patients with APS and those with  
226 SN-APS [75]. Zohoury *et al.* showed that about 35% of SN-APS patients have detectable non  
227 classical aPL [76]. Hence, some consider that SN-APS is a proof that classic aPL tests are not  
228 sufficient to diagnose APS, arguing for an update to the current criteria incorporating non-  
229 conventional aPL and consensual technical procedures in diagnosis criteria [77]. Recently, four  
230 profiles of non-criteria APS were proposed : 1) clinical non-criteria APS, 2) laboratory non-criteria  
231 APS, 3) incomplete laboratory APS: patients fulfilling clinical criteria, plus positive aPL, but not  
232 fulfilling the classification criteria, and 4) seronegative APS: patients with persistently negative  
233 antiphospholipid antibodies despite fulfilling clinical criteria [78].

234 Our actual knowledges on non-classical aPL highlight their importance in the APS physiopathology.  
235 Among them, antibodies targeting the PS/PT complex can display LA activity [79–81] and seems to  
236 be of strong clinical relevance. Their presence, together with anti- $\beta$ 2GPI-DmI, correlate with vascular  
237 and obstetrical complications, and their combination have 100% predictive value for the diagnosis of  
238 APS [82–84]. In 14 triple positive patients also positive for anti-PS/PT (so called "tetra positive APS  
239 patients"), from whom different type of aPL were affinity purified, anticoagulant activity was largely  
240 attributable to anti-PS/PT antibodies [85]. Anti-PS/PT detection is not influenced by anticoagulant  
241 treatment and could be considered to confirm the presence of LA. Concerning other non-classical aPL,  
242 among a cohort of patients with thrombosis, anti-PE antibodies were found in 15%, being the sole aPL  
243 detected in 67% of the cases [86]. Antibodies targeting phosphatidic acid (PA), or phosphatidylinositol

244 (PI) may be the only one detectable in women with recurrent pregnancy losses [87,88], although their  
245 binding to antigen and their pathogenicity may be  $\beta$ 2GPI-dependent. Lastly, IgA against  $\beta$ 2GPI are  
246 significantly associated with TEs, especially in SLE patients [89]. Despite the clinical interest of non-  
247 conventional aPL, a lack of standardization in the assays used for their detection still remains [90].

#### 248 *Specific aPL for distinct phenotypes?*

249 Vascular occlusions belong to the classical clinical pictures of APS, and include deep venous  
250 thrombosis, pulmonary embolism or arterial TEs such as strokes [91]. In addition to this thrombotic  
251 APS, some patients present with an “obstetric APS” phenotype and suffer from pregnancy morbidity,  
252 including recurrent early miscarriage but also late (after 10 weeks) pregnancy loss and premature birth  
253 (<34 weeks) related to preeclampsia or placental insufficiency [92]. Patients with APS may also  
254 experience a myriad of additional features, usually branded as “extra criteria”, including neurological  
255 (epilepsy, chorea...) and hematological manifestations (thrombocytopenia and hemolytic anemia),  
256 livedo reticularis, nephropathy and valvular heart disease [93]. Catastrophic antiphospholipid  
257 syndrome (CAPS) is the most severe form of the disease and characterized by widespread small vessel  
258 thrombosis with multiorgan failure presenting over a short period of time and is associated with a  
259 mortality higher than 30% [91][94]. Despite published association with drugs, malignancies and a  
260 wide spectrum of autoimmune diseases [95], secondary APS is mainly associated with SLE, in about  
261 20-30% of cases [2,5]. aPL positive patients with SLE have higher rates of arthritis, cytopenia,  
262 hypocomplementemia and severe renal disease than those without SLE [96].

263 Relying these homogenous clinical phenotypes to serological characteristics remains challenging.  
264 Patients with single obstetrical APS tend to have lower rate of multiple aPL positivity [9,97]. In series  
265 on the Serbian national cohort, patients with secondary APS had more frequent multiple aPL positivity  
266 than those with PAPS, whereas LA alone was more frequently reported in the latter [98]. However,  
267 these results were not found in the large Euro-Phospholipid cohort. Additionally, in this cohort of  
268 1000 European patients with APS, no differences were found in the clinical presentation of PAPS, at  
269 its onset and during a 10-year follow-up period, according to the presence or absence of anti-CL or LA  
270 [5,91]. In 2016 data from the CAPS Registry, LA was present in 83% of episodes, anti-CL IgG in

271 81%, IgG anti- $\beta$ 2GPI in 78% whereas anti-CL and anti- $\beta$ 2GPI of IgM isotype were less frequent (50%  
272 and 58% respectively) [99]. Recently, three studies on large cohort aimed to aggregate patients into  
273 subgroups sharing common clinical and specific classical aPL profiles [97,100,101]. The main results  
274 are depicted in **Table 2**. Nonetheless, these studies do not consider extra criteria aPL. Using multiplex  
275 line immunoassay, Volkov *et al.* evaluated the presence of non-criteria aPL in a cohort of 130 APS  
276 patients. Whereas arterial thrombosis was associated with multiple aPL positivity ( $>7$ ), venous TE was  
277 not linked to any specific aPL and inversely correlated with anti-phosphatidic acid IgM detection. The  
278 presence of anti-CL IgG and anti-PS IgG was linked with pregnancy morbidity [102]. These results  
279 suggest that evaluating a broad spectrum of aPL may enable to better define APS phenotype.

### 280 *Prognostic markers and risk assessment*

281 Identifying the risk of clinical events in patients with APS is challenging due to the clinical  
282 heterogeneity, the different sets of aPL and their fluctuation in the disease course. Multiple positivity  
283 for anti-CL, anti- $\beta$ 2GPI or LA is associated with higher rate of TEs than single test positivity  
284 [103,104]. Subjects with triple positivity for these criteria aPL have an annual incidence of TEs of  
285 5,3%, with a cumulative risk of 37% after ten years [105]. LA positivity is associated with the highest  
286 risk for both arterial and venous TEs [106] whereas results are controversial for anti-CL [106–108].  
287  $\beta$ 2GPI antibodies seems to confer a moderate increase of the risk, with an odd ratio from 1.6 to 2.4 for  
288 venous TEs and of 2.3 for ischemic strokes [109–111].  $\beta$ 2GPI-dependent LA activity assay correlated  
289 two times better with TEs than the classic LA assay [112]. The relevance of anti-CL/ $\beta$ 2GPI IgM  
290 antibodies has been debated. Most studies were unable to demonstrate a correlation between IgM  
291 positivity and TEs, but Chayoua *et al.* recently provided evidence for an independent association with  
292 pregnancy morbidity [113]. Among non-criteria aPL, anti- $\beta$ 2GPI-DmI positively correlates with the  
293 Global Antiphospholipid Score (GAPSS) [114,115], a validated thrombotic risk score [116]. Hence,  
294 anti- $\beta$ 2GPI-DmI seems to represent a robust biological criterion, in association with classical aPL, to  
295 better apprehend the thrombotic risk. Other works showed diverse clinical association with reactivity  
296 to different epitopes on  $\beta$ 2GPI and suggested that classification of anti- $\beta$ 2GPI into subgroups  
297 regarding epitope specificity would be of clinical relevance [117–120]. As an example, in a cohort of

298 135 women with persistently positive anti- $\beta$ 2GPI IgG, anti- $\beta$ 2GPI-DmI positivity was associated with  
299 triple positivity, thrombosis and predictive of late pregnancy morbidity, whereas anti-domain IV/V  
300 (anti- $\beta$ 2GPI-DmIV/V) antibodies were not associated with clinical APS manifestations [118].  
301 Conversely, DmIV/V are preferentially recognized by anti- $\beta$ 2GPI antibodies from one-year-old  
302 healthy children born to mothers with systemic autoimmune diseases and from subjects with non-APS  
303 conditions [119], and the ratio anti- $\beta$ 2GPI-DmI to anti- $\beta$ 2GPI-DmIV/V IgG has been proposed as a  
304 helpful tool to discriminate patients with systemic autoimmunity from those carrying less pathogenic  
305 anti- $\beta$ 2GPI antibodies [120].

306 Regarding other non-classical aPL, it is widely accepted that prothrombin is a common antigenic  
307 target for aPL, but assays may detect distinct populations of autoantibodies which have different  
308 clinical implications. Positivity for anti-PT, detected by directly coating prothrombin on ELISA plates,  
309 does not seem to be such a strong risk factor for APS [121]. However, the cumulative incidence rate of  
310 thrombotic events is significantly higher when anti-PS/PT, which recognize a conformational epitope,  
311 are detected among aPL carrier patients [122]. Additionally, the risk of pregnancy morbidity seems to  
312 be associated with the presence of anti-PE, PA, PS and PI [90].

313 Based on the global aPL profile, different models, such as the Antiphospholipid Score, have been  
314 tested to evaluate the risk of having APS and for predicting thrombosis [123]. The use of antibody  
315 profiles as prognostic markers is however delicate for several reasons. High inter-laboratory variability  
316 and the widespread of retrospective designs may explain the large discrepancies between the finding  
317 of clinical studies. Moreover, conventional cardiovascular risk factors should be considered to assess  
318 the thrombotic risk. The GAPSS proposed by Sciascia *et al.*, based on the profile of four aPL and the  
319 presence of dyslipidemia or arterial hypertension, has been proven to represent the probability of  
320 having TEs or pregnancy loss in patients with SLE and is an example of risk model that takes in  
321 account such factors [116]. EUREKA is another algorithm that predicts the risk of pregnancy  
322 morbidity considering aPL titres and profiles [124].

323

## 324 **5. Management of APS and B cells: paradox and remaining challenges**

325 Despite the widespread of targeted therapies in autoimmune diseases, anticoagulation remains the  
326 corner stone of management in patients with APS. Briefly, low dose aspirin (LDA) is recommended  
327 for asymptomatic patients with high risk aPL profiles whereas patients with thrombotic APS should  
328 have long term treatment with vitamin K antagonists. The use of direct oral anticoagulants (DOACs) is  
329 highly debated [92,125]. Patients with obstetric APS only should receive prophylactic dose low-  
330 molecular-weight heparin (LMWH) and LDA throughout pregnancy. The European League Against  
331 Rheumatism (EULAR) recently developed evidence-based recommendations for the prevention and  
332 treatment of adults with APS [126]. Considering the crucial role of autoantibodies in the definition and  
333 the pathophysiology of the disease, data on the use of therapies directed towards B cells are lacking  
334 and somehow disappointing in APS.

### 335 ***B cell targeting therapies***

336 The use of anti-CD20 therapies has been supported by strong evidences in other autoimmune  
337 diseases[127], but also by clinical and/or serological beneficial effects in case reports concerning  
338 PAPS, secondary APS, or CAPS [128]. In the retrospective literature review by Erre *et al.*, eight out of  
339 12 patients with classical aPL treated with rituximab, a chimeric monoclonal antibody targeting CD20,  
340 showed normalization or reduction of aPL titers. Some of them were however concurrently treated  
341 with immunosuppressors [129]. Results on TEs recurrence are debated and limited to small series or  
342 case reports [130]. In a phase II trial, safety and efficacy of rituximab was evaluated in 19 patients  
343 with APS criteria [131]. Rituximab was efficient for some non-criteria manifestations, especially  
344 thrombocytopenia (response rate of 50%) cognitive dysfunction (80%) and skin ulcers (100%), but  
345 failed to change significantly aPL profiles [131]. Such effect on extra thrombotic manifestations,  
346 including thrombocytopenia, was confirmed in other studies [133,134]. There are concerns about  
347 thrombosis exacerbation since severe acute thrombotic events were described in two APS/SLE  
348 patients receiving rituximab [132]. In the study from Erkan *et al.*, 12 serious adverse events were  
349 recorded including neurological complications (n = 5), infections (n = 3) and recurrence of TEs (n = 1)  
350 [131]. According to recommendations, rituximab may be considered in CAPS, where aPL production  
351 is associated with systemic inflammatory response syndrome [94]. In a retrospective review, 13 of 20

352 patients with severe/relapsed CAPS treated with rituximab recovered. aPL became negative in fifty  
353 percent of those with follow-up (4/8). Comparing 30 patients treated with rituximab versus  
354 contemporaneous patients in the CAPS registry, an *ad hoc* analysis provided an odd's ratio for  
355 survival of 0.41 (95% CI 0.15, 1.11) [133]. However, evidences are not strong enough to use  
356 rituximab as a first line therapy in CAPS and it is considered as an alternative treatment in severe or  
357 refractory cases [92]. Novel anti-CD20 therapies such as obinutuzumab, a human monoclonal anti-  
358 CD20 antibody, induce different cellular response and stronger antibody-dependent cell-mediated  
359 cytotoxicity (ADCC) [134]. Their uses in autoimmune diseases are under evaluation and they could  
360 provide a second life to anti-CD20 therapy in APS.

361 Because anti-CD20 treatment does not affect plasma cells, the insufficient therapeutic outcome may be  
362 caused by the survival of aPL producing plasma cells. *Ex vivo* experiments showed that aPL producing  
363 CD20 negative B cells circulate in peripheral blood of APS patients [28]. This rational is sustained by  
364 the successful use of bortezomib on TEs recurrence and aPL titers in case reports [135,136]. As an  
365 alternative, anti-CD38 monoclonal antibodies, such as daratumumab, enter the field of autoimmune  
366 diseases therapy with impressive response in post-transplant patient with autoimmune cytopenia  
367 [137,138] and at least in 2 patients with SLE, in association with belimumab [139].

### 368 *Cytokines*

369 BAFF blockade has shown therapeutic potential in preclinical models. BAFF antagonists reduced  
370 renal and cardiac infarcts and prolonged survival in NZW×BXSB F1 mice although it did not prevent  
371 anti-CL development [140]. Interestingly, in human, BAFF level is increased in PAPS and secondary  
372 APS [24,30]. Belimumab, a monoclonal antibody against BAFF, was found to reduce anti-CL and  
373 anti-β2GPI IgM and IgG titers treatment in 12 SLE/APS patients independently of  
374 hydroxychloroquine treatment [141]. However, in a recent post-hoc analysis on two large randomized  
375 SLE-controlled trials focusing on anti-CL titers, intravenous belimumab showed a significant  
376 reduction of anti-CL IgA and IgG only in patients with concomitant antimalarial treatment [142].  
377 Similarly, Bettiol *et al.*, in another recent post hoc analysis of 249 patients with SLE from the BLISS-  
378 SC trial, observed a significant reduction of anti-β2GPI IgA and anti-CL IgM titres in the

379 belimumab+antimalarials group as compared with the placebo+antimalarials group, suggesting that  
380 the two treatments may exert beneficial synergistic effect [143]. The independent role of belimumab  
381 on aPL titre reduction needs to be further evaluated, and, so far, such data are lacking in PAPS.

382 Alternative therapeutic approaches might consider inflammatory mediators inhibitors such as type I  
383 IFN antagonists. Anifrolumab, that blocks the type I interferon receptor subunit 1, has proven  
384 efficiency on disease activity in SLE patients [144]. The high type I IFN signature observed in about  
385 40% of patients with PAPS [145], together with the positive correlation between plasma cells  
386 expansion and type I IFN upregulation in APS [28], support the evaluation of such antagonists.

### 387 *aPL targeting*

388 Plasmapheresis and intravenous immunoglobulins (IVIg) could thwart aPL pathogenic effects by  
389 increasing their clearance, inhibiting their action or through the control of anti-idiotypic antibodies. In  
390 CAPS, they are recommended as first line treatment in combination with steroids and heparin [126].  
391 Agostinis *et al.* identified a single-chain fragment variable directed against  $\beta$ 2GPI-DmI that induced,  
392 in mice, thrombosis and fetal losses in a complement dependent manner. Interestingly, a variant of this  
393 antibody lacking the CH2 domain, required for C1q binding and complement activation, compete with  
394 human anti- $\beta$ 2GPI IgG and prevent blood clot formation and fetal loss [146].

395 The use of peptide therapies recently provided another approach to inhibit aPL pathogenicity. The  
396 rationale is to block binding to target cells or receptors using small synthetic peptides. No clinical trial  
397 has been developed to date but such molecules, including synthetic analogs of  $\beta$ 2GPI-DmI or A1  
398 (ApoER2 ligand-binding domain) reduced thrombus formation in animal models [147,148]. Peptides  
399 therapy, as well as CH2-deleted antibody, could represent an innovative approach potentially useful to  
400 treat refractory APS patients.

### 401 *Beyond B cells*

402 Other conventional or targeted therapies could overcome the apparent limited effectiveness of B  
403 therapies and work synergistically to counterbalance pro-coagulation state. Some of them may exert  
404 indirect action on aPL or autoreactive B cells development. *In vitro*, hydroxychloroquine reverses the

405 disruption of AnxA5 shielding induced by aPL, and thereby restore the binding of AnxA5 to  
406 phospholipids [16]. Besides decreasing aPL titers in patients with secondary APS, hydroxychloroquine  
407 reduces LA activity [142,149]. Sirolimus may also contribute to lower aPL titers in SLE patients  
408 [130]. Statins exert multiple anti-inflammatory effects, including reduction on tissue factor expression  
409 by monocytes and cytokine secretion, but little is known about B cell implication. In accordance with  
410 the hypocomplementemia and increased levels of complement activation in APS patients [150], anti-  
411 C5 antibody prevents the prothrombotic activity of aPL *in vitro*, and precludes aPL-mediated  
412 thrombosis in some murine models [38]. This therapy showed promising results as adjunction therapy  
413 in CAPS, but results are limited to cases reports and do not enable conclusion on aPL titers [130,151].  
414 Further studies should elucidate the place of these therapies in the APS therapeutic plan and their  
415 specific effect on B cells and autoantibodies.

416

## 417 **6. CONCLUSION**

418 The central role of B cells in APS is highlighted by strong evidence of antibody mediated  
419 pathogenesis, specific B cell phenotype abnormalities, and by the development of the disease in  
420 patients with inborn errors of immunity involving B cell ontogeny. Recent findings questioned the  
421 links between B cell tolerance breakdown, genetic defects, and commensal microorganisms. However,  
422 the origin and characteristics of pathogenic aPL producing clones remain largely unknown. Despite  
423 the discrepancies in clinical trial results, therapies targeting B cells should remain an important  
424 therapeutic approach in APS. A broad range of immunomodulatory drugs has recently emerged with  
425 serious rationale to modulate the inflammatory response and vascular damages in APS. Further studies  
426 should determine if this new therapeutic area could provide a second wind to the field of B cell  
427 targeting in APS and which homogeneous groups of patients should benefit from it.

428

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436

#### 437 **CONFLICTS OF INTEREST**

438 Declarations of interests: none.

439

#### 440 **AUTHORSHIP**

441 Y.D, A.G., A-S.K. and V.G. wrote the manuscript.

442 Y.D, A.G., V.P., P.S.L, T.M., A-S.K. and V.G. reviewed or edited the manuscript before submission.

443 Y.D, A.G., V.P., P.S.L, T.M., A-S.K. and V.G. contributed to researching data for the article and  
444 discussions of the article content.

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**Table 1: aPL and/or phospholipid/aPL complex-mediated prothrombotic and proinflammatory changes**  
[9,12,19,21].

<b>Actors</b>	<b>Binding</b>	<b>Consequences</b>
<b>Anticoagulant mediators</b>		Inhibition of protein C activation inhibition of the tissue factor pathway inhibitor Inhibition of $\beta$ 2GPI anticoagulant activity Inhibition of antithrombin activity Disruption of AnxA5
<b>Endothelial cells</b>	$\beta$ 2GPI AnxA2 TLR2 TLR4	Expression of adhesion molecules (E-selectin, ICAM-1, VCAM-1) Increase of tissue factor expression PP2A activation, decreased NO production inhibition of the TF pathway inhibitor and of the C reactive protein activity Proliferation through mTOR pathway activation IL-1, IL-6, IL-8, and TNF- $\alpha$ release
<b>Platelets</b>	ApoER2 GpIbA	Platelet activation Increase of GPIIb-IIIa expression Thromboxan A2 synthesis
<b>Monocytes</b>	AnxA2 TLR2 TLR4	IL-1, IL-6, TNF $\alpha$ secretion Increase of tissue factor expression Increased intracellular ROS
<b>Neutrophils</b>	TLR4 C5a receptor	Increased NET release IL-8 secretion
<b>Complement</b>		Classical complement pathway activation Increased C5b-9 deposition on the cell surface
<b>Trophoblast</b>	$\beta$ 2GPI	Increased trophoblast apoptosis Decreased trophoblast proliferation and migration Decreased production of hCG defective secretion of growth factors
<b>Decidua</b>		Inflammatory response in decidual cells Defective angiogenesis and spiral artery development

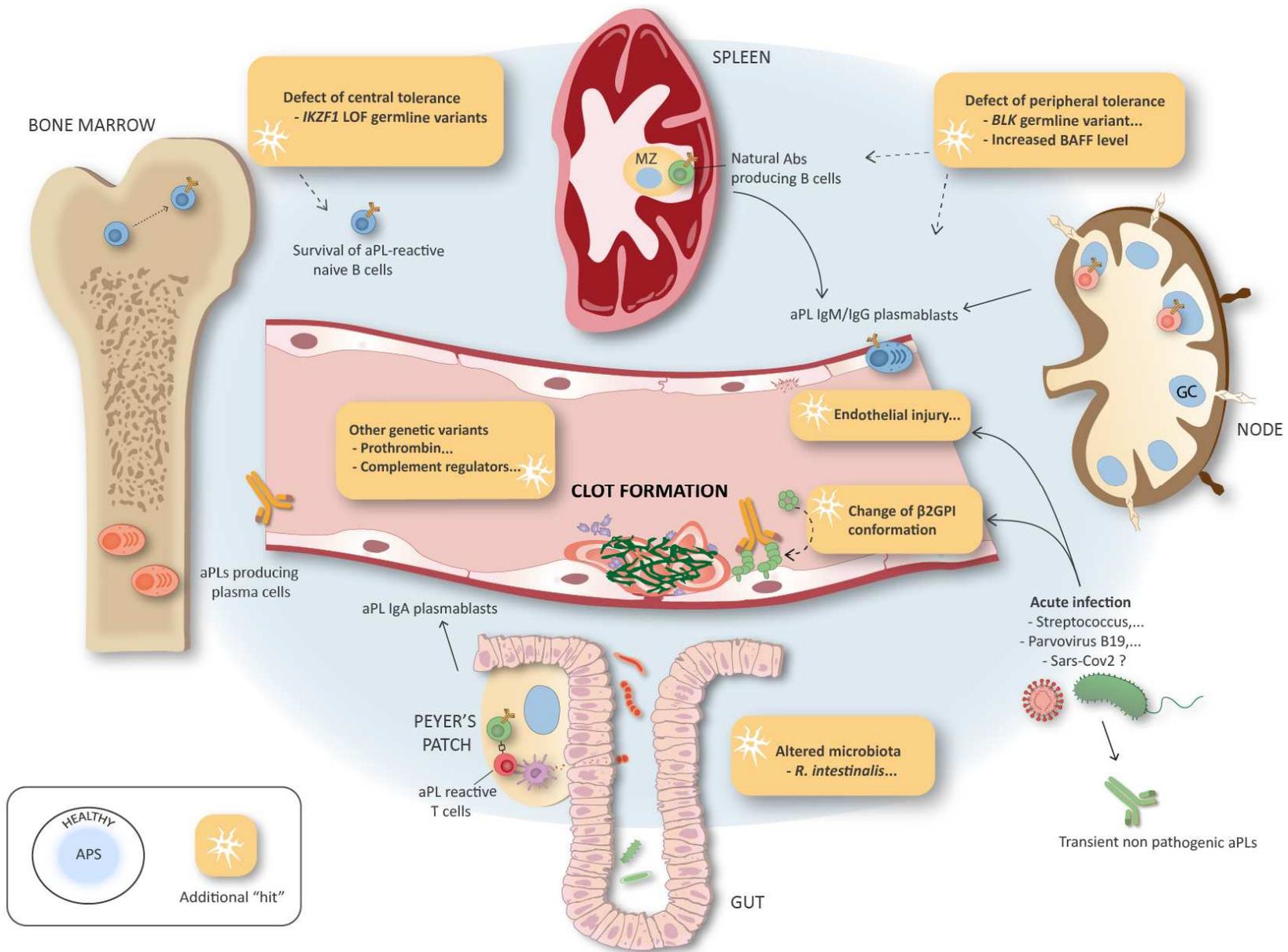
1 Anx, Annexin;  $\beta$ 2GPI,  $\beta$ 2 glycoprotein I; GP, Glycoprotein; hCG, Human chorionic gonadotropin; ICAM, Intercellular adhesion  
2 molecule; IL, Interleukine; NET, Neutrophil extracellular traps; NO, Nitric oxide; ROS, reactive oxygen species; TF, Tissue factor; TLR,  
3 Toll-like receptor; VCAM, Vascular cell adhesion molecule.

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**Table 2: Summary of aPL profiles according to identified clusters.**

Study	Subjects	Ethnicity	Thrombosis	Pregnancy morbidity	APS with SLE	Healthy aPL carriers	Comments
<b>Sciascia (97) 2019</b>	n = 486 - 257 PAPS - 96 SLE/APS - 133 aPL carriers	Caucasian	- high prevalence of anti-CL IgG/M (69%) 85% with multiple aPL positivity - high rate of triple positivity (35%)	- low rate of anti-β2GPI (23%) - low rate of multiple aPL positivity (30%) - low rate of triple positivity (4%)	- high rate of multiple aPL positivity (84%) - 22% with triple positivity	- high prevalence of anti-CL IgG/M (61%) - multiple aPL positivity in 57% of patients - no patient with triple positivity	<u>One additional cluster:</u> - defined APS (42%) and high rate of cytopenia (42%) - low rate of triple positivity (5%)  No data on extra criteria aPL
<b>Zuily (100) 2020</b>	n = 497 - 324 PAPS - 173 aPL/APS associated with other autoimmune disease	Various	<u>In females with frequent VTE:</u> - high rate of anti-CL (93%) - high rate of β2GPI (77%) - high rate of triple positivity (55%)  <u>In men with frequent ATE, heart valve disease, CV risk factors:</u> - anti-β2GPI :53% - triple positivity: 40%	<u>In females with isolated PM:</u> - high frequency of LA (80%) - high frequency of anti-CL (68%) - lower frequency of anti-β2GPI (40%)	- high prevalence of LA (85%) - low rate of anti-anti-β2GPI (14%) - low rate of triple positivity (7%)	<u>Asymptomatic aPL-positive females:</u> - high prevalence of anti-CL (85%) or anti-β2GPI (64%)	No data on extra criteria aPL
<b>Alijotas-Reig (101) 2019</b>	n = 1000 - 1000 OAPS - 76 OAPS with SLE	Caucasian	/	- high frequency of single LA positivity (35%) - low rate of double (18%) and triple positivity (11%)	/	/	<u>Recurrent miscarriage:</u> - high rate of single anti-CL IgG/M positivity (27%) - 26% with multiple aPL positivity <u>Fetal loss:</u> - high rate of single LA positivity (31%)  No data on extra criteria aPL

anti-CL, Anticardiolipin antibody; aPL, Antiphospholipid antibody; ATE, Arterial thromboembolic event; CV, Cardiovascular; LA, Lupus anticoagulant; OAPS, Obstetric antiphospholipid syndrome; PAPS, Primary antiphospholipid syndrome; PM, Pregnancy morbidity; SLE, Systemic lupus erythematosus; VTE, venous thromboembolic events.



## FIGURE LEGEND

**Figure 1: From B-cell tolerance breakdown to thrombosis in APS : a multistep process toward B cell maturation.** Predisposal genetic background, chronic inflammation or continuous antigen exposure through host-commensal interactions represent crucial “hits” that could favor the escape of transient or anergic aPL reactive clones to tolerance checkpoints, their expansion and maturation, and lead to aPL-secreting plasma cells accumulation. Specific epitope exposure due to additional factors (infection, inflammation...) thereafter provide the trigger leading to clinical events, such as thrombosis, defining APS.

Abs, antibodies; aPL, Antiphospholipid antibody; APS, Antiphospholipid syndrome; BAFF, B-cell activating factor; CMV, cytomegalovirus; GC, Germinal center; IgA/G/M, Immunoglobulin A/G/M; LOF, Loss of function; MZ, Marginal zone.