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Independent association of low IFNλ1 gene expression and type I IFN score/IFNλ1 ratio with obstetric manifestations and triple antiphospholipid antibody positivity in primary antiphospholipid syndrome

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ABSTRACT

Recent data suggest an important role of type I interferons (IFN) in antiphospholipid syndrome (APS). Here we aimed to evaluate the interplay of type I and type III (or IFNλs) IFNs in APS and potential clinical and serological associations. Our findings suggest that patients with primary APS (PAPS) and systemic lupus erythematosus (SLE)/APS displayed increased type I IFN scores but decreased IFNλ1 gene expression levels compared to healthy individuals, as assessed with real-time qPCR analysis in isolated peripheral blood mononuclear cells (PBMCs). Type I IFN score/IFNλ1 ratio was remarkably higher in patients with PAPS and SLE/APS as well as in SLE patients with or without antiphospholipid antibodies (aPL) *vs* controls. In conclusion, our results reveal an association between low IFNλ1 expression and obstetric APS. Moreover, the type I IFN score/IFNλ1 ratio seems to be a potential marker of high risk APS given its associations with triple aPL positivity.

1. Introduction

Antiphospholipid syndrome (APS) is characterized by recurrent arterial or venous thrombotic events or pregnancy morbidity in association with persistent antiphospholipid antibodies (aPL) [1]. Though APS pathogenesis is not entirely elucidated, orchestrated immune responses against β2-glycoprotein I (β2GPI) seem to be crucial in endothelial activation and generation of thrombogenic events [2]. A growing body of emerging data imply activation of type I IFN system in APS, in line with previous observations in other systemic and organ specific autoimmune diseases [3,4].

In more detail, over the last few years an increasing number of reports revealed upregulation of several type I interferon (IFN) inducible genes –the so called type I IFN signature- in peripheral blood mononuclear cells (PBMCs) [5–9] of APS patients [5–9]. Impaired endothelial progenitor repair, a mechanism previously viewed as proatherogenic [6], anti-β2GPI antibody positivity [6,9], earlier disease onset and preeclampsia [8], have all been related to type I IFN signature in the setting of APS. In the presence of systemic lupus erythematosus (SLE), type I IFN signature was found to be associated with aPL positivity in patients of African American ancestry [10]. Treatment with hydroxychloroquine [5,9] or statins [5] was shown to dampen type I IFN related transcripts, implying a potentially immunomodulatory role for these medications in the context of APS.

Beyond type I IFNs, emerging data support a potential role of type III IFNs (Lambda IFNs or IFNλs) in the pathogenesis of autoimmune diseases including SLE and Sjogren's syndrome [11–13]. IFNλs are the newest members of the IFN family of antiviral cytokines; though earlier studies implied shared biological properties to type I IFNs, it is increasingly appreciated that type III IFNs display also distinct anti-inflammatory functions [14,15]. Of interest, in recent studies IFNλs have been shown to prevent neutrophil pro-inflammatory activation *in vivo*

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[14], and inhibit neutrophil extracellular traps (NETs) formation *in vitro* [16], a mechanism previously shown to get involved in the pathogenesis of both arterial and venous thrombotic events in the general population as well as in the context of APS [17–19]. No data are available about IFNλ gene expression and its association with type I IFN signature in APS.

The aim of the current study was to quantitate the IFNλ gene expression levels and type I IFN/IFNλ ratio in patients with APS in comparison with those with SLE with or without aPL and healthy controls, and explore any potential associations with APS-related clinical and serological phenotypes.

2. Patients and methods

2.1. Study population

Consecutive adult patients with primary APS (PAPS, n = 48), age-matched patients with SLE/APS (n = 26), SLE patients with positive aPL (SLE/apL+, n = 18), SLE patients with negative aPL (SLE, n = 24), and 26 healthy individuals of similar age and sex distribution were included in the study. Patients with APS (either PAPS or SLE/APS) fulfilled the updated Sapporo classification criteria [1] and patients with SLE met the updated ACR classification criteria for SLE [20]. All patients were regularly followed-up at the Rheumatology Unit of the First Department of Propaedeutic Internal Medicine, Laikon Hospital, National and Kapodistrian University of Athens, Greece. Sera, plasma and whole blood samples were collected. Clinical and laboratory characteristics were recorded in all patients as previously described [9]. APS clinical (thrombotic and obstetric) and laboratory characteristics were defined according to the updated Sapporo classification criteria for APS [1]. All immunological tests were performed at Laikon Hospital. Anticardiolipin and antiβ2GPI antibodies (both of IgG and IgM isotype) were measured using a standardized ELISA assay, while lupus Anticoagulant was tested according to the Scientific Standardisation Subcommittee on Lupus Anticoagulant/Phospholipid Antibodies recommendations. The study protocol was approved by the local IRB ("Laikon Hospital Scientific Council") and all participants provided written informed consent.

2.2. PBMC isolation-Quantitation of type I IFN score

Isolation of PBMCs from whole blood samples was performed by Lymphoprep (Stem Cell Technologies) according to the manufacturer's instructions and total RNA was extracted from PBMCs using Trizol reagent (Ambion, Life Sciences, USA). Total RNA was transcribed into cDNA by Superscript III (Thermoscientific, USA) and mRNA expression of three type I IFN inducible genes including myxovirus (influenza virus) resistance 1 (MX-1), interferon- induced protein with tetratricopeptide repeats 1 (IFIT-1) and interferon- induced protein 44 (IFI44) was quantitated by real time qPCR; subsequently type I IFN score was calculated as previously described [21]. Briefly, the mean and SD level of each IFN inducible gene in the healthy control group were used to standardize expression levels of each gene for each study sample. Type I IFN gene expression score was calculated as the sum of each participant's relative expression for each of three type I IFN-inducible genes tested. These experiments were performed in the Department of Physiology, School of Medicine, National and Kapodistrian University of Athens. Type I IFN score was considered high if exceeded the mean + 2SD value of the control group.

2.3. Quantification of IFNλ transcripts in PBMCs

Quantification of IFNλ transcripts (IFNλ1-IL29, IFNλ2-IL-28A, IFNλ3-IL-28B) in PBMCs was performed by real time qPCR. Total RNA from PBMCs was transcribed into cDNA using M-MLV Reverse Transcrptase (PROMEGA, USA). The primer sequences for IFNλ1-IL29 were Forward: 5′-GGACGCCTTGGAAGAGTCACT-3′ Reverse: 5′-AGAAGCCTCAGGTCCCAATTC-3′ and for hIL28AB Forward: 5′-CTGCCACATAGCCCAGTTCA-3′ Reverse: 5′-AGAAGCGACTCTTCTAAGGCATCTT-3′. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as the internal control and normalization gene used for the calculations. Each sample's threshold cycle values for the target and housekeeping genes were subtracted from the corresponding values of the reference sample. Finally, the target gene values were divided by the housekeeping gene values for each sample, and the result was the relative expression value ($2^{-\Delta\Delta Ct}$) for each unknown sample. These experiments were performed in the Laboratory of Immunobiology, Center for Clinical, Experimental Surgery and Translational Research, Biomedical Research Foundation of the Academy of Athens (BRFAA). The median value of the distribution was implemented as a cut-off value with high and low IFNλ1 gene expression levels defined accordingly. Moreover, the type I IFN score/IFNλ1 ratio was constructed and the mean + 2SD value of the control group was used as a cut-off point.

2.4. Statistics

Statistical analysis was performed with SPSS v.22 software. Continuous data were assessed using Mann-Whitney *U* test. Categorical data were assessed using Fisher's exact test. Results were considered significant when p-value < .05. Bonferroni correction was applied to test for multiple comparisons. Multivariate models were also constructed in order to assess the presence of independent associations between low IFNλ1 levels and high type I IFN/IFNλ1 ratio. Adjusted was performed for variables shown to be significant in the univariate analysis.

3. Results

3.1. Type I IFN scores and IFNλ1 transcript levels in PBMCs from patients with APS and SLE

As shown in Fig. 1, patients with APS (PAPS and SLE/APS) demonstrated increased type I IFN scores but decreased IFNλ1 mRNA levels on the PBMC compartment compared to healthy individuals (Fig. 1A & B). The latter was not observed in SLE patients compared to controls, though type I IFN scores were upregulated in this group, in line with previous observations [22]. As a result, type I IFN score/IFNλ1 ratio was significantly higher in patients with APS (PAPS and SLE/APS), as well as in SLE with or without the presence of aPL (Fig. 1C). Levels of IFNλ2 and IFNλ3 were almost undetectable and therefore they were not further analyzed. When Bonferroni correction was applied, the p-value remained significant for type I IFN between SLE/apl + and HC groups, for the type I IFN/IFNλ ratio between SLE/APS and HC groups, while the statistical significance for IFNλ was lost.

3.2. Clinical and serological associations in patients with PAPS

When patients with PAPS were classified according to median of the distribution (0.000008221095483) IFNλ1 gene expression levels in PBMCs into high and low IFNλ1 groups, triple aPL positivity and obstetric APS manifestations were found to occur more frequently in the low IFNλ1 gene expression group (Table 1). The association with obstetric APS remained significant after adjustment for age, sex and triple aPL positivity (OR 95% [CI]:7.7 [1.2–49.1]).

To further explore whether type I IFN score/IFNλ1 ratio could serve as a marker of clinical or laboratory APS related features, patients with PAPS were divided into high and low type I IFNscore/IFNλ1 ratio groups, as described in the *Methods* section. As depicted in Table 2, patients with PAPS in the high ratio group experienced significantly higher rates of antiβ2GPI antibodies and triple aPL positivity compared to those in the low ratio group. Although borderline significant, an increased frequency of obstetric APS complications and lupus

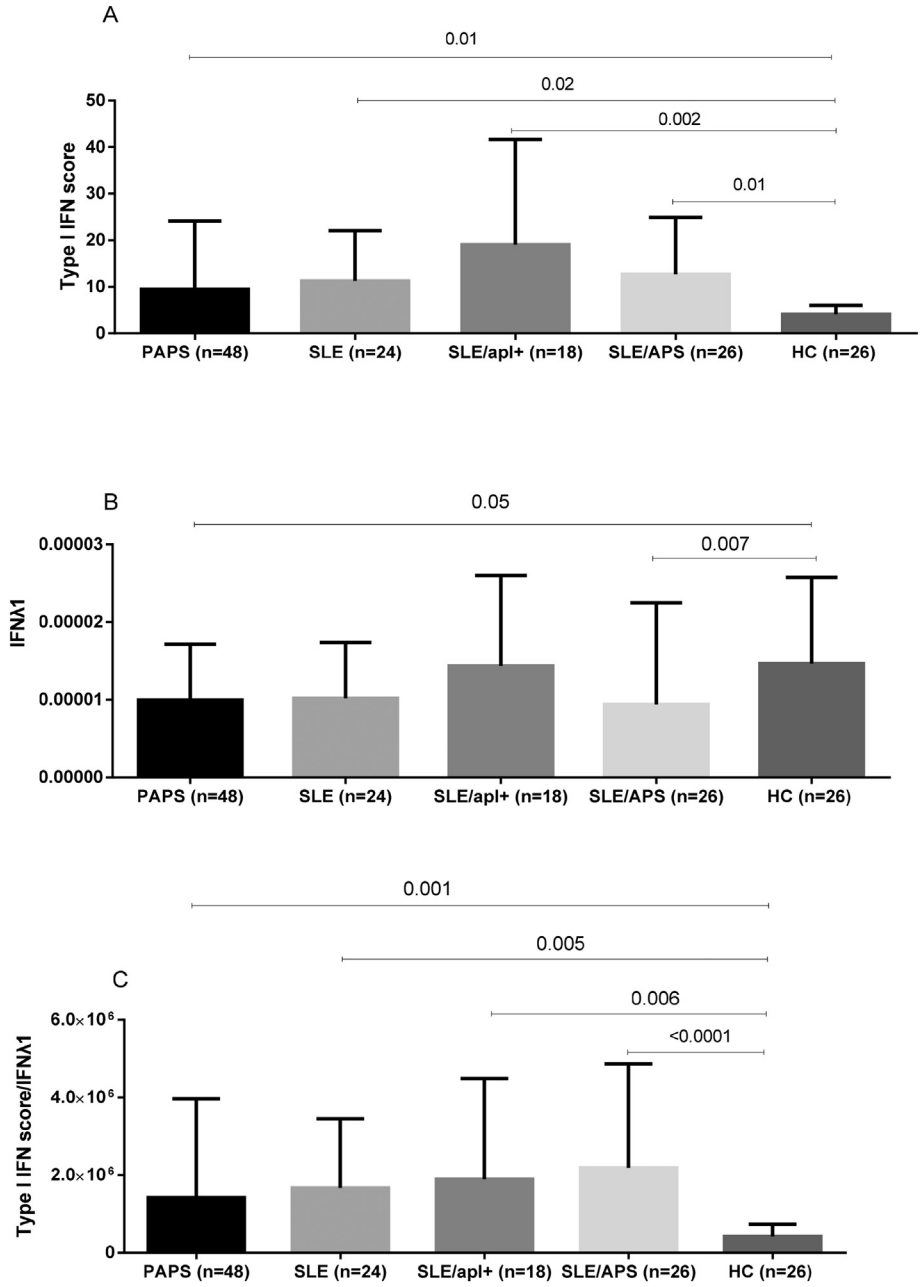

Fig. 1. Type I IFN score, IFNλ and IFN score/IFNλ1 ratio in patients with antiphospholipid syndrome (APS). A, B. Increased type I IFN scores but decreased IFNλ1 mRNA levels in PBMCs derived from APS patients compared to HC, C. Significantly higher type I IFN score/IFNλ1 ratio in both APS and SLE patients compared to HC. PAPS: Primary antiphospholipid syndrome, SLE: Systemic lupus erythematosus, apl+: presence of antiphospholipid antibodies, HC: healthy controls.

anticoagulant was also observed in the high *vs* the low type I IFN score/IFNλ1 group. Following adjustment for age, sex and anti-β2GPI positivity, triple positivity was found to be independently associated with type I IFN score/IFNλ1 ratio (OR 95% [CI]: 6.8 [1.9–24.7]).

3.3. Clinical and serological associations in SLE

In the setting of SLE, either in the presence or absence of aPL, low IFNλ1 transcript levels were found to be associated with advanced age and statin use (Suppl Table 1). No statistically significant differences were detected in SLE patients according to the type I IFN score/IFNλ1 ratio (Suppl Table 2). Moreover, no statistically significant associations between type I IFN score and autoantibody/complement levels (Suppl Table 3) or lupus nephritis (Suppl Fig. 1) were observed. Though not statistically significant, a borderline association between type I IFN score and SLEDAI was detected (Suppl Table 3).

4. Discussion

This is the first study to our knowledge evaluating the IFNλ1 gene expression and type I IFN score/IFNλ1 ratio in patients with APS (PAPS or SLE/APS). We found an increased type I IFN score but decreased IFNλ1 gene expression in PBMCs compared to healthy individuals. As a result, the type I IFN score/IFNλ1 ratio is remarkably higher in patients with APS (PAPS or SLE/APS), as well as in patients with SLE with or without aPL. Moreover, an independent association of low IFNλ1 gene expression and APS-related obstetric manifestations, as well as of high type I IFN score/IFNλ1 ratio with triple aPL positivity in the setting of PAPS, was demonstrated.

The association of type I IFN with autoantibodies in several systemic

Table 1
Clinical and serological characteristics according to IFNλ1 gene expression levels in patients with primary antiphospholipid syndrome.

	High IFNλ1 gene expression levels (n = 24)	Low IFNλ1 gene expression levels (n = 24)	p-value	OR 95% (CI)	OR 95% (CI[a])
Age (years, mean ± SD)	43.3 ± 14.7	44.5 ± 11.5	NS		
Females (%)	58.3	66.7	NS		
Arterial thrombotic events (%)	62.5	62.5	NS		
Venous thrombosis (%)	45.8	50	NS		
Non-criteria APS manifestations (%)	39.1	29.2	NS		
Recurrent thrombotic events (%)	33.3	50	NS		
Obstetric APS manifestations (%)	13.3	58.8	0.008	9.3(1.6 – 54.8)	7.7 (1.2 – 49.1)*
Anticardiolipin positivity (%)	75	70.8	NS		
Anti-β2GPI positivity (%)	37.5	58.3	NS		
Lupus anticoagulant positivity (%)	70.8	91.7	NS		
Triple aPL positivity (%)	25	58.3	0.019	4.2 (1.2 – 14.4)	
Hydroxychloroquine use (%)	54.2	45.8	NS		
Aspirin use (%)	54.2	50	NS		
Statin use (%)	25	8.3	NS		

[a] Associations with low IFNλ1 gene expression group after adjustment for age, sex, triple aPL positivity.

and organ specific autoimmune disorders including SLE, SS, dermatomyositis, systemic sclerosis, and autoimmune thyroiditis has been already well established [22–26] and seems to be attributed to type I IFN related B-cell activation either through excessive B cell signaling, B-cell activation factor (BAFF) induction by dendritic cells or IgG switching [27–29].

In the setting of APS, production of potentially pathogenic antibodies [2], as a result of pronounced B cell activity, is a central event given the previously demonstrated link between titers/type of autoantibodies and thrombotic risk [30]. In a recent report, increased plasmablasts as a source of aPL have been shown to occur in association with a TLR7 genetic variant and heightened type I IFN inducible genes [31]. Moreover, increased BAFF levels -previously shown to correlate with both lupus disease activity [32] and lupus related subclinical atherosclerosis [33]- have been also recently documented in patients with PAPS [34] in association with the adjusted global APS score, a validated tool for thrombotic risk assessment. Taken together, these data support a role for type I IFN in inducing B cell hyperactivity in the setting of APS, as revealed by the previously demonstrated associations between type I IFN score and anti-β2GPI antibodies [6,9,35]. Of interest, the ratio of type I IFN/IFNλ1 seems to be an even more informative biomarker for autoantibody production in the context of PAPS, since it was shown to be independently associated with triple aPL positivity rather than a single aPL type.

The independent association between low IFNλ1 transcript levels in PBMCs derived from PAPS patients and obstetric complications found in the present study is intriguing. IFNλ1 expression has been previously shown to be present in fetal membranes, representing a potential mechanism for combating infectious invaders [36–38]. APS-related obstetric complications include among others recurrent pregnancy loss, premature delivery and intrauterine growth restriction [39]. In a previously described animal model of APS induced fetal loss, tissue factor expression in neutrophils was shown to mediate respiratory burst, trophoblast injury and ensuing fetal loss [40]. Given the recently demonstrated diminishing role of IFNλ1 in neutrophil activation and NET release, and the amount of cytoplasmic tissue factor in neutrophils [14], it is possible that attenuation of IFNλ1 related anti-inflammatory activity mediates fetal injury.

However, our study has some limitations. Our results about type I IFN and IFNλ1 were drawn from the corresponding expression levels in mRNA isolated from PBMCs. We understand that PBMCs represent a heterogeneous population and the percentage of each cell subset in it cannot be directly determined. The contribution of the various cell subsets on IFNs may differ, so further studies with isolated cell subpopulations are needed to clarify our observations. Moreover, the size of our patient and control populations are relatively small, leading to some results with marginally significant differences.

Taken together, these findings reveal an association between low IFNλ1 expression levels and obstetric complications in the context of APS, possibly as a result of impaired neutrophil control. Moreover, the type I IFN score/IFNλ1 ratio seems to be a potential marker of high risk APS given its associations with triple aPL positivity [41]. However, larger studies are needed to confirm these findings.

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Table 2
Clinical and serological associations of type I IFN score/IFNλ1 ratio in patients with.

	High type I IFN score/IFNλ1 ratio (n = 19)	Low type I IFN score/IFNλ1 ratio (n = 29)	p-value	OR 95% (CI)
Age (years, mean ± SD)	41.4 ± 12.6	45.6 ± 13.4	NS	
Females (%)	73.7	55.2	NS	
Arterial thrombotic events (%)	52.6	69	NS	
Venous thrombosis (%)	52.6	44.8	NS	
Non-criteria APS manifestations (%)	26.3	39.3	NS	
Recurrent thrombotic events (%)	47.4	37.9	NS	
Obstetric APS manifestations (%)	53.3	23.5	NS	
Anticardiolipin positivity (%)	84.2	65.5	NS	
Anti-β2GPI positivity (%)	73.7	31	0.004	6.2(1.7 – 22.6)
Lupus anticoagulant positivity (%)	94.7	72.4	NS	
Triple aPL positivity (%)	68.4	24.1	0.002	6.8 (1.9 – 24.7)[a]
Hydroxychloroquine use (%)	52.6	48.3	NS	
Aspirin use (%)	47.4	55.2	NS	
Statin use (%)	5.3	24.1	NS	

[a] Associations with high type I IFN score/IFNλ1 ratio after adjustment for age, sex, anti-β2GPI positivity.

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Declaration of Competing Interest

The authors have nothing to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2019.108265.

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