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**Utilizing type I interferon expression in the identification of antiphospholipid syndrome subsets**

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**Keywords:** Antiphospholipid Antibodies, Antiphospholipid Syndrome, Interferon, Interferon Signature, Type I Interferon.

## Article highlights:

- The type I interferon pathway may be a central player in APS pathogenesis and development
- Type I interferon signature have shown promising results in discriminating distinct APS phenotypes
- The translational application of the type I interferon signature in routine clinical practice, although fascinating, needs further investigation
- Heterogeneous methodological approaches in type I interferon signature quantification represent a major limitation for its translational applicability in APS setting
- The use of therapeutic agents directly or indirectly targeting interferon production and functions is promising, especially for those aPL-positive patients who presented higher IFN-I signature

## Abstract

*Introduction:* Antiphospholipid Syndrome (APS) is a systemic autoimmune disease with a complex multifactorial pathogenesis, combining genetic background, traditional cardiovascular risk factors, disease-specific features such as the presence of antiphospholipid antibodies (aPL), and an imbalance of various immune system functions. Recent data support the role of interferons (IFN), especially type I IFN (IFN-I), in the onset and development of APS clinical manifestations, including thrombotic events and obstetric complications. *Areas covered:* In this review we aimed to discuss the growing body of evidences on the relevance of IFN-I pathways in APS, both from a basic mechanistic perspective, focusing on its possible use in disease/patients stratification. The IFN-I signature has shown promising, although preliminary, results in segregating aPL-positive subjects by aPL profile, association with other autoimmune conditions such as lupus, age at onset, and current treatment, among others. *Expert opinion:* To date, the scarce available data as well as methodological and technical heterogeneity among studies limit the comparability of the results, thus requiring further validation to translate these findings to routine clinical practice. Therefore, further research is required in pursuit of more nuanced patient profiling and the development of new immunomodulatory therapeutic strategies for APS beyond anti-coagulant and anti-platelet agents.

## 1. Introduction

The Antiphospholipid Syndrome (APS) is clinically defined by the occurrence of vascular thromboses, both venous and arterial, and by pregnancy morbidity events, such as recurrent unexplained early miscarriages, foetal deaths and late obstetrical complications in patients persistently positive for antiphospholipid antibodies (aPL), including lupus anticoagulant (LA), anti-cardiolipin (aCL), and anti- $\beta_2$ -glycoprotein I (a $\beta_2$ GPI) antibodies [1]. Commonly, affected patients manifest exclusively one of the two forms, either thrombotic or obstetric APS, and only a minority of subjects have characteristics of both, suggesting the existence of pathogenic mechanisms that are only partially overlapping [2].

While aPL positivity is necessary to confirm a diagnosis of APS, their mere presence seems not to be sufficient to cause the full APS pathophysiology, and the hypothesis of a second trigger, therefore called “second hit”, is usually suggested [3]. Furthermore, since its first description, the clinical spectrum of APS has been expanded, including a wide range of pathologic features, the so-called “extra criteria manifestations”, such as cardiac valve disease, cognitive impairment and haematological abnormalities, which cannot be explained solely by the hypercoagulable state typical of the disease [4]. In parallel, new autoantibody specificities and pathogenic pathways have been identified and their role in APS pathogenesis is emerging with variable degrees of relevance to APS clinical scenarios [5–11]. Overall, these advances reflect the complex multifactorial nature of APS pathogenesis and support the difficulties encountered by physicians in managing this systemic condition [12].

Interferons (IFNs) are a large family of proteins with autocrine and paracrine actions. IFNs are mostly secreted by infected cells in response to viral infections but they also play numerous roles in modulating innate and adaptive responses, including in cancer protection and autoimmunity. Based on their receptors, IFNs have been categorized into three main distinct groups: IFN type I (including IFN- $\alpha$  and IFN- $\beta$  among others), II, and III. These groups are further divided into subtypes based on their structural and antigenic characteristics [13].

Type I IFN (IFN-I) exert several crucial functions, such as balancing the innate immune response and favouring immunologic memory and antigen-specific T and B cells

1 responses. Nowadays, IFN-I is also considered a key player in the initiation, progression  
2 and treatment response of a wide number of immune-rheumatic diseases, such as  
3 systemic lupus erythematosus (SLE), Sjögren's syndrome, systemic sclerosis, rheumatoid  
4 arthritis (RA) and myositis [14].

5 In this review, we aimed to summarise the available evidence regarding the pathogenic  
6 pathways and mechanisms associated with IFN-I activation in APS, as well as potential  
7 clinical and therapeutic implications.

## 8 9 **2. Interferon activation and signature in APS patients: pathogenic pathways and** 10 **mechanisms**

### 11 **2.1 Sources of type I interferons in APS**

#### 12 **2.1.1 Cells producing type I interferons**

13 Plasmacytoid dendritic cells (pDCs) recognize viruses via toll like receptors (TLRs) and  
14 release IFN-I, thus modulating anti-viral responses [15,16]. pDCs are also known to be  
15 major producers of the specific IFN-I, interferon alpha (IFN- $\alpha$ ) in many autoimmune  
16 diseases [17–22]. Beyond pDCs, fibroblasts [23], epithelial cells [24], endothelial cells  
17 [25], and neutrophils [26,27] can be primed to produce IFN-I. Which cells are the most  
18 important producers of IFN-I in APS remains to be determined.

#### 19 **2.1.2 TLR-triggered generation of type I interferon**

20 In 2011, Prinz et al. [28] demonstrated increased expression of TLR7/8 by peripheral  
21 blood mononuclear cells (PBMCs) in the specific population of APS patients. In this  
22 study exposing pDCs to either total IgG fractions from patients or non-cofactor-  
23 dependent monoclonal aPL promoted IFN- $\alpha$  expression in a TLR7-dependent manner;  
24 this only occurred in the presence of traditional TLR7 ligands such as single-stranded  
25 RNA, suggesting that aPL sensitize pDCs to these ligands [28]. This ligand dependence  
26 is somewhat reminiscent of lupus, where IFN-I induction likely depends on the presence  
27 of both RNA and ribonucleoprotein-binding antibodies [29–33]. From a mechanistic  
28 standpoint, aPL appeared to prime pDCs to internalize RNA, while also promoting  
29 translocation of TLRs from the endoplasmic reticulum to the endosome [28]. All these  
30 events were downstream of endosomal NADPH activation and superoxide production,  
31 as inhibiting either blocked aPL-mediated effects [28]. Based on these findings, it has

1 been suggested that, irrespective of the concomitant presence of SLE, aPL are able to  
2 directly maximize the sensitization to TLR7. This could represent, therefore, an  
3 additional pathogenic factor in APS and in aPL- positive SLE patients. In the context of  
4 SLE, a large amount of data indicates that different TLRs, mainly TLR2-5 and TLR7-9,  
5 are involved in SLE susceptibility, onset, and progression [34]. Regarding the  
6 production of IFN-I in SLE patients, it is recognized that pDCs are induced via TLR9  
7 stimuli to produce IFN- $\alpha$ , increasing the production of B cell activating factor (BAFF),  
8 which in turns activates B cells autoreactivity [35]. In addition, IFN- $\alpha$  levels are  
9 reported to correlate with TLR5, TLR7, and TLR9 mRNA in PBMCs of SLE patients  
10 [36].

11 It is worth mentioning that other TLRs, such as TLR2 and TLR4, which are not directly  
12 linked to IFN-I, are also involved in APS pathogenesis. They have been shown to have  
13 a central role in thrombosis, endothelial dysfunction, and inflammatory cytokine and  
14 chemokine production, with their activation mediated by the presence of aPL, especially  
15 a $\beta$ 2GPI IgG and a $\beta$ 2GPI IgG/ $\beta$ 2GPI immune complexes [37].

### 16 **2.1.3 Potential connections between $\beta$ <sub>2</sub>-glycoprotein I and type I interferon**

17  $\beta$ <sub>2</sub>GPI, a circulating phospholipid-binding protein with roles in innate immunity and  
18 apoptotic cell clearance, is the most studied antigen for aPL [38–41]. Using a lupus  
19 mouse model that relies on duplication of TLR7, Giannakopoulos et al. [42] found  
20 impaired apoptotic cell clearance when  $\beta$ <sub>2</sub>GPI was knocked out. The knockout mice also  
21 demonstrated higher IFN-I signatures, higher autoantibody titers, and an exaggerated  
22 lupus phenotype as compared with the  $\beta$ <sub>2</sub>GPI-expressing mice [42]. The Authors  
23 speculated on a TLR7 inhibitory function of  $\beta$ <sub>2</sub>GPI in this model, which might be  
24 disrupted by aPL [42]. To this end, several studies have found a correlation between the  
25 presence of a $\beta$ <sub>2</sub>GPI antibodies and the IFN-I signature in primary APS patients [43–45].  
26 One study showed that while IFN-I levels in patients with primary APS, SLE only, and  
27 SLE with APS were similar, and a $\beta$ <sub>2</sub>GPI positivity was correlated with higher IFN-I  
28 score in primary APS patients. This suggests there may be different and independent  
29 pathways that drive disease pathogenicity [46].

30 An area for potential future research is the fact that basal levels of IFN-I appear to be  
31 maintained by commensal bacteria [47–50]. Interestingly, a recent study hinted that the  
32 commensal bacterium *Roseburia intestinalis*, which has homologous sequences to

1  $\beta_2$ GPI, may be a nidus of autoimmunity in APS patients [51]. The extent to which the  
2 microbiome may be contributing to aPL and/or IFN-I production in APS is an area we  
3 expect to see investigated in the coming years.

#### 4 **2.1.4 MicroRNA and type I interferon**

5 MicroRNAs are short non-coding RNA molecules that bind to the 3' end of untranslated  
6 mRNAs and reduce their translation, thereby regulating gene expression [52]. There is  
7 ample evidence that specific microRNAs destabilize and thereby suppress IFN-pathway  
8 transcripts in different cell types; not surprisingly, dysregulation of these microRNAs  
9 have been associated with autoimmune diseases [52–59]. In a recent study, global pDC  
10 microRNA expression was found to be downregulated in APS patients (both primary  
11 and associated with SLE), where it tracked with the IFN-I signature [60]. Pathway  
12 analysis of the downregulated microRNA target genes demonstrated “pDC activation”  
13 as a regulated pathway, thereby hinting at interplay between pDCs, microRNAs, and  
14 IFN-I [60]. Interestingly, related studies have found APS patient microRNAs to be  
15 associated with accelerated atherosclerosis and oxidative stress, as well as the  
16 prothrombotic phenotype inherent to many APS patients [61,62].

#### 17 **2.1.5 Neutrophil extracellular traps and type I interferon**

18 Neutrophil extracellular traps (NETs) are extracellular webs of DNA and microbicidal  
19 proteins that have the potential to cause tissue damage, trigger endothelial activation,  
20 and serve as a source of autoantigens [63–65]. As compared with healthy neutrophils,  
21 APS neutrophils release NETs in an exaggerated fashion [64,66], while administration  
22 of aPL to mice triggers large thrombi enriched in NETs [67]. APS patients also appear  
23 to have increased circulating numbers of so-called low-density granulocytes  
24 (LDGs)[68], which have a lower threshold to release NETs as compared with normal-  
25 density neutrophils. In lupus, it is well established that NETs activate pDCs via TLRs  
26 and thereby induce production of IFN-I, which in turn primes neutrophils to release  
27 more NETs [64,69,70]. Interestingly, recent data showed that while augmented NET  
28 release and an increase in LDG numbers were observed in those SLE patients who  
29 display higher IFN-I signatures, LDG numbers did not associate with IFN-I signatures  
30 in APS [71]. These findings, although preliminary, further strengthen the idea of APS  
31 and SLE as distinct pathological entities, with different pathogenic pathways and

underlying mechanisms. Nevertheless, whether NETs are a driver of IFN-I production in APS setting is still an area deserving of future research [72].

An interesting study related to this topic reported that SLE patients with a single nucleotide polymorphism (SNP) in the neutrophil cytosolic factor 1 gene, which encodes a subunit of NADPH oxidase 2, not only have higher IFN-I activity (as compared to SLE patients with the normal genotype), but are also more likely to have high levels of circulating aPL and to actually develop APS [73]. While overall NET production in patients with the implicated SNP was apparently impaired due to reduced production of cellular reactive oxygen species (ROS), neutrophil expression of mitochondrial ROS was enhanced [73], with mitochondrial ROS being known to trigger NETs that are particularly interferogenic [74]. Another study profiling gene expression of primary APS neutrophils as compared with control neutrophils found IFIT1 (an IFN-I regulated gene) as the most upregulated gene [75], hinting at a potential bidirectional relationship between neutrophils and IFN-I in the context of APS. Many questions still remain including the extent to which IFN-I may regulate neutrophils in APS.

## **2.2 Downstream effects of type I interferon in APS**

### **2.2.1 Vascular damage and type I interferon**

IFN-I are a well-known driver of the endothelial dysfunction and premature atherosclerosis inherent to SLE [76,77]. Accelerated endothelial damage and atherosclerosis have also been recognized in many APS patients [78–81]. In a cohort of patients with primary APS, Grenn et al. [43] evaluated the association between the IFN-I signature and endothelial progenitor cell (EPC) dysfunction, which have been linked together in other autoimmune diseases [82–86]. They found that both classic EPCs and circulating angiogenic cells (CACs, which are myeloid-derived and have proangiogenic properties) from APS patients, failed to differentiate into mature endothelial cells [43]. This defect did not correlate with the presence of aPL, but rather with levels of IFN-I. Furthermore, blockade of the IFN-I receptor *in vitro* rescued the differentiation into healthy endothelial cells [43]. In pursuit of other mechanisms that could lead to vascular damage in APS, a different group found IFN-I expression to correlate with both increased tissue factor expression and increased intermediate and non-classical monocytes, the latter having been associated with cardiovascular disease in SLE and RA [44].



### 2.2.2 Type I interferon and adaptive immunity

As a bridge between innate and adaptive immunity, IFN-I activate antigen-presenting cells (APCs), increase the expression of CD86 and MHC class I and II molecules on APCs, and enable B cell differentiation [87–89]. Specific to humoral immunity, IFN-I upregulate BAFF on DCs [90] and efficiently trigger BAFF mobilization from intracellular stores to the cell surface of lupus monocytes [91]. B cells and pDCs from SLE patients demonstrate increased BAFF expression, which can be decreased by anti-IFN-I agents [91–93]. In APS, clinical studies have found a correlation between BAFF levels and higher adjusted global APS scores [94]. Higher levels of both BAFF and IFN- $\alpha$  have also been appreciated in APS patients with adverse pregnancy outcomes, albeit without a clear correlation between the two cytokines [95]. Whether disrupting IFN-I activity in APS may also negate BAFF expression is an area deserving of future research [96], including disease stratification to guide treatment-decision making.

When focusing on pregnancy complications in the context of APS, a very limited number of studies have observed an association between obstetric events, mainly preeclampsia and higher IFN-I levels [97], which is in line with previous data from SLE patients suggesting a role for IFN- $\alpha$  in inducing angiogenic imbalance [98]. Further studies are needed to elucidate the possible role of IFN-I in pregnancy morbidity in APS patients.

As discussed above, ligands of TLR7 trigger IFN-I production by pDCs, with subsequent differentiation of B cells into antibody-producing plasmablasts [99]. Recently, a study linked a specific TLR7 SNP to higher IFN-I production from APS PBMCs; the patients also demonstrated increased plasmablast numbers along with decreased memory and regulatory B cell populations [100]. When studied *ex vivo*, it appeared that CD20-negative B cells (a population that includes plasmablasts) were the most important producers of aPL [100]. These results are notable given that similar derangements in B cell populations have been found in SLE, where circulating plasmablasts are predictive biomarkers in at least a subset of patients [100–102].

## 3. IFN signature and APS: from different laboratory techniques to diagnostic and therapeutic implications

1 Due to the rapidly growing body of evidence supporting a role for IFN-I in the  
2 immunopathogenesis of APS, and other systemic diseases, there has been a parallel  
3 interest in capturing the activation of the IFN-I pathway from a translational  
4 perspective. The assessment of the IFN-I pathway activation through the so-called ‘IFN  
5 signature’ has been hypothesized to provide an added value in diagnosis, patient  
6 stratification and treatment decisions in APS. However, despite several efforts, some  
7 obstacles still exist before considering the IFN signature as a biomarker to be used in  
8 the routine clinical setting. To date, the cross-sectional nature of the majority of the in  
9 vivo studies limits clinical validation. Moreover, the heterogeneity of assays used to  
10 measure the IFN signature and IFN activation described in the literature represents a  
11 further challenge to be addressed. In the following section, a summary of the  
12 approaches to capture the IFN signature in APS together with their potential clinical  
13 relevance are discussed.

14 A major challenge to assess the IFN activation is the great diversity of IFN ligands and  
15 their relatively short half-lives, which hinder our ability to capture IFN activation at the  
16 protein level. Although recent developments such as ultrasensitive single molecule  
17 assays (Simoa) [20] or dissociation-enhanced lanthanide fluorescence immunoassay  
18 (DELFI)[103] seem to partially overcome these issues, there is limited evidence on  
19 their actual added value. Besides, their use in the setting of APS needs to be explored.  
20 Consequently, most of the available studies focused on the assessment of the cellular  
21 response to IFN-I, that is, the assessment of IFN-inducible gene expression, usually in  
22 whole blood or sorted immune cell populations. These approaches have been conducted  
23 by qPCR classical methods or by large-scale microarrays. Pre-analytical, technical-  
24 related, and methodological limitations can be also observed for this approach, the  
25 choice of the candidate IFN-inducible genes and the calculation of the score or signature  
26 being the most evident ones. With a similar rationale, the quantification of IFN-induced  
27 gene encoded proteins in serum has been also explored in APS. Additionally, the use of  
28 cellular assays to measure IFN activity using different cell substrates has been also  
29 proposed. Finally, the identification of epigenetic fingerprints to the IFN signature has  
30 added another layer of complexity to this scenario. In sum, a considerable number of  
31 distinct approaches have been described to measure the IFN signature in APS, although  
32 they cover a notable variety of biological processes and readouts. Consequently, there is  
33 a relatively solid evidence about the presence of the IFN-I pathway in APS, but its

1 clinical implication needs to be considered with caution. Although grouped under a  
2 common umbrella, these methods likely provide a different, and probably  
3 complementary, information for the clinical setting.

4 Microarray studies have revealed that a number of IFN-related genes are differentially  
5 expressed in APS as compared with healthy controls and/or SLE patients, either in  
6 whole blood or in sorted cell subsets (monocytes or neutrophils)[43,44,75,104,105].  
7 Actually, IFN-related genes were ranked among the most upregulated genes [43,104].  
8 Although a significant overlap was observed with SLE and SLE-associated with APS,  
9 current evidence suggests that differential, APS-specific components can be found in  
10 the IFN signature, which reinforces differential pathogenic substrates among these  
11 conditions [104], as previously discussed. Interestingly, this specific imprint was related  
12 to mitochondria biogenesis and function and oxidative stress, hence underlining the  
13 links between IFN and mitochondrial activity in APS. Nevertheless, although  
14 interesting from a basic perspective, in order to relate individual genes to complex  
15 pathogenic pathways, the added value of microarray studies for clinical and therapeutic  
16 decisions is uncertain.

17 Most of the studies analysing the IFN signature in APS are based on gene expression  
18 analyses by qPCR methods. However, a large variation in the number of genes assessed  
19 (from 3 to 41 genes) and a lack of consistent methods to select the target genes  
20 (candidate genes vs unsupervised methods) and score calculations (averages,  
21 normalizations, z-scores, etc.) make difficult to compare the results obtained across  
22 studies [44,106–109]. In general terms, an IFN signature can be distinguished in APS  
23 patients, and both quantitative and qualitative differences seem to emerge compared to  
24 SLE or secondary APS patients. In fact, an IFN signature can be observed in about a  
25 half of the APS patients (38-49%)[44,106], whereas a higher activation could be found  
26 in SLE (70-90%)[44,106,110]. Additionally, not all genes exhibited similar alterations  
27 in APS compared to SLE or secondary APS patients, thus reinforcing their potential  
28 disease-specific involvement. Interestingly, the genes more consistently analysed were  
29 those ubiquitously found to be elevated in systemic diseases, such as MX1, IFI44,  
30 IFI44L or IFIT1. Although the increase in the IFN signature in APS is clear, there is  
31 limited evidence on their actual value as a diagnostic biomarker, and whether it should  
32 be considered a patient subset feature rather than a disease feature is still under debate.  
33 Since the former will be more relevant for patient stratification whereas the latter will be

for disease diagnosis, this represents a point of major interest for the clinical setting. In patients with established disease, some studies have highlighted an association between the IFN signature and diverse disease outcomes, such as antibody status (a $\beta$ <sub>2</sub>GPI positivity and triple positivity)[43,106], obstetric events [108] or age at onset [108], but replication studies are needed.

Of note, a recent study from Flessa et al. have moved beyond IFN-I and tried to explore the significance of a new member of the IFN family, the type III IFNs (IFN $\lambda$ , particularly IFN $\lambda$ 1) which is thought to play an additional role in the pathogenesis of several autoimmune conditions, such as APS [111]. Interestingly, lower IFN $\lambda$ 1 gene expression in PBMCs showed an independent association with obstetric APS and pregnancy complications. This data might suggest an impaired function of IFN $\lambda$ 1 in modulating neutrophil activation and NETs formation leading to foetal damage in the affected patients. Moreover, the study reported higher levels of IFN-I score/IFN $\lambda$ 1 ratio in those primary APS patients who presented with triple aPL positivity and a $\beta$ <sub>2</sub>GPI [111]. Overall, these evidences seem to confirm both the pivotal role of IFNs in APS pathogenesis and its link with autoantibodies production.

Finally, IFN signature by qPCR has been reported to be influenced by pharmacological agents, such as hydroxychloroquine (HCQ) [44,106] and statins [44].

On the one hand, these findings may suggest that the IFN signature could be a targetable mechanism in APS, as proposed in other conditions. On the other hand, these results highlight that treatments can be a significant confounding factor for the IFN signature, hence requiring a dedicated analysis, especially since studies addressing the very early stage of the disease in treatment-naïve cohorts are lacking.

Detection of IFN signature in APS at the protein level, including IFN $\alpha$  or IFN-induced proteins detection [43,107], as well as functional assays using cell-reporter lines have also been used in APS [43]. Although some studies showed promising results, the findings are far from providing a clear added value for the clinical setting. Finally, a recent genome-wide methylation study has characterized several differentially methylated sites between APS and controls [112]. Among them, the hypomethylation of the IFI44L locus was found to discriminate between APS and SLE.

## 4. Conclusions

In conclusion, available data suggest that aPL have the ability to potentiate TLR activity, which results in IFN-I production by pDCs and possibly other cell types. This process is likely to be modulated by microRNAs and NETs, with hints that bidirectional relationships may be at play. Regarding pathogenesis, elevated levels of IFN-I may contribute to endothelial damage and increased in plasmablast numbers, the latter likely to be an important source of aPL. In Table 1 and Figure 1 the main mechanisms involved in IFN-I production and its effects are presented, along with main therapeutic immunomodulatory approaches and possible future treatment targets in the APS context.

While IFN-I represent a fascinating and promising tool in APS clinical setting, both for diagnostic, clinical management, and prognostic purposes, the high heterogeneity in pre-analytical and methodological approaches along with the relatively low number of available studies (and lack of long-term trials), have limited researchers to reach solid conclusions negatively impacting the translation of these findings into routine clinical practice. Nevertheless, current literature has pointed out the existence of a distinct IFN-I signature when comparing APS patients with healthy controls and SLE with or without APS, along with its correlation with clinical and disease-specific serological features, thus supporting its potential role in identifying different subsets of the disease.

## 5. Expert opinion

### *5.1 How can type I interferon expression help us in identifying APS subsets?*

To date, IFN-I have been extensively proven to be relevant in SLE setting, for diagnosis, patient characterization and disease activity assessment [24,113,114]. Subsequently, fewer studies have evaluated the ability of the IFN-I signature and activation to identify specific subgroups of aPL-positive subjects in larger SLE cohorts with promising results [107]. In fact, Iwamoto et al. reported an independent correlation between aPL positivity and IFN $\alpha$  activation in SLE African-American patients. Moreover, this correlation was not observed in either Hispanic-American or European-American subjects, supporting the link between IFN pathways not only with disease-specific features, such as autoantibody production, but also with genetic background, which is well known to play a central role in APS pathogenesis [12,115]. Interestingly,

a recent attempt by Idborg et al. to characterize a cohort of SLE patients from a molecular perspective based on their autoantibody profile, have identified two distinct main subgroups, one associated with the presence of SSA/SSB positivity and the other characterized by LA positivity [116]. Higher degrees of IFN-I activation were found in the SSA/SSB+ subgroup as compared with the aPL-positive patients where complement activation and systemic inflammation seemed to be more prominent mechanisms, with possible therapeutic implications [116]. Furthermore, recent experiences have specifically addressed the role of IFN-I in primary APS showing clear associations with specific clinical manifestations and autoantibody profiles [45,108]. Moreover, different degrees of global activation of the IFN-I signature along with distinct activation patterns of IFN-I-regulated gene networks have been appreciated among healthy controls, aPL-carriers, primary APS patients, and SLE patients with and without APS; furthermore, a positive association has been detected between IFN-I signatures and the presence of the anti-phosphatidylserine/prothrombin IgG isotype, an autoantibody specificity of emerging importance in APS [117,118]. Taken together, these limited data support the possible use of the IFN-I signature in APS characterization and disease subset identification, with potential implications for more effectively personalizing prognosis and therapeutic strategies. Moreover, the identification of disease mechanisms occurring at the earliest stages of the disease will broaden the understanding about disease-initiating pathogenic mechanisms, with potential relevance for disease prevention and/or early management.

## *5.2 How can type I interferon expression play a role in guiding the therapeutic strategies in APS?*

Evaluating the potential associations between the IFN-I signature and the clinical, laboratory and treatment characteristics of patients with primary APS is crucial in pursuit of identifying potential new approaches directly or indirectly targeting IFN pathways by using both highly-specific anti-IFN antibodies or therapeutic approaches that can modulate IFN production and functions. In this context, the use of HCQ, which is largely employed in both thrombotic and obstetric APS as well as a prophylactic strategy in high-risk aPL-positive subjects, has been shown to be associated with lower degrees of IFN-I activation in patients with primary APS [45], in line with its ability to modulate NETs formation and TLR signalling [44]. Monoclonal anti-IFN $\alpha$  agents, such as sifalimumab and rontalizumab, have been developed and tested in SLE cohorts, with

heterogeneous results [119,120]. Other monoclonal antibodies with a potentially wider spectrum of anti-IFN-I functions, such as anifrolumab, have demonstrated more promising results [121].

Overall, given the likely role of the IFN-I signature in APS pathogenesis and progression, as well as the complex interplay between the various cellular functions and pathologic mechanisms regulated by IFN-I, the employment of therapeutic agents able to directly or indirectly target the IFN-I-associated pathways represents an interesting treatment option both in SLE and APS. Further prospective studies are however needed in order to elucidate clinical effectiveness and safety profiles, especially in APS.

### *5.3 How can type I interferon signature can be useful in APS patients stratification and profiling?*

Although the presence of the IFN-I signature in APS patients has been demonstrated by different and complementary approaches, a number of knowledge gaps still remain that prevent their implementation in routine clinical practice at present. First, the lack of consensus about how to best quantify the IFN-I signature is a major impediment. Standardized recommendations are therefore warranted. Furthermore, large, well-designed studies, with an adequate appraisal of confounding factors and long-term follow-up, are needed to delineate the added value of the IFN-I signature especially for disease activity assessment and individual risk stratification. In fact, the concept of an APS disease activity index represents an unmet need giving the lack of reliable biomarkers which directly correlate with the development of discrete thrombotic and obstetric complications as well as organ damage over time. On the other hand, numerous attempts have been made with the aim of identifying homogeneous groups of subjects at greater risk of developing clinical manifestations of the syndrome which however seem inadequate to cover the entire spectrum of the disease [122–124]. Scoring systems have been also developed and validated over the years combining disease specific features and traditional cardiovascular risk factors, which have a pivotal role in clot formation [125,126]. Two recent studies, in which a clustering-analysis approach was employed, confirmed the presence of relatively homogeneous subgroups of aPL-positive subjects sharing similar clinical and serological characteristics corresponding to known and defined nosological entities [127,128]. At the same time, both studies also demonstrated the extreme heterogeneity of APS manifestations and the existence of an interesting subgroup of aPL-positive patients showing intermediate

characteristics between SLE and primary APS, particularly with lower risk for developing thrombotic events and higher rates of systemic features such as anti-nuclear antibodies (ANA) positivity (97%) and cytopenia (mainly thrombocytopenia), supporting the idea of a *continuum* in APS clinical spectrum [128]. In line with these data, the employment of an approach based on patient profiling, rather than its mere categorization into discrete disease groups, should allow for a more real-life, precise, and personalized management of these patients. Interestingly, given its crucial role in anti-viral responses, IFN-I has been further studied in the context of SARS-CoV-2 infection. Based on the available data, the dysregulation of IFN functions has been associated with the most severe forms of the disease [129], through an exaggerated enhancement of pro-inflammatory factors that lead to tissue damage, a hypercoagulable state and autoimmune reactions, including the production of autoantibodies, especially in susceptible individuals [129–131]. These data would seem to both confirm the complexity of IFN pathways in disease pathogenesis and progression, while also emphasizing that we must move beyond a dichotomous perspective and rather employ more sophisticated approaches that will enable more insightful clinical application.

Available data suggest that the IFN-I signature may represent an additional tool not only to better define APS patients, but also to better characterized aPL-positive patients who do not fulfil traditional classification criteria, in particular aPL asymptomatic subjects, patients who are refractory to standard treatment and those patients who present with intermediate and “systemic” forms of APS who could benefit from alternative therapeutic approaches beyond anti-coagulant and anti-platelets agents (with anti-IFN-I agents being one consideration). In addition, a special focus should be made in the early stages of the disease, in order to assess the role of IFN-I signature in early diagnosis and to evaluate drivers of its fluctuations during follow-up.

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Factors potentially associated with increased IFN- $\gamma$ in APS			
Factor	Possible mechanism	Study design	References

1            <https://pubmed.ncbi.nlm.nih.gov/32733003/>.

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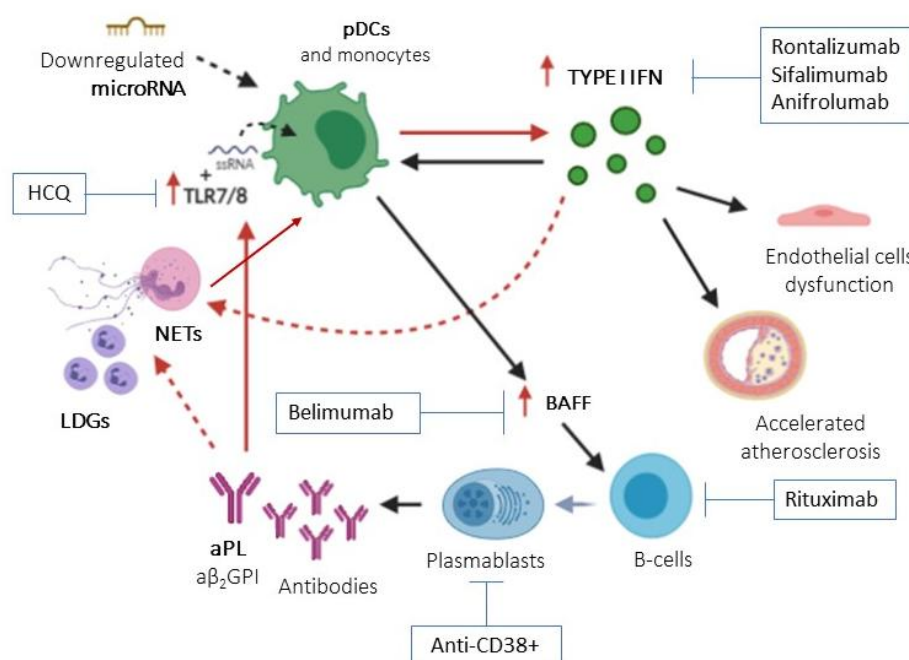
6

7    **Table 1.** Influence and regulation of type I interferon in antiphospholipid syndrome

<b>aPL</b>	<ul style="list-style-type: none"> <li>Associated with IFN-I signature in clinical studies</li> <li>Increase IFN-I transcript expression by pDCs</li> <li>Prime pDCs to internalize RNA</li> <li>Promote translocation of TLRs from endoplasmic reticulum to endosome</li> <li>Sensitize cells to TLR ligands</li> <li>Possibly inhibit the anti-inflammatory function of <math>\beta_2</math>GPI</li> <li>Microbiome (<i>Roseburia intestinalis</i>) due to the presence of homologous sequences to <math>\beta_2</math>GPI can play a role in IFN-I production</li> </ul>	Human <i>in vivo</i> , <i>ex vivo</i> , <i>in vitro</i> and animal models [27,37-40], PAPS [38], SLE, SAPS and PAPS [39,40]	[28,42–45]
<b>TLRs</b>	<ul style="list-style-type: none"> <li>Increased expression in APS</li> <li>pDCs produce IFN-I in a TLR7-dependent pathway</li> <li>TLR7 ligands needed to produce IFN-I</li> <li>Interferogenic TLR7 SNP associated with increased plasmablast numbers</li> </ul>	Human <i>in vivo</i> , <i>ex vivo</i> , <i>in vitro</i> and animal models, PAPS, SAPS [92]	[28,100]
<b>pDCs</b>	<ul style="list-style-type: none"> <li>Quintessential producers of IFN-I</li> </ul>	Human <i>in vivo</i> , <i>ex vivo</i> , <i>in vitro</i> and animal models [16-19]	[17–22]
<b>Potential downstream effects of IFN-I in APS</b>			
<b>Endothelial injury</b>	<ul style="list-style-type: none"> <li>IFN-I linked with EPC dysfunction</li> <li>Increased pro-atherosclerotic monocyte subsets</li> </ul>	Human <i>in vivo</i> , <i>ex vivo</i> assays, PAPS, SAPS and SLE patients [38, 39]	[43,44]
<b>Increased BAFF</b>	<ul style="list-style-type: none"> <li>Potential correlation between IFN-I and BAFF in APS patients</li> </ul>	<i>In vivo</i> assays, aPL positive PAPS, SAPS and SLE patients [88, 89]	[94,95]
<b>Potential bidirectional effects</b>			
<b>NETs</b>	<ul style="list-style-type: none"> <li>APS patients have increased number of LDGs, which produce more NETs</li> <li>NETs are a source of antigens that can stimulate TLRs on pDCs; as discussed above, TLRs and pDCs can enhance IFN-I production</li> <li>APS NETs have enhanced expression of mitochondrial ROS, which are more interferogenic than cytosolic ROS</li> <li>The IFN-I regulated gene group was the most significantly enriched in APS neutrophils</li> <li>In SLE, IFN-I primes neutrophils to form NETs</li> </ul>	Human <i>in vivo</i> , <i>ex vivo</i> , <i>in vitro</i> and animal models, PAPS, SAPS and SLE patients [64,66,68,70,73–75]	[64,66,68,70,73–75]
<b>Plasmablasts</b>	<ul style="list-style-type: none"> <li>A SNP causing increased TLR7 and IFN-I production was associated with increased plasmablast numbers</li> <li>CD20-negative B cells (includes plasmablast population) produce aPL, which can then increase IFN-I expression as discussed above</li> </ul>	Human <i>in vivo</i> and <i>ex vivo</i> assays, PAPS and SAPS patients	[100]
<b>Unclear association</b>			
<b>microRNA regulation</b>	<ul style="list-style-type: none"> <li>Downregulated microRNA expression and IFN-I signature linked in APS</li> </ul>	Human <i>in vivo</i> and <i>ex vivo</i> assays, PAPS, SAPS and SLE patients	[60]

*aPL* means antiphospholipid antibodies; APS, antiphospholipid syndrome;  $\beta_2$ GPI,  $\beta_2$ glycoprotein I; BAFF, B cell activating factor; EPC, endothelial progenitor cell; IFN-I, type I interferon; NETs, neutrophil extracellular traps; LDGs, low-density granulocytes; pDCs, plasmacytoid dendritic cells; SLE, systemic lupus erythematosus; ROS, reactive oxygen species; SNP, single nucleotide polymorphism; TLRs, toll like receptors; PAPS, primary APS patients; SAPS, secondary APS patients.

**Figure 1.** Main sources and effects of type I interferon in antiphospholipid syndrome.



pDCs are recognized as the main source of IFN-I. Although not completely elucidated, it is known that a number of complex processes causes an increased production of IFN-I in APS setting, leading to endothelial dysfunction, accelerated atherosclerosis, and higher levels of BAFF which are responsible for an increased production of autoantibodies. APL, especially  $\alpha\beta_2$ GPI, are thought to induce IFN-I production through the hyperactivation of TLR. Moreover, IFN-I production seems to be modulated by microRNA, which global expression have been reported to be downregulated in pDCs of APS patients, and by NETs. It is still debated whether the increase in NETs formation and LGDs number are directly responsible for higher IFN-I

1 levels, as it has been reported in SLE pathogenesis. This figure also shows the main  
2 treatment agents with immunomodulant properties employed in APS, along with  
3 possible future therapeutic targets including those with direct anti-IFN-I effects.

4 *pDCs means plasmacytoid dendritic cells; IFN-I, type I interferon; BAFF, B-cell*  
5 *activating factor; aPL, antiphospholipid antibodies;  $\alpha\beta$ 2GPI, anti- $\beta$ 2-glycoprotein I*  
6 *antibodies; LDGs, low-density granulocytes; NETs, neutrophil extracellular traps; TLR,*  
7 *toll like receptor; HCQ, hydroxychloroquine; APS, antiphospholipid syndrome.*

8 Created using BioRender.com.