Department of Clinical and Biological Sciences

PhD School "Health and Life Sciences"

PhD Programme "Complex Systems for Life Sciences"

PhD Thesis

Integrated Analysis of Serological Biomarkers for the Early Detection of Pregnancy Morbidity in Connective Tissue Diseases

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2022-2023

SUMMARY

GENERAL INTRODUCTION

Connective tissue diseases (CTDs) are a heterogeneous group of chronic systemic conditions mainly affecting young women of childbearing age [1]. Despite recent advances, pregnancy morbidity still represents a challenge due to the multi-factorial elements influencing aspects, including disease and organ damage, disease activity, ongoing therapies, age, previous pregnancy history, and additional risk factors, such as the presence of specific autoantibodies such as antiphospholipid antibodies (aPL).

With the aim of filling this gap, this three-year PhD project aimed at developing new tools for patients' profiling, characterization, and risk-based stratification in the context of CTDs and antiphospholipid syndrome (APS), leading to a better understanding of these complex diseases and to an early detection of pregnancy complications and consequently tailor management.

First, previous experiences from our group and others have demonstrated the importance of testing for a wide range of conventional and newly identified antinuclear antibody (ANA) specificities in women suffering from obstetric complications in order to improve patients' outcomes [2]. Rooting from these results, I focused on the potential differences existing when stratifying patients for the presence or absence of ANA in a large cohort of aPL positive subjects registered in the APS ACTION International Clinical Database and Repository (the largest available prospective cohort for this condition).

Afterwards, based on the central role of the complement cascade in pregnancy and since an abnormal complement activation has been associated with poor obstetric outcomes in systemic lupus erythematosus (SLE) patients [3], I focused on assessing the importance of complement fluctuation monitoring in a large cohort of lupus patients, derived from four prospective studies, through a network meta-analysis approach.

In parallel, in order to explore different approaches to profiling patients with CTDs and based on the central role of type I interferons (IFN) in the pathogenesis, disease activity, and evolution of several autoimmune conditions [4], we decided to design a cross-sectional study evaluating the differential expression of IFN regulated genes (IRGs) among different subsets of aPL positive subjects (aPL carriers, PAPS, secondary APS – SAPS) and SLE patients. We employed different analytic approaches, such as correspondence and network analyses, in order to be able to truly capture different gene expression programs across the entire APS spectrum.

By using novel biomarkers, innovative laboratory techniques, and alternative analytic approaches, we were able to identify different risk profiles for pregnancy morbidity in women with CTDs and APS.

The systematic implementation of our finding into clinical practice might help physicians identify women who benefit from tailored monitoring due to their immunological signature. The results of this research might help treating clinicians ameliorate the care of pregnant patients suffering from CTDs.

Clinical and Serological Characteristics of ANA-positive versus ANA-negative Antiphospholipid Antibody-positive Patients Without Other Systemic Autoimmune Diseases: Results from the APS ACTION Clinical Database and Repository

ABSTRACT

Background: APS ACTION is an international Clinical Database and Repository of persistently antiphospholipid antibody (aPL)-positive subjects, collecting demographic, medical history, and aPL data. This study focused on the prevalence of antinuclear antibodies (ANA) in aPL positive patients without a defined concomitant autoimmune disease. The objective of this study was to evaluate potential differences when stratifying patients by ANA, and to better phenotype aPL positive patients.

Patients and Methods: Data from aPL positive patients with or without APS classification criteria were retrieved from the APS ACTION Database. Patients with a diagnosis of systemic lupus erythematosus (SLE) or other connective tissue disease were excluded. Subjects who tested positive for anti-double stranded DNA and/or for anti-Smith antibodies were also excluded from the study, based on their high specificity for SLE diagnosis. Patients were divided in two groups (ANA+ and ANA-), based on ANA status at registry entry. Subsequently, demographic, clinical (including 1997 ACR SLE classification criteria), and serological data were compared between the two subgroups.

Results: A total of 430 individuals were included in the analysis [mean age 52.2 years (S.D. ±13), 71% females]. Among them, 240 (56%) patients were found positive for ANA testing, whereas 190 (44%) subjects were negative. ANA positivity was significantly associated with previous history of hematological manifestations as a whole, including hemolytic anemia, thrombocytopenia, and leukopenia (16.6% ANA+ vs. 7% ANA-, $p = 0.006$). A positive association was also observed for multiple aPL positivity in ANA+ subgroup ($p = 0.02$), along with low C3 and C4 levels ($p = 0.05$ and $p = 0.009$, respectively), and higher positivity for extractable nuclear antigens (ENA), such as anti-Ro ($p < 0.001$), anti-La ($p = 0.023$), and anti-RNP ($p = 0.014$) antibodies. The proportion of patients who experienced previous arterial events was comparable among the two subgroups, as well as the number of arterial thromboses, previous venous occlusions and number of venous events. Among female patients who have experienced at least one pregnancy, 113 were ANA+ and 96 were ANA-. When comparing the two subgroups, we have found a statistically significant difference in the number of pregnancies (mean 3.1 ±1.65 vs. 2.26 ±1.64 in the ANA- and ANA+ subgroups respectively, $p = 0.018$), and number of live births (mean 1.69 ±1.1 vs. 1.35 ±0.9, ANA- and ANA+ subgroups

respectively, p =0.014). Conversely, no difference has been noted when computing adverse obstetric outcomes between ANA+ and ANA- subjects. Finally, a wider proportion of ANA+ patients were reported to be treated with hydroxychloroquine (HCQ) (p <0.001). When evaluating ANA positivity in aPL carriers and PAPS individually, the association between ANA+ and previous hematologic manifestations remained significant for both groups, as well as low complement levels, multiple aPL positivity, ENA positivity, and HCQ use. Additionally, among aPL carriers, ANA+ patients presented with a higher rate of arthritic manifestations (p =0.006).

Conclusions: In this large international cohort, the presence of a positive ANA test was associated with a higher rate of hematologic manifestations, multiple aPL positivity, lower complement levels, ENA positivity, and articular involvement. Moreover, patients with ANA positivity were more often treated with hydroxychloroquine. Those aPL positive subjects with a negative ANA test showed a higher rate of pregnancies and live births, suggesting a possible link between these autoantibodies and decreased fertility.

INTRODUCTION

Antiphospholipid syndrome (APS) is defined by the persistent presence of at least one antiphospholipid antibody (aPL) positive test among anticardiolipin (aCL) IgG/IgM, anti-β2-glycoprotein-I (aβ2GPI) IgG/IgM, and lupus anticoagulant (LA), and at least one clinical manifestation such as thrombosis and pregnancy complications (at least one unexplained fetal death at and beyond 10 weeks of gestation and/or premature birth before 34 weeks of gestation due to eclampsia, severe pre-eclampsia or placental insufficiency, and/or at least three unexplained consecutive abortions before the 10^{th} week of gestation) [5].

While some patients experienced both vascular and pregnancy morbidity events, many of them presented with pure thrombotic or obstetric forms of the syndrome and emerging data are suggesting that the occurrence of fetal and maternal complications in the presence of aPL might constitute a distinct disease [6,7]. It is also known that APS can present either as an isolated pathologic entity, namely "primary APS" (PAPS), or in association with other rheumatic conditions, mainly systemic lupus erythematosus (SLE), to which it refers as "secondary APS" (SAPS) [5,8]. Although often associated, a significant proportion of aPL positive lupus patients do not develop the full APS phenotype during the follow up and, *vice versa*, PAPS patients do not necessarily evolve toward SLE during their life [9–13], even if frequently found positive for anti-nuclear (ANA) and anti-double stranded DNA (anti-dsDNA) [14]. Indeed, these two conditions, although closely linked, do not completely overlap in terms of pathogenic pathways and represent two distinct entities, whose association further complicate the clinical management [15]. Moreover, while it is known that subjects who tested persistently positive for aPL, not fulfilling the classification criteria for the syndrome and often referred as "aPL carriers", are at higher risk for the clinical manifestations of APS when compared to the general population, especially those with multiple aPL positivity, it remains a challenge for the treating physician to clearly identify those who will develop an adverse event [16–18]. Over the last years, researchers have also described the existence of additional aPL-related features, called "extra-criteria", such as cytopenia, *livedo reticularis*, and aPL-nephropathy, whose pathogenesis, epidemiology and clinical significance still need further elucidation [19].

The increased complexity of APS is mirrored by the recent attempts in understanding the disease from alternative perspectives, trying to overcome classification criteria [20–22], which prevent us from capturing the full APS spectrum, with the concrete risk of underdiagnosing. Among these, two recent studies from Zuly and Sciascia, in which a cluster analysis approach was employed, have highlighted the heterogeneity among aPL positive subjects [23,24]. In particular, Sciascia has demonstrated the existence of a subgroup of patients with intermediate characteristics between pure PAPS and SLE, with a higher rate of systemic features, including ANA positivity, which was found in up to 97% of the

patients, along with cytopenia, especially thrombocytopenia, and at lower risk for developing thrombotic events. Indeed, these findings might have important implications for the treatment and the overall management of patients.

Rooting from these results, we designed this study with the main aim of evaluating if any difference exists in a large cohort of aPL positive patients, without a concomitant diagnosis of defined connective tissue disorder (CTD), when stratifying for the presence of ANA.

METHODS

APS ACTION Registry

The REDCap (Research Electronic Data Capture) [25,26], a global web-based platform, is used to collect information on patient demographics along with clinical and serologic data on aPL positive patients. Inclusion criteria for registry entry are: a) age between 18 and 60 years; and b) persistent (at least 12 weeks apart) aPL-positivity within 12 months prior to screening. In detail, aPL positivity is defined as aCL IgG/IgM/IgA isotypes (> 40 GPL/MPL/APL, medium-to-high titer, and/or greater than the 99th percentile), aβ2GPI IgG/IgM/IgA isotypes (> 40 units, medium-to-high titer). LA test is considered positive *as per* the International Society on Thrombosis and Hemostasis [27]. Patients regular follow up is performed (every 12 ±3 months) with clinical data and blood collection. For the purpose of this study, only data entered at the time of inclusion in the registry by each individual Center were used.

Cohort

Patients with a diagnosis of SLE or other defined CTDs were excluded from the analysis. Likewise, those subjects who tested positive for anti-dsDNA and/or for anti-Smith (anti-Sm) antibodies at the time of the inclusion in the Registry were also excluded from the study, based on their high specificity for SLE diagnosis [28]. Therefore, only PAPS, both thrombotic and obstetric, and aPL carriers were included in the analysis and compared after separating the cohort based on ANA status at registry entry [5].

For the sake if this study, we considered the following as "extra-criteria" manifestations: aPL-related nephropathy, *livedo reticularis*, superficial vein thrombosis, heart valve disease, leukopenia, hemolytic anemia, thrombocytopenia, transient ischemic attack, skin ulcers, chorea, and cognitive impairment. We also retrieved data on pregnancy outcomes, additional clinical manifestations such as photosensitivity, arthritis and serositis, among others, as well as on additional serological features including extractable nuclear antigens (ENA) test results and complement fractions levels.

Analysis

Categorical variables are presented as number (%) and continuous variables are presented as mean (S.D.). The significance of baseline differences was determined by the chi-squared test, Fisher's exact test or the unpaired t-test, as appropriate. A two-sided P-value <0.05 was statistically significant. All statistical analyses were performed using SPSS version 28.0 (IBM, Armonk, NY, USA).

RESULTS

A total of 477 patients classified as aPL carriers and PAPS [5], were identified among all patients entered in the APS ACTION Database. Forty-seven subjects were excluded from the analysis based on the lack of ANA test results. A total of 430 individuals were therefore included in the final analysis [mean age 52.2 years (S.D. ±13), 71% females]. Among them, 79 were aPL carriers, and 351 were diagnosed with PAPS. A positive ANA test (ANA+) was reported in 240 (56%) patients, whereas 190 (44%) subjects were found negative for ANA (ANA-) testing. The complete demographic, clinical, and serological characteristics of the cohort at the time of registry entry are displayed in Table 1.

When focusing on the ANA+ subgroup, we observed that, as expected, the vast majority of the patients were female (174, 72.5%), and the mean age was 52.3 years (S.D. ±13). APS was diagnosed in 191 patients, among whom 139 (73%) suffered from previous thrombotic events, 23 (12%) manifested a pure obstetric phenotype (obstetric APS), and 29 patients (15%) experienced both thromboses and pregnancy complications.

When looking at the ANA- population [mean age at inclusion 52.2 years (S.D. ±13), 70% females], we observed that 30 (16%) subjects were aPL carriers and 160 (84%) were PAPS, including 113 thrombotic (70.6%), 22 obstetric (14%), and 25 patients who exerted both clinical phenotypes (15.6%).

In our cohort, aPL were distributed as follows: LA positivity was observed in 181 out of 240 (75.4%) ANA+ patients and in 129 out of 190 (68%) in the ANA- subgroup; aCL tested positive in 72% of ANA+ and 64% of the ANA- patients; aβ2GPI positivity was reported in 145 (60%) ANA+ subjects and in 101 (53%) ANA- patients. No statistically significant difference was observed when aPL distribution was computed separately for each autoantibody specificity. However, when considering the aPL profile, significant differences were observed among the two groups. In fact, ANA+ subjects presented a higher rate of triple aPL positivity when compared to ANA- subgroup (42% vs. 31% respectively, p =0.02). Conversely, single aPL positivity was found in a higher proportion of ANA- subjects (38% ANA- vs. 27% ANA+, p =0.017). A similar representation of double aPL positivity was found among the two groups (26% ANA+ vs. 26.3% ANA-).

	ANA-	ANA+	p-value
Total number of patients, n (%)	190 (44)	240 (56)	-
Demographic features			
Age, years, mean (±S.D.)	52.2 (±13)	52.3 (±13)	n.s.
Sex, females, n (%)	(70)	174 (72.5)	n.s.
Ethnicity, Caucasians, n (%)	123 (64.7)	162 (67.5)	n.s.
APS classification following Sidney criteria			
PAPS*, n (%)	160 (84)	191 (79.6)	n.s.
Thrombotic PAPS*, n (%)	113 (70.6)	139 (73)	n.s.
Obstetric PAPS*, n (%)	22 (14)	23 (12)	n.s.
Thrombotic and obstetric PAPS*, n (%)	25 (15.6)	29 (15)	n.s.
aPL carriers*, n (%)	30 (16)	49 (20)	n.s.
Serologic features			
aPL positive, n (%)	190 (100)	240 (100)	-
aCL positive (IgG/IgM), n (%)	122 (64)	173 (72)	n.s.
aβ2GPI positive (IgG/IgM), n (%)	101 (53)	145 (60)	n.s.
LA positive, n (%)	129 (68)	181 (75)	n.s.
Triple aPL positive, n (%)	60 (31)	103 (42)	**0.02**
Double aPL positive, n (%)	50 (26)	63 (26)	n.s.
Single aPL positive, n (%)	72 (38)	64 (27)	**0.017**
Low C3 levels, n (%)	3 (1.6)	16 (6.6)	**0.02**
Low C4 levels, n (%)	5 (2.6)	19 (8)	**0.03**
ENA anti-SSA/Ro positive, n (%)	1 (0.5)	17 (7)	**<0.001**
ENA anti-SSA/La positive, n (%)	0 (0)	5 (2)	**0.023**
ENA anti-RNP positive, n (%)	0 (0)	6 (2.5)	**0.014**
Traditional cardiovascular risk factors			
Arterial hypertension (on medication), n (%)	62 (32)	65 (27)	n.s.
Dyslipidemia (on medication), n (%)	53 (27.8)	53 (22)	n.s.
Diabetes mellitus, n (%)	7 (3.6)	7 (2.9)	n.s.
Smoking, n (%)	64 (33.6)	92 (38)	n.s.
Additional clinical features			
aPL nehropaty, n (%)	5 (2.5)	6 (2.6)	n.s.
Hematological manifestations, n (%)	14 (7)	40 (16.6)	**0.006**
Livedo reticularis/racemosa, n (%)	21 (11.5)	35 (14.5)	n.s.
Superficial vein thrombosis, n (%)	10 (5)	13 (5)	n.s.
Heart valve disease, n (%)	9 (4.7)	18 (7.5)	n.s.
Transient ischemic attack, n (%)	19 (10)	21 (8.7)	n.s.
Skin ulcers, n (%)	10 (5)	10 (4)	n.s.
Chorea, n (%)	2 (1)	3 (1)	n.s.
Cognitive impairment, n (%)	5 (2.6)	10 (4)	n.s.
Photosensitivity, n (%)	5 (2.6)	10 (4)	n.s.
Arthritis, n (%)	4 (2)	13 (5)	n.s.
Serositis, n (%)	1 (0.5)	2 (0.8)	n.s.

Table 1. Demographic, clinical, and laboratory characteristics of the cohort based on anti-nuclear antibodies status.

ANA means anti-nuclear antibodies; PAPS, primary antiphospholipid syndrome; aPL, antiphospholipid antibodies; aCL, anti-cardiolipin antibodies; aβ2GPI, anti-β2 glycoprotein I antibodies; ENA, extractable nuclear antigens.

**Sidney APS classification criteria (S Miyakis, et al. J Thromb Haemost, 2006)*

The proportion of patients who experienced at least one arterial thrombotic event was similar among the two subgroups as well as the number of arterial thrombotic events. Similarly, no difference has been observed when comparing ANA+ and ANA- patients for the occurrence of venous thromboses and for the overall number of venous events.

Among female patients who have experienced at least one pregnancy, 113 were ANA+ and 96 were ANA-. When comparing the two subgroups, we have found a statistically significant difference in the number of pregnancies (mean 3.1 ±1.65 vs. 2.26 ±1.64 in the ANA- and ANA+ subgroups respectively, $p = 0.018$), and number of live births (mean 1.69 ±1.1 vs. 1.35 ±0.9, ANA- and ANA+ subgroups respectively, $p = 0.014$). A similar proportion of patients among both subgroups have experienced at least one pregnancy morbidity event during their life (68% ANA+ vs. 74% ANA-), as well as a similar rate of unexplained fetal death beyond 10 weeks of gestation (32% ANA+ vs. 36% ANA-), premature births before 34 weeks of gestation (19% ANA+ vs. 17% ANA-), unexplained spontaneous abortions before the 10th week of gestation (35% ANA+ vs. 41% ANA-), and three consecutive unexplained spontaneous abortions before the 10th week of gestation (8% ANA+ vs. 11% ANA-).

Moreover, a significant proportion of patients (45.6%) experienced at least one extra-criteria manifestation among the ones listed in the Methods section. No statistically significant difference was observed in the overall rate of extra-criteria manifestations, when computed as a whole, between ANA+ and ANA- subgroups (48.3% vs. 42%, respectively). However, ANA+ patients presented a significantly higher rate of hematological manifestations, including leukopenia, hemolytic anemia and thrombocytopenia (16.6% ANA+ vs. 7% ANA-, $p = 0.006$). No other differences have been noted when comparing the two subgroups for additional extra-criteria manifestations.

Interestingly, additional differences have been found among the two groups, including: a wider proportion of ANA+ patients were reported to be treated with hydroxychloroquine (HCQ) ($p < 0.001$), presented higher percentage of hypocomplementemia, both for C3 and C4 fractions ($p = 0.05$ and $p = 0.009$, respectively), higher positivity for ENA, such as anti-Ro ($p < 0.001$), anti-La ($p = 0.023$), and anti-RNP ($p = 0.014$) antibodies.

When further separating between aPL carriers and PAPS, the association with hematologic manifestations and ANA positivity remains significant for PAPS patients (16.7% ANA+ vs. 7.5% ANA-, $p = 0.004$), as well as the lower levels of C3 and C4 fractions, for both PAPS and aPL carriers, and the use of hydroxychloroquine for ANA+ PAPS subjects ($p < 0.001$). Among aPL carriers, ANA+ patients presented with a higher rate of arthritic manifestations ($p = 0.006$). Moreover, when focusing on pregnancy outcomes, ANA- patients, both aPL carriers and PAPS, have a higher number of

pregnancies and live births, with stronger significance for the latter group (p =0.022 and p =0.016, respectively).

DISCUSSION

The APS ACTION Clinical Database collects the largest, well-characterized, international cohort of aPL positive patients, therefore representing a unique resource to better understand this disease, which, despite being considered rare in most of the countries due to its low prevalence, is in parallel described as the most common form of acquired thrombophilia [29]. Affected patients are often female at a young age, potentially experiencing life-threatening manifestations such as arterial thrombotic events, as well as venous occlusions and pregnancy complications, with severe negative impact on morbidity, mortality, and quality of life. Indeed, since its first description, APS has been often associated with SLE and the presence of ANA has been extensively described in the context of APS [30]. ANA antibodies are considered the hallmark of various systemic autoimmune conditions, but their precise clinical and prognostic value in APS setting needs further elucidation. Therefore, following numerous efforts to profile aPL positive individuals, we performed this study in order to characterize patients according to ANA status.

First, we highlight the fact that a significant proportion of aPL carriers and PAPS patients included in the analysis was found positive for ANA test (56%), although in the absence of a concomitant diagnosis of defined CTD or other autoimmune systemic condition. Moreover, in line with a recent retrospective study by Ricard and co-workers [31], our findings confirmed the association between ANA presence and a high risk aPL profile, especially triple positivity. Interestingly, in our cohort, this evidence was not associated with a higher rate of arterial events, as emerged in previous studies [32,33]. While it is well recognized, that triple aPL positive patients are more likely to experience thromboembolic relapses [34], the design of this study, which relies solely on data gathered at the time of registry entry, might have prevented us from observing the occurrence of first thrombotic event in aPL carriers as well as recurrences in PAPS patients during the follow-up period. Moreover, those patients who exhibit a positive ANA test seem to exert a tendency toward extra-criteria and systemic manifestations, particularly cytopenia, low complement circulating levels and ENA antibodies positivity, as already observed in other studies [30,32,35], which, aside from their diagnostic value, might be crucial for prognostic and therapeutic purposes [36]. Of note, the occurrence of additional clinical features, such as articular involvement, became relevant in our cohort only when focusing on individuals without the overt form of the syndrome. Over these years, whether aPL positive patients with and without additional clinical and serological features beyond thrombosis and pregnancy complications, such as arthritis, *livedo reticularis* and cytopenia, might develop SLE during their life, has

been already investigated by several authors, with conflicting results [37–41]. Indeed, the presence of intermediate clinical characteristics between CTDs and APS, which cannot be explained only by the existence of a pro-coagulant state, might justify the significantly higher proportion of ANA+ subjects, both aPL carriers and PAPS, treated with HCQ, that has been observed in our cohort. HCQ, which has been proven to be effective in preventing thrombotic recurrences in PAPS patients [42], is extensively used in lupus setting both for prophylaxis and treatment strategies, due to its immunomodulatory properties, its optimal safety profile and availability [43]. Data have also suggested that HCQ might be effective in delaying the progression toward SLE in ANA positive subjects through the inhibition of early mediators such as B cells activating factor and interferon pathway [44].

During the last decades, the role of the immune system has dramatically grown and it is no longer confined to host defense against infections. The complex interplay between immunity and the reproductive system has become a central topic and an important field of medical research. A recent systematic review and metanalysis by Ticconi, Inversetti and colleagues [45], performed to evaluate the significance of ANA in female fertility, subfertility, and pregnancy complications, has reported that the available literature does not support the role of ANA in late maternal and fetal complications, such as preterm births, stillbirths and preeclampsia/hypertensive disorders, mainly due to the lack of large, well-designed, comparable studies, and for the heterogeneity in ANA positivity cut-off values. In addition, the absence of data on the relationship between ANA positivity and infertility, does not allowed the authors to draw any conclusion. However, solid evidence seems to exist regarding the unfavorable effect of ANA presence on in-vitro fertilization (IVF) procedures, in terms of implantation failure, miscarriage after implantation, and overall low probability of IVF success [45]. When focusing on recurrent early pregnancy loss (RPL), the authors have found a higher risk of RPL in ANA+ compared to ANA- patients, when considering both definitions of RPL (> two and > three pregnancy losses) [45]. In our cohort, ANA- female patients presented a higher number of pregnancies and live births when compared to the ANA+ subgroup. Interestingly, no statistical difference has been observed when focusing on gestational complications, such as stillbirths, premature births, pregnancy losses before the 10^{th} week of gestation, and RPL. Although limited, these data suggest an association between the presence of ANA and infertility in aPL positive patients, irrespectively of being or not diagnosed with APS. Moreover, the larger use of HCQ in ANA+ group that has been observed in our cohort might have had a favorable influence on pregnancy outcomes in these patients, as already reported by numerous authors [46–48], consistently decreasing the risk of experiencing adverse events during gestation.

This study suffers from some limitations, such as the low number of aPL carriers included in the analysis which have hampered potential observations in this specific subgroup. Moreover, ANA testing

and consequently ANA status (positive or negative) have been performed locally and reported by each center at the time of the enrollment in the APS ACTION Database, thus implying a certain heterogeneity in laboratory techniques and cut-off values. In addition, since the dense fine speckled 70kDa molecular weight (DFS-70) antibody represents a type of ANA often found in healthy individuals and in those patients, who will not develop a systemic autoimmune disorder during their life [49], the assessment of anti-DFS-70 antibody status in our cohort, would have increase the specificity of our analysis. Finally, the lack of follow-up data has limited our ability to observe the clinical course of our patients, and therefore to asses the prognostic value of ANA.

On the other hand, this study has been developed using real word data collected and entered by international centers with great experience in the field of CTDs and APS. In addition, the exclusion of patients who were found positive for anti-dsDNA and anti-Sm antibodies, which are highly specific for SLE diagnosis, increase the reliability of our findings by limiting the analysis to aPL carriers and PAPS patients without a concomitant diagnosis of defined CTD.

CONCLUSIONS

In conclusion, ANA antibodies are a common hallmark of autoimmunity and our study showed that the presence of ANA in aPL positive subjects, irrespectively from being diagnosed or not with APS, is associated with a higher rate of systemic features, such as hematological manifestations, as well as low C3 and C4 circulating levels, ENA positivity, and arthritis. Moreover, ANA+ patients presented a higher proportion of multiple aPL positivity, therefore increasing the risk of thrombotic relapses during the follow-up. The study also highlighted that among female aPL positive patients who experienced at least one pregnancy, the absence of ANA is associated to a higher number of both pregnancies and live births, perhaps suggesting a link between these antibodies and decreased fertility.

Indeed, further studies are needed in order to assess the precise prospective role of ANA positivity in aPL positive subjects, with important implications for patients' monitoring and treatment.

Complement Levels During the First Trimester of Gestation Predict Disease Flare and Adverse Pregnancy Outcomes in Systemic Lupus Erythematosus: A Network Meta-Analysis on 532 Patients

ABSTRACT

Background: Complement levels have been proposed as candidate biomarkers of disease activity and obstetric risk in pregnancies, but their reliability has been questioned due to the physiologic fluctuations of complement during gestation. Thus, this study aimed at assessing the clinical significance of complement fluctuations in lupus pregnant women.

Methods: Corresponding authors of 19 studies meeting inclusion criteria were invited to contribute with additional data including C3 and C4 levels [before pregnancy, at conception, in every trimester (T) and 3 months after delivery]; data were pooled together in a network meta-analysis.

Results: A total of 532 women with SLEs from four studies were included in the analysis. In these women, C3 and C4 increased progressively during gestation: levels remained stable during T1 and peaked in T2 to decrease in T3. Patients with previous lupus nephritis (LN) and those who experienced flares during pregnancy had significantly lower mean levels of C3 and C4 at all timepoints. The lowest levels of complement were observed, particularly during T1, in patients with LN and gestational flare. Both reduction and the lack of increase of C3 and C4 levels at T1 versus conception were associated with gestational flares, particularly in LN patients. Pregnancies with flare had a statistically significant higher rate of maternal and fetal complications.

Conclusions: Low complement levels, particularly in T1, were associated with a higher frequency of gestational flare. Either reduction or smaller increase of C3 and/or C4 levels, even within normal range, might predict flares especially in early gestation.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototypical immune complex-mediated disease, characterized by a wide spectrum of phenotypes with heterogeneous courses and progression, varying from persistently low, relapsing-remitting, to persistently high disease activity [50,51]. The epidemiology of SLE, which mainly presents in young women of childbearing age [1,52], accounts for the fact that clinicians assist lupus patients very often in their journey towards motherhood. To explain such epidemiological female predominance, several hypotheses have been formulated: candidate risk genes for SLE map on the X chromosome, and estrogens favour autoimmunity by promoting B-cell maturation, antibody production, Th2 responses, and survival of autoreactive cells [53,54]. As expected, pregnancy can impact SLE disease activity, and in turn SLE may affect obstetric outcomes. Pregnancy in women with SLE has always been regarded as at high risk; however, thesignificant advancements made in the overall disease management have led to a net improvement of both maternal and fetal outcomes [55,56]. Nevertheless, pregnancy still represents a challenge in women with SLE, especially in those with renal involvement, due to the risk of disease flare, gestational diabetes and placenta-related disorders including pre-eclampsia (PE), as well as fetal complications such as miscarriages, fetal loss, intrauterine growth restriction (IUGR), prematurity, and neonatal lupus [57,58]. Reliable biomarkers to stratify the risk of a disease flare during pregnancy and to early detect adverse pregnancy outcomes (APO) in pregnant lupus women are still lacking. Complement levels have been proposed as candidate biomarkers of disease activity and of obstetric risk in lupus pregnancies, but their reliability has been questioned due to the physiologic fluctuation of complement levels during gestation [3,59]. In order to optimize the interpretation of available data on the fluctuation of complement levels during SLE pregnancy, we performed a network meta-analysis to assess the fluctuations of C3 and C4 levels from preconception period, throughout pregnancy, and up to 3 months after delivery and to evaluate the association of complement levels with the occurrence of disease flares and/or APO.

METHODS

Systematic literature review

A detailed literature search strategy has been developed a priori to identify articles that reported findings from available prospective studies investigating pregnancies in patients with SLE from January 2002 to December 2020. Key words and subject terms included:(("longitudinal studies"[MeSH Terms] OR ("longitudinal"[All Fields] AND "studies"[All Fields]) OR "longitudinal studies"[All Fields] OR "prospective"[All Fields] OR "prospectively"[All Fields]) AND ("lupus vulgaris"[MeSH Terms] OR ("lupus"[All Fields] AND "vulgaris"[All Fields]) OR "lupus vulgaris"[All Fields] OR "lupus"[All Fields]

OR "lupus erythematosus, systemic"[MeSH Terms] OR ("lupus"[All Fields] AND "erythematosus"[All Fields] AND "systemic"[All Fields]) OR "systemic lupus erythematosus"[All Fields]) AND ("pregnancy"[MeSH Terms] OR "pregnancy"[All Fields] OR "pregnancies"[All Fields] OR "pregnancy s"[All Fields])) AND (1000/1/1:2021/6/15[pdat]).

The search strategy was applied to Ovid MEDLINE, In-Process and Other Non-Indexed Citation from January 2002 to December 2020. Figure 1 resumes the search strategy. Retrieved papers were further screened upon additional inclusion criteria in order to refine the search strategy. Inclusion criteria included: a) prospective design, b) a sample size of at least 50 lupus patients, c) exclusion ofmiscarriages before 12 weeks of gestation as obstetric outcome.

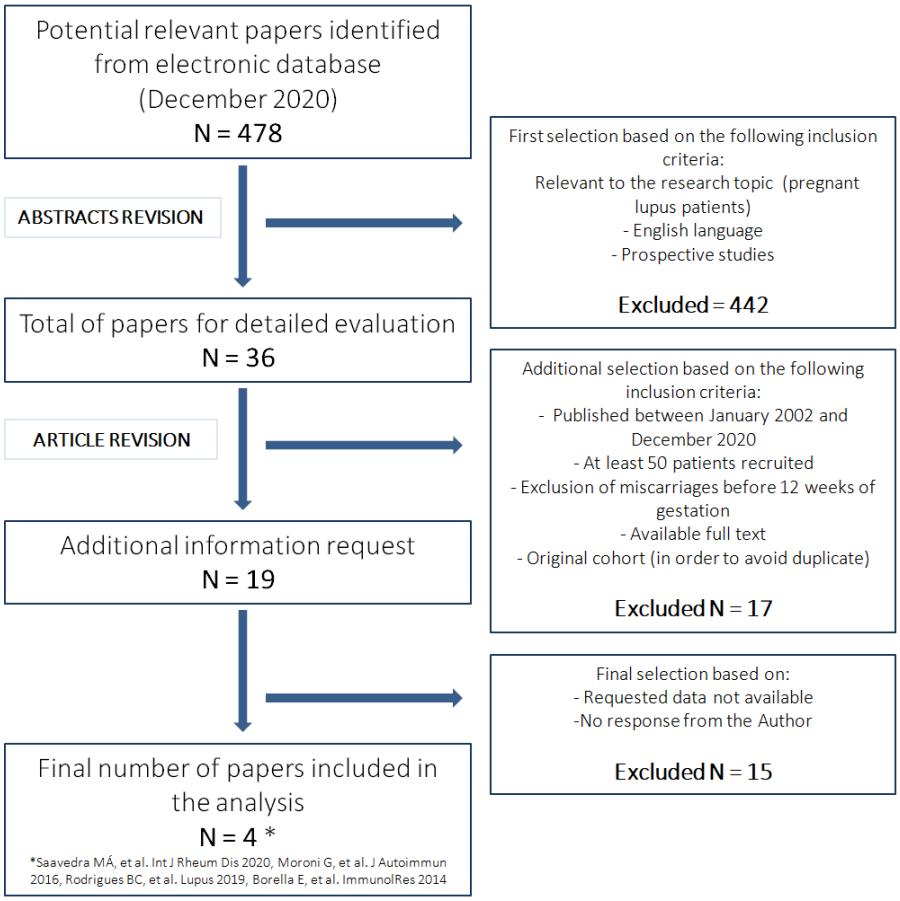


Figure 1. Literature search strategy

Data collection

Two review Authors (M.R. and I.C.) independently assessed studies for inclusion. One review author completed data extraction, which was checked by a second review author. A total of 19 studies were finally selected for data request. Each corresponding author of the selected manuscripts was invited to contribute with additional data that were not presented in the published manuscript, including complement levels, C3 and C4 separately, at 6 months before pregnancy, at conception, during the first trimester (T1), during thesecond trimester (T2), during the third trimester (T3), and 3 months after delivery (post-partum, PP). Further details on the number of pregnancies, patients' classification, diagnosis at conception, treatment during pregnancy, occurrence of flares during gestation, as well as maternal and fetal outcomes were also recorded. We performed a network meta-analysis within a Bayesian framework as previously descrive [60].

Statistical analysis

Categorical variables are presented as numbers (%) and continuous variables are expressed as mean ± standard deviation (SD). The significance of baseline differences was determined by the chi-squared test, Fisher's exact test or the unpaired t-test, as appropriate. Correlation analysis, linear regression, and Odds Ratio (OR) were also performed. Missing data were approached with mean substitution system. A two-sided p-value <0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 26.0 (IBM, Armonk, NY, USA).

Study variables definitions

SLE, lupus nephritis (LN), and antiphospholipid syndrome (APS) diagnosis and classification were based upon each study definition [5,61,62]. SLE flare was defined by the need of new immunosoppressive therapy or increase in the dosage of prednisone ≥10 mg/day.

APO were defined as follows:

a) fetal death after 12 weeks' gestation in the absence of chromosomal abnormalities, anatomic malformations, or congenital infections;

b) neonatal death before hospital discharge due to complications related to prematurity or placental insufficiency (e.g., abnormal fetal surveillance test results, abnormal Doppler flow velocimetry waveform analysis suggestive of fetal hypoxemia, or oligohydramnios, or both);

c) pretermdelivery or pregnancy loss at less than 36 weeks due to gestational hypertension, PE, or placental insufficiency;

d) small-for gestational-age neonate, defined as one with a birthweight below the 5th percentile without anatomical or chromosomal abnormalities.

The fluctuation of C3 and C4 levels between T1 and conception was defined as ΔC3T1-conception and ΔC4T1-conception. When the decrease in C3 levels between T1 and conception was below 2 mg/dl or the increase in C3 at T1 versus conception was below 4 mg/dl (defined using two standard deviations from mean, as per Westgard rules), ΔC3T1–conception was considered as clinically not relevant.

RESULTS

C3 and C4 levels progressively increased during gestation in women with SLE

A total of 532 SLE women from 4 studies were included in the analysis [63–66]. APS had been diagnosed in 68 women (12.8%), while 82 patients (15.4%) were positive for antiphospholipid antibodies (aPL) without overt clinical manifestations of APS (referred as "aPL carriers"). As detailed in Table 1 and visually presented in Figure 2A, both C3 and C4 levels increased progressively in women with SLE during gestation. In particular, C3 and C4 levels remained stable duringT1 and peaked at T2, then decreased during T3. At 3 months after delivery, a different behavior was noted for C3 and C4: C3 continued to decrease whereas C4 levels in the PP period were higher than those registered in T3.

Patients with flares during pregnancy displayed significantly lower levels of complement compared to patients without gestational flare

A flare during pregnancy was observed in 170 patients (32%). Levels of both C3 and C4 were lower at all timepoints in subjects who experienced flares during pregnancy (C3 at T1 78.3±22.8 versus 100.5±20.7, $p<0.001$; C3 at T2 94.2±13.4 versus 115.7±12.3, $p<0.001$; C3 at T3 99±18.6 versus 111.4±16, $p<0.001$; C3 at PP 92.4±15.7 versus 102.6±13.4, $p<0.001$; Table 1 and Figure 2B).

The physiological increase in complement levels throughout gestation was rather marked among patients who did not experience a disease flare while pregnant. Complete data on complement levels fluctuation at all time-points in patients experiencing a gestational flare versus those who did not presented a disease flare while pregnant are listed in Table 1 and illustrated in Figure 2B.

Patients with LN displayed significantly lower levels of complement compared to patients without renal involvement

LN had been diagnosed in 237 women (44.5%). Patients with LN had significantly lower levels of complement when compared to patients without renal involvement (C3 at T1 84.6±32.2 versus

98.4±14.1, p<0.001; C3 at PP 93.4±12 versus 103.1±15.4, p<0.001; C4 at T1 15±7.8 versus 16.3±2.8, p<0.001; C4 at PP 16.2±4.3 versus 19.8±6.9, p<0.001, Table 1 and Figure 2C).

Patients with previous LN and flare during pregnancy displayed the lowest complement levels

A flare during pregnancy was observed in 73 women with a previous diagnosis of LN. The lowest levels of complement, both for C3 and C4, were observed in patients with a previous diagnosis of LN who experienced a flare during pregnancy. Complete data are listed in Table 1 and visually represented in Figure 2C.

The fluctuations of C3 and C4 levels at T1 versus conception displayed the highest clinical significance in predicting disease flares

When analyzing the fluctuations of complement levels between different timepoints, the variations in both C3 and C4 between levels assessed at T1 versus those recorded at conception emerged as the most clinically significant. Indeed, the differential values in both C3 and C4 at T1 versus at conception (defined as ΔC3T1–conceptionand ΔC4T1–conception, respectively) were significantly lower in patients with LN when compared to patients without renal involvement (ΔC3 0.5±53 versus 16.6±34.3, p<0.001; ΔC4 1.5±9.1 versus 4.5 ±6.3, p <0.001).

Women who experienced a flare during pregnancy had lower ΔC3T1–conception and ΔC4T1–conception (ΔC3T1–conception -6.7±48.8 versus 18.8±37.6, p<0.001; ΔC4 1.2±8.1 versus 4.4±7.1, p <0.001). The lowest levels of ΔC3T1–conception and ΔC4T1–conception were reported in patients that were diagnosed with LN and experienced flares during pregnancy (ΔC3T1–conception -36.1±42.6; ΔCT1–conception -1.1±8.5).

A decrease in ΔC3T1–conception yielded an OR for flare during pregnancy of 3.1 (CI 95% 2.1-4.8) when below 5 mg/dL, an OR that increased up to 3.9 (CI 95% 2.5-6) when below 15 mg/dL.

Similar figures emerged when assessing the association between ΔC3T1–conception and a prior diagnosis of LN: ΔC3T1–conception ≤ 5 mg/dL conveyed an OR for a prior diagnosis of LN of 6.1 (CI 95% 3.9-9.6) while ΔC3T1–conception ≤ 10 mg/dL conveyed an OR of 7.2 (CI 95% 4.5-11.7). Interestingly, even the lack of clinically relevant changes in the complement levels between T1 and conception was associated with both previous LN diagnosis (OR 2.2; CI 95% 1.3-3.6) and development of flare during pregnancy (OR 5.2; CI 95% 2.9-9.3). Table 2 resumes the results of the coefficient of risk conveyed by different ΔC3T1–conception levels upon LN diagnosis or presence of flare.

The fluctuations of C3 and C4 levels at T1 versus conception displayed the highest clinical significance in predicting APO

Preterm delivery or miscarriage at less than 36 weeks were more frequent in women with a previous diagnosis of APS (39.7% versus 23%; p=0.003), in patients that developed flares during pregnancy irrespectively of a concomitant diagnosis of LN (42.5%versus 28%; p= 0.01 in patients with LN and 34% versus 17.2%; p= 0.01 in those without a diagnosis of LN). Additionally, fetal death was more frequent in patients with a diagnosis of LN and positive aPL (4 out of 30 versus 6 out of 206; p=0.008).

When computing all APO together, higher rates of complications were reported in patients with a previous diagnosis of APS (88.2% versus56%; p< 0.0001) as well as LN (67.9% versus 53.9%; p<0.0001) and occurrence of flare during pregnancy (91.2% versus 45.6%; p<0.0001).

ΔC3T1–conception ≤ 5 mg/dL and no changes of ΔC3T1–conception were both associated with higher rate of overall APO (63.4% versus 45.6%; p=0.003 and 58.5% versus 72.8%; p=0.02, respectively).

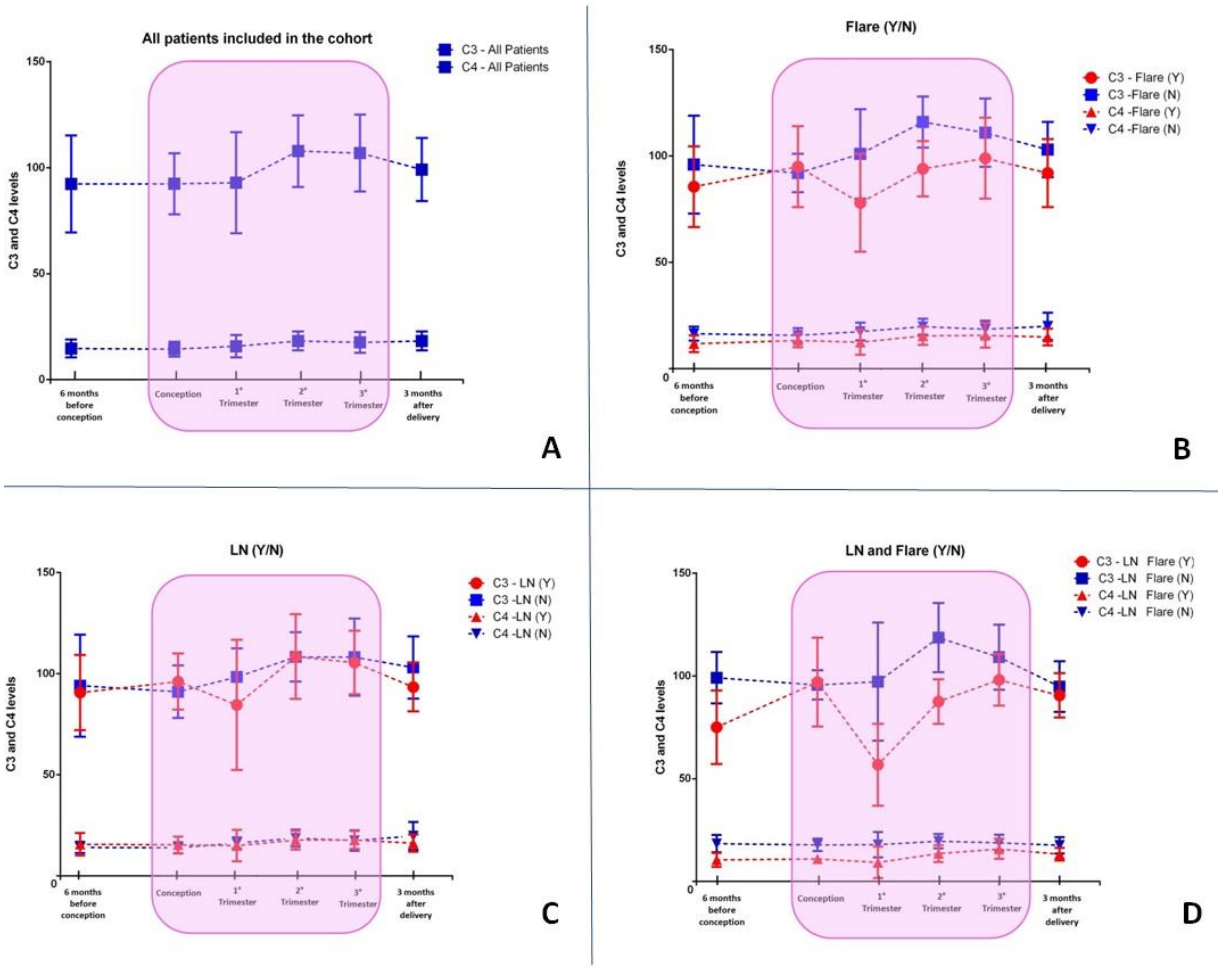


Figure 2. Complement levels fluctuations over 6 time points (before conception, at conception, during each trimester of pregnancy, and after delivery)

Panel 2A. Linear representation of the complement levels overtime in the entire cohort of systemic lupus erythematosus (SLE) patients. Panel 2B. Linear representation of the fluctuations of complement levels during pregnancy in patients with SLE with and without the occurrence of flares during pregnancy. Panel 2C. Linear representation of complement levels during time in patients with and without lupus nephritis (LN). Panel 2D. Linear representation ofcomplement levels during time in patients with and without LN and presence, or absence, of flare during pregnancy. *LN means lupus nephritis.*

	All SLE patients (N=532)	Patients with LN (N=237)	Patients without LN (N=295)	Patients with flare (N=170)	Patients without flare (N=362)	Patients with LN and flare (N=73)	Patients with LN and without flare (N=164)
C3 6 months before pregnancy	92.3±22.9	90.7±18.6	94.1±25.2	**85.6±19.1**	**95.6±23.3**	**75 ±17.9**	**99.1±12.5**
C3 at conception	92.4±14.4	**96.1±13.9**	**91.1±13**	**95.3±19.5**	**91.8±9.1**	97 ±21.6	95.6±7.1
C3 T1	92.9±23.8	**84.6±32.2**	**98.4±14.1**	**78.3±22.8**	**100.5±20.7**	**56.8 ±19.9**	**97.2±28.7**
C3 T2	107.8±16.9	108.5±21	108.3±12.2	**94.16±13.4**	**115.7±12.3**	**87.5 ±10.9**	**118.6±16.8**
C3 T3	106.9±18.1	105.5±15.7	108.2±19.1	**98.97±18.6**	**111.4±16**	**98.1 ±12.6**	**109.1±15.8**
C3 3 months PP	99.1±14.9	**93.4±12**	**103.1±15.4**	**92.4±15.7**	**102.6±13.4**	**90.5 ±10.8**	**94.8±12.3**
C4 6 months before pregnancy	14.7±4.2	**15.7±5.5**	**14.1±2.8**	**11.8±3.9**	**16.5±3.3**	**10.5±3.4**	**18.4±4.2**
C4 at conception	14.4±3.5	**15.4±4.1**	**13.9±2.8**	**13.3±3.2**	**15.7±3.4**	**11±1.3**	**17.8±3**
C4 T1	15.8±5.3	**15±7.8**	**16.3±2.8**	**12.5±5.9**	**17.5±4.2**	**9.3±7.6**	**17.9±6.2**
C4 T2	18.3±4.4	**17.7±4.7**	**18.7±4.2**	**15.5±4.3**	**19.8±3.7**	**13.6±4.1**	**19.6±3.5**
C4 T3	17.6±4.9	17.8±4.4	17.5±5.1	**15.7±5.8**	**18.6±4**	**15.8±4.8**	**18.8±3.9**
C4 3 months PP	18.3±6.2	**16.2±4.3**	**19.8±6.9**	**14.9±3.9**	**20±6.4**	**13.3±3.1**	**17.6±4**
ΔC3 (ΔC3T1–at conception)	10.3±43.2	**0.5±53.2**	**16.6±34.3**	**-6.7±48.8**	**18.8±37.6**	**-36.1±42.6**	**17.3±49.1**
ΔC4 (ΔC4T1–at conception)	3.4±7.6	**1.5±9.1**	**4.5±6.3**	**1.2±8.1**	**4.4±7.1**	**-1.1±8.5**	**2.8±9.1**

Table 1. Complement levels at the six different timepoints (values expressed as mean ±SD), according to diagnosis of lupus nephritis (LN) or presence of a disease flare during pregnancy

Results highlighted in bold are statistically significant. *SLE means systemic lupus erythematosus; LN, lupus nephritis; T1, 1st trimester of gestation; T2, 2nd trimester of gestation, T3, 3rd trimester of gestation; PP, post-partum period (up to 3 months after delivery)*

	FLARE OR	FLARE CI 95%	LN OR	LN CI 95%	LN & FLARE OR	LN & FLARE CI 95%
ΔC3 ≥15 mg/dL	0.3	0.2-0.5	1.2	0.7-2.5	0.06	0.02-0.3
ΔC3 ≥10 mg/dL	0.5	0.3-0.7	0.4	0.3-0.5	0.03	0.01-0.1
ΔC3≥5 mg/dL	0.4	0.3-0.6	0.2	0.1-0.3	0.02	0.01-0.07
ΔC3 no change defined as [-2;+4] g/dL	1.1	0.6-1.9	**2.2**	**1.3-3.6**	**5.2**	**2.9-9.3**
ΔC3 ≤5 mg/dL	**3.1**	**2.1 -4.8**	**6.1**	**3.9-9.6**	**6.5**	**3.9-11.2**
ΔC3 ≤10mg/dL	**3.3**	**2.2-5.1**	**7.2**	**4.5-11.7**	**5.6**	**3.3-9.7**
ΔC3 ≤15mg/dL	**3.9**	**2.5-6**	**6.4**	**4-10.3**	**6.2**	**3.6-10.7**

Table 2. **Odds Ratios according to LN diagnosis or presence of flare and different ΔC3 levels (first trimester –at conception)**

Bold results are statistically significant. *LN means lupus nephritis.*

DISCUSSION

The present network meta-analysis, which includes more than 500 pregnant lupus patients from 4 international independent studies, allowed us to clearly assess the clinical relevance of complement monitoring during gestation to predict both disease flares and APO.

Levels of C3 and C4 emerged as reliable biomarkers to identify those women who are at higher risk of developing disease flares and APO, even in case of a concomitant diagnosis of LN [67,68]. These findings are extremely relevant from a clinical perspective given that, despite the substantial improvements accomplished in the management of SLE patients, 50% of lupus women might develop a flare during gestation, with severe organ involvement occurring in up to 25% of cases [69–72]. Unfortunately, the current lack of reliable biomarkers and validated tools for the assessment of disease activity during pregnancy limits our ability to predict which subjects will experience disease worsening and/or APO. In the last few decades, a number of scoring systems have been developed to assess lupus activity and the risk of flare during pregnancy. Most of these tools, such as the LAI in Pregnancy (LAI-P), the SLE-Pregnancy Disease Activity Index (SLEPDAI), and the modified SLAM (m-SLAM) [73], include hypocomplementemia (C3 and C4). These clinimetric instruments have been created modifying existing lupus activity indexes in order to differentiate between disease-specific features and physiologic changes occurring during gestation. Although promising, these pregnancy-adapted scores have not been extensively validated in large prospective cohorts and therefore their current employment in clinical practice is strongly limited. Similarly, C3 and C4 levels should be carefully evaluated in pregnant lupus women as complement serum levels rise throughout the course of normal gestation [74]. This

study confirms that complement levels fluctuate over gestational course even in SLE women: values of C3 and C4 remained stable at early stages of pregnancy, to progressively increase during the second trimester of gestation; once reached the highest levels, both C3 and C4 showed a decline with discrepant behaviors after delivery, resulting in a constant rise of C4 values and a progressive decrease of C3. Interestingly, we observed that lupus patients who experienced a clinical flare during pregnancy had significantly lower mean values of C3 and C4 throughout the entire gestation compared with patients with stable disease activity. If our data confirm the relevance of complement as a monitoring tool of lupus disease activity even during gestation, it should be mentioned that the consensus about the reliability of complement in predicting SLE flare is not unanimous. Indeed, its relevance has been questioned by few studies [75–78], most likely due to the methodological challenges of accurately measuring circulating complement levels as well as to the inappropriate designs of clinical studies [79]. Nevertheless, despite these inconsistencies, it is universally accepted that complement activation in SLE is mirrored by a secondary decline of circulating complement levels and a parallel increase in complement split products and circulating levels of complement proteins (C3 and C4) are extensively used in clinical practice for classification and diagnostic purposes, monitoring of disease activity and follow-up [28]. Similarly, the clinical significance of low C3 and C4 circulating levels as biomarkers for LN is still matter of research [80]. If a significant drop in C4 levels can be observed even two months prior to renal flare occurrence, a decline in C3 was shown to be influenced by genetic variants of factor H, which regulates C3-convertase in the alternative pathway. In addition, elevated titers of autoantibodies directed against C1q have been described as better predictors of renal involvement in SLE patients compared to C3 and C4, although with inconclusive results [81,82]. Further analysis of our data revealed significantly lower levels of C3 and C4 in pregnant patients with flare at all time-points considered, from conception throughout the entire pregnancy and until 3 months following delivery, as shown by other authors [83]. Most importantly, this study also highlights that those patients with history of LN and disease flare during gestation had the lowest complement levels, suggesting that decreased levels of C3 and C4 before conception can serve as predictor of flare during pregnancy in this high-risk group of patients [84].

In this network meta-analysis, to better evaluate the fluctuation of C3 and C4 minimizing the confounding effect of cut-off variability and inter-assay heterogeneity among the four different cohorts, as well as the potential influence of genetic variants, the analysis also assessed the differential levels of circulating C3 and C4 values (ΔC3 and ΔC4) between different trimesters of gestation, rather than the mere absolute levels or the dichotomous categorization into hypocomplementemia versus normocomplementemia. This approach allowed us to determine that the most informative data in clinical practice consists in the lack of physiological increase in C3 and C4 values in the first trimester

of gestation compared to conception: women who experienced lupus flare during gestation displayed the lowest ΔC3 and ΔC4 during the first trimester versus at conception. In addition, the less pronounced is the increase in C3 levels from conception throughout the first trimester of gestation, the higher the risk of developing disease flare with an OR up to 3.9 when ΔC3 is below 15 mg/dl. The same conclusions can be extrapolated to pregnant women with renal involvement and the occurrence of flare during gestation, a subset of patients where a poor ΔC3 carried an even higher risk of disease flare (OR 5.2). Despite the significance of C4 variations during pregnancy in predicting both APO and disease flare, we decided to emphasize the results obtained when focusing on C3 variations. In fact, from a practical point of view, and based on the more extended range of C3 values, ΔC3 might be easier to assess and more informative for the treating clinicians.

The data gathered in this meta-analysis allowed us to investigate also the role of complement levels in predicting obstetric morbidity among lupus women. Women with lower levels of both C3 and C4 prior to conception and during the entire gestation are more likely to experience poor maternal outcomes: a ΔC3 below 5 mg/dl between the first trimester and at conception as well as no changes in ΔC3 at these time-points were associated with an overall higher rate of adverse pregnancy outcomes. These findings are consistent with available literature, which traditionally enlists hypocomplementemia, together with active LN at conception, previous history of LN, aPL positivity and high disease activity before conception, as major determinants of poor maternal and fetal outcomes in lupus women [3,57,58]. The relationship between complement levels and APO should not be surprising, given the multifaceted role of the complement cascade in pregnant lupus women. On one hand, the complement system, with more than 30 plasma proteins and receptors, represents a key element of the innate immunity response that contributes to the progression of SLE through the stimulation of inflammation and the removal of immune complexes, cells, and apoptotic debris [85]. Importantly, SLE onset, disease activity and organ damage have all been linked to complement activation and consumption, as well as to complement deficiencies [86]. On the other hand, a consistent stream of data has progressively demonstrated that the complement cascade exerts a pivotal role throughout all stages of physiologic gestation (conception, embryo implantation, placentation, fetal growth, and labor) and the fine tuning of the expression of complement factors, receptors and inhibitors during gestation, with their increased hepatic synthesis, is mandatory to ensure pregnancy success [87].

This study presents some limitations that should be acknowledged. First, the limited number of included studies does not encompass the whole prospective experience in lupus pregnancy available in the literature. Second, the geopolitical representation of the included cohorts does not comprehend North America, Asia or Africa, thus reducing the generalizability of our conclusions. Third, since SLE is an extremely heterogeneous condition, the inclusion of patients with distinct clinical profile might

limit the reproducibility of the observed results. Fourth, given the nature of the study, the lack of a control group (e.g. healthy subjects) represents another limitation. Despite the acknowledged limitations, our study has indeed some strengths: the high number of included patients, the prospective design of the considered studies, and lupus diagnosis assessed with homogeneous criteria across different cohorts [5,61,62]. Moreover, despite the absence of complement levels adjustment for gestational state or trimester [55,88–90], cut-off values for circulating levels C3 and C4 were comparable among different cohorts.

CONCLUSIONS

This network meta-analysis supports the role of C3 and C4 in monitoring disease activity in lupus pregnancy and in identifying those patients at higher risk for experiencing APO. Our findings further suggest the inclusion of complement evaluation in the careful multidisciplinary counseling and individual risk assessment that every lupus woman should undergo before embarking on a pregnancy as well as in the longitudinal gestational follow-up.

In particular, the data presented in this study point out that the lack of increase in C3 and C4 levels, especially during the first thirteen weeks of gestation, is a strong biomarker of the risk of developing lupus flare during gestation, even in case of a prior diagnosis of LN.

Type I Interferon Pathway Activation Across the Antiphospholipid Syndrome Spectrum: New Insights into the Systemic Antiphospholipid Syndrome

ABSTRACT

Background: While type I interferon (IFN-I) pathway is crucial in autoimmunity, its role in antiphospholipid antibodies (aPL) positive subjects, including aPL carriers and antiphospholipid syndrome (APS) patients, is poorly understood. The existence of an intermediate condition between pure primary APS (PAPS) and defined connective tissue disease with more pronounced general features, known as systemic APS, needs further clarification from a molecular standpoint. The aim of this study was to evaluate the differential expression of IFN regulated genes (ISGs) among aPL positive subjects.

Methods: A total of 112 patients, including 29 aPL carriers, 31 PAPS, 25 secondary APS (SAPS), 27 systemic lupus erythematosus (SLE) patients without aPL, and 44 healthy controls (HCs), was recruited. Complete demographic, clinical, and laboratory data were collected at the time of the inclusion. IFI6, IFI44, IFI44L, MX1, IFI27, OAS1 and RSAD2 gene expression was evaluated by RT-PCR in whole blood, and a composite index (IFN score) was calculated. Kruskal-Wallis tests, cluster, correspondence and network analyses were performed.

Results: An overall activation of the IFN-I pathway was observed across the entire APS spectrum, with differences among genes based on the specific disease subset. The composite score revealed quantitative differences across APS subsets, being elevated in aPL carriers and PAPS patients compared to HCs (both $p<0.050$) and increasing in SAPS ($p<0.010$) and SLE patients ($p<0.001$). An unsupervised cluster analysis identified three clusters and correspondence analyses demonstrated that cluster usage differed across APS subsets ($p<0.001$), thus correlating with different clinical status. Network analysis revealed different patterns characterizing different subsets. The associations between IFN-I pathway activation and clinical outcomes (especially triple positivity, ANA and aPS/PT antibodies) differed across APS subsets. Although no differences in gene expression were observed in systemic APS, network analyses revealed specific gene-gene patterns, and a distinct distribution of the clusters previously identified was noted ($p=0.002$).

Conclusions: IFN-I pathway activation is a common hallmark among aPL positive individuals as well as in systemic APS patients. Qualitative and quantitative differences across the APS spectrum can be

identified, leading to the identification of distinct IFN-I signatures with different clinical value beyond traditional categorization.

INTRODUCTION

The clinical definition of antiphospholipid syndrome (APS) relies on the finding that individuals persistently positive for antiphospholipid antibodies (aPL), including lupus anticoagulant (LA), anti-β2 glycoprotein I (aβ2GPI) and anti-cardiolipin (aCL) antibodies. APS patients are more likely than the general population to develop arterial and venous thrombotic events, especially at young age [5,91]. Moreover, women with APS can experience recurrent pregnancy losses along with several fetal and maternal complications, such as preterm delivery, intrauterine growth restriction and preeclampsia [5]. Indeed, these phenotypes, thrombotic and obstetric, which can coexist within the same subject, constitute a distinct clinical entity known as "primary APS" (PAPS). The association between APS and other autoimmune conditions, such as systemic lupus erythematosus (SLE), which further complicates the management of these patients, is commonly called "secondary APS" (SAPS). Although this nosological approach is useful to categorize individuals into discrete disease subgroups based on a number of shared clinical and serological features, compelling evidence suggest that it does not encompass the entire clinical spectrum of the disease, thus leaving a not negligible part of patients uncovered and/or underdiagnosed. In fact, over the years, a deeper understanding of the syndrome has led to the identification of a wide range of overlapping additional clinical manifestations as well as novel potential biomarkers, mirroring the complexity of APS pathophysiology, which seems far from being fully elucidated [92].

Several attempts have been made to overcome the conventional classification of the syndrome, both from a clinical and biological standpoint, with the aim of profiling rather than categorizing patients. Among them, two recent publications [93,94] have described the existence of a bridging condition, often encountered in clinical practice, between pure thrombotic APS and lupus, characterized by a higher rate of general features such as cytopenia and anti-nuclear antibodies (ANA) positivity and a lower risk of vascular occlusions, which was termed as "systemic APS". The correct identification of these aPL positive individuals, with or without previous thrombotic events, who do not fulfil the diagnostic criteria for a defined connective tissue disorder despite presenting a tendency toward a more systemic involvement, might led to alternative therapeutic strategies, such as the use of immunomodulant agents, monitoring and prognosis.

From a molecular perspective, type I interferons (IFN-I) have been associated with breakdown of tolerance and perpetuation of autoimmune responses. Although extensive data has supported their involvement in a number of systemic autoimmune conditions, a recent systematic review has revealed

that APS has received limited attention [95,96] Emerging data has suggested the importance of IFN-I in the pathogenesis of APS [97].

However, whether IFN-I pathway activation underlies the earliest stages of the disease and its clinical significance have not been explored yet. Evidence from other conditions, such as SLE [98–102], as confirmed considerable promise for IFN-I pathway activation to improve disease monitoring and patient stratification, as well as to drive disease profiling approaches. Methodological challenges and the low number of studies available pose additional challenges to understand the potential use of IFN-I pathway activation in APS. Taken together, we hypothesize that IFN-I pathway activation may help in the profiling of the APS spectrum and gain insight into their clinical classification. The aims of this study were (i) to assess the IFN-I pathway activation in a cohort of aPL positive individuals, including patients affected by well-described nosological entities such as PAPS and SAPS, as well as lupus patients, (ii) to evaluate the associations between the degree of activation and the structure of the IFN-I pathway activation with clinical outcomes across the APS spectrum, and (iii) to characterize the IFN-I pathway activation in the systemic APS subset.

METHODS

Ethical approval

The study protocol was performed in compliance with the Declaration of Helsinki and approved by the Institutional Review Boards from the University of Turin and the University of Oviedo (reference CEImPA 2021.126). All participants gave written informed consent prior enrolment.

Study participants

This cross-sectional study included consecutive patients attending the San Giovanni Bosco Hospital in Turin (Italy), from January 2019 to December 2022. We enrolled patients who met one of the following inclusion criteria:

1) tested persistently positive for at least one criteria aPL, in the absence of clinical manifestations of APS ("aPL carriers") [5];

2) diagnosis of PAPS defined *as per* Sydney criteria [5];

3) diagnosis of SAPS defined *as per* Sydney criteria [5,28];

4) diagnosis of SLE following the 2019 EULAR/ACR classification criteria, tested persistently negative for criteria aPL as well as for anti-phosphatidylserine/prothrombin (aPS/PT) antibodies (IgG and/or IgM isotypes) [28].

For the purpose of the study we also included age- and sex-matched subjects as healthy controls (HCs).

Systemic APS subset was defined, following previous papers [93], by the presence of 1) persistent aPL positivity with or without clinical manifestations of APS [5], and 2) ANA positivity tested with immunofluorescence on Hep-2 cells at a titer ≥1:80, and 3) at least one additional clinical manifestation (including cytopenia as a whole, hemolytic anemia, leukopenia and thrombocytopenia, hypocomplementemia, arthritis, serositis, Raynaud's phenomenon, photosensitivity, *livedo reticularis*, neuropsychiatric and mucocutaneous manifestations related to the presence of an autoimmune condition), and 4) not fulfilling classification criteria for a defined connective tissue disorder. Demographic, clinical and laboratory characteristics were collected at the time of the enrolment.

Patients and controls were tested for complete aPL profile, including criteria aPL (LA, aCL IgG/IgM and aβ2GPI IgG/IgM), and anti-phosphatidylserine/prothrombin (aPS/PT, IgG/IgM isotypes) antibodies. The aCL, aβ2GPI and aPS/PT were semi-quantitatively assayed using a commercial ELISA kit by Inova Diagnostics, Inc (San Diego, CA, United States). Plasma samples were tested for the presence of LA according to the recommended criteria from the International Society on Thrombosis and Haemostasis Subcommittee on Lupus Anticoagulant/Phospholipid-Dependent Antibodies [103].

The cumulative Global Antiphospholipid Syndrome Score (GAPSS) was calculated for each patient as previously reported by adding together all points corresponding to the score risk factors [104]. Briefly, 5 points for aCL (IgG/IgM), 4 points for LA and aβ2GPI (IgG/IgM), 3 points for aPS/PT (IgG/IgM) and hyperlipidemia and 1 point for arterial hypertension.

RNA isolation and PCR assays

Whole blood samples were processed immediately after extraction by using RNA Stabilization Reagent for Blood/Bone Marrow (Roche, Germany) for stabilization, according to the protocol provided by the manufacturer and stored at -20ºC. Samples were then thawed at room temperature in batches and mRNA was isolation by using the mRNA Isolation Kit for Blood/Bone Marrow (Roche), following the manufacturer's instructions. Reverse transcription was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche).

IFN-stimulated genes (ISGs) expression was evaluated as previously described [105]. In brief, gene expression was assessed with TaqMan pre-designed assays for the following genes: IFI6 (interferon

alpha inducible protein 6, ref. Hs00242571_m1), IFI44 (interferon induced protein 44, ref. Hs00197427_m1), IFI44L (interferon induced protein 44 like, ref. Hs00915292_m1), MX1 (MX dynamin like GTPase 1, ref. Hs00895608_m1), IFI27 (interferon alpha inducible protein 27, ref. Hs01086373_g1), OAS1 (2'-5'-oligoadenylate synthetase 1, ref. Hs00973635_m1) and RSAD2 (radical S-adenosyl methionine domain containing 2, ref. Hs00369813_m1). These candidate genes were selected based on previous evidence supporting their IFN-I dependency and being reported in APS and SLE studies [106,107]. Real-Time quantitative PCR reactions were carried out in an ABI Prism HT7900 (Applied Biosystems, Germany). All samples were assayed by triplicate. Ct values were evaluated with the software SDS 2.3®, and expression levels were evaluated by the $2^{-\Delta\Delta}$Ct method, using the GAPDH gene expression as a housekeeping

Statistical analysis

Variables were summarized as median (interquartile range) or n (%) as appropriate. Z-scores were calculated for each ISG. Differences among groups were assessed by Mann Withney U, Kruskal-Wallis (with Dunn-Bonferroni correction for multiple comparisons) or chi-squared tests. Correlations were analysed by Spearman ranks test. Principal Component Analysis (correlation method) was used to evaluate collinearity among individual ISGs. A composite index for IFN-I pathway activation (IFN-stimulated gene expression score, IFN score) was calculated by averaging all ISGs per individual. Network analyses were generated to analyze the correlations among ISGs across different subsets. Centrality measures (betweenness, closeness, strength and expected influence) were computed. Unsupervised cluster analysis was performed based on squared euclidean distances and Ward's Minimum Variance Method. Correspondence Analyses were used to explore the simultaneous associations among categorical variables (clusters vs subsets). A p-value >0.050 was considered as statistically significant. Statistical analyses were performed in SPSS 27.0, R 4.1.3 and GraphPad Prism 8.4 for Windows.

RESULTS

Patients' characteristics

A total of 112 patients, including 29 aPL carriers, 31 PAPS, 25 APS patients with a concomitant diagnosis of SLE (SAPS), 27 SLE patients without aPL positivity, were recruited. Mean age at inclusion was 48.5 years (S.D. ±13.5 years), with an expected female predominance (75%). In addition, a total of 44 HCs was included in the analysis. Complete demographic, clinical and laboratory characteristics at the time of inclusion in the study and at sample collection, are displayed in Table 1.

	HCs	aPL carriers	PAPS	SAPS	SLE
Total number of patients	44	29	31	25	27
Demographic features					
Age, years, mean (±SD)	50 (11)	46.5 (13)	54.5 (13)	49 (12)	41 (10)
Sex, females, n (%)	39 (89)	22 (75)	25 (81)	14 (56)	23 (85)
Ethnicity, Caucasians, n (%)	44 (100)	29 (100)	30 (96)	23 (92)	27 (100)
Clinical features					
Thrombosis (arterial and/or venous), n (%)	0	0	28 (90)	25 (100)	3 (11)
Thrombotic recurrences, n (%)	0	0	6 (19)	5 (20)	1 (3)
Obstetric complications (APS criteria*), n (%)	0	0	5 (16)	1 (4)	0
Serologic features					
aPL positive, n (%)	0	29 (100)	31 (100)	25 (100)	0
aCL positive (IgG/IgM), n (%)	0	14 (48)	25 (81)	14 (56)	0
aβ2GPI positive (IgG/IgM), n (%)	0	10 (34)	21 (68)	12 (48)	0
LA positive, n (%)	0	20 (69)	25 (81)	19 (76)	0
aPS/PT positive (IgG/IgM), n (%)	0	13 (45)	15 (48)	16 (64)	0
Hypocomplementemia (C3 and/or C4 fractions), n (%)	-	11 (38)	5 (16)	12 (48)	18 (67)
ANA positive, n (%)	-	16 (55)	17 (31)	25 (100)	27 (100)
Anti-dsDNA positive, n (%)	-	1 (3)	0	16 (64)	22 (81)
ENA positive, n (%)	-	7 (24)	1 (3)	8 (32)	14 (52)
Traditional cardiovascular risk factors and GAPSS					
Arterial hypertension, n (%)	9 (20)	9 (31)	17 (31)	10 (32)	8 (30)
Dyslipidemia, n (%)	8 (18)	4 (14)	13 (42)	10 (32)	2 (7)
Diabetes mellitus, n (%)	1 (2)	0	6 (19)	2 (8)	0
Smoking (ongoing), n (%)	5 (11)	3 (10)	7 (22.5)	9 (36)	4 (15)
GAPSS**, value, mean (±SD)	-	9 (5)	9 (5)	12 (5)	-
Treatment (at the time of sample collection)					
Prednisone or equivalent ≤ 5 mg/day, n (%)	0	9 (31)	6 (19)	12 (48)	17 (63)
Prednisone or equivalent > 5 mg/day, n (%)	0	1 (3)	0	2 (5)	4 (15)
HCQ (200-400 mg/day), n (%)	0	9 (5)	8 (25)	16 (64)	12 (44)
LDA (100 mg/day), n (%)	0	14 (48)	20 (64.5)	14 (56)	4 (15)
Vitamin K antagonists, n (%)	0	3 (10)	20 (64.5)	11 (44)	1 (4)

DOACs, n (%)	0	0	4 (13)	4 (16)	0
Other immunosuppressive treatment, n (%)	0	2 (7)	2 (6)	10 (32)	10 (37)

Table 1. Description of study participants

Demographic, clinical and serological features of study participants. *HCs means healthy controls; aPL, antiphospholipid antibodies; PAPS, primary antiphospholipid syndrome; SAPS, secondary antiphospholipid syndrome; SLE, systemic lupus erythematosus; aCL, anti-cardiolipin antibodies; aβ2GPI, anti-β2-glycoprotein I antibodies; LA, lupus anticoagulant; aPS/PT, anti-phosphatidylserine/prothrombin antibodies; ANA, anti-nuclear antibodies; anti-dsDNA, anti-double stranded DNA antibodies; ENA, extractable nuclear antigens; GAPSS, Global Antiphospholipid Syndrome Score; HCQ, hydroxychloroquine; LDA, low dose aspirin; DOACs, direct oral anticoagulants. * Miyakis S, et al. J Thromb Haemost, 2006. ** Sciascia S, et al. Rheumatology (Oxford), 2013.*

IFN pathway activation across the APS spectrum

The analysis of ISGs expression, either individually (Figure 1A) or as a composite score (IFN score) (Figure 1B) revealed a significant IFN-I pathway activation across the APS spectrum, although differences were noted among genes and subsets. Interestingly, the expression of some ISGs, such as IFI44, IFI44L, MX1, OAS1 and RSAD2, was increased already in the aPL carriers' subset compared to HCs (Figure 1A). Of note, this group exhibited a significant heterogeneity. On the contrary, other ISGs were found to be increase only in SLE or SAPS subsets, such as IFI6 or IFI27. Although no changes were observed between aPL carriers and PAPS subsets in any of the genes analysed, IFI44 and OAS1 were found to be elevated in aPL carriers compared to HCs, whereas the same cannot be applied to their PAPS counterparts. Similarly, certain ISGs (IFI44, IFI44L, IFI27 and RSAD2) showed differences between PAPS and SAPS subsets. Finally, a significant number of ISGs (IFI6, IFI44L, MX1, OAS1 and RSAD2) exhibited differences between SAPS and SLE patients.

As expected, all ISGs showed a high degree of correlation. This was confirmed by means of a PCA (matrix determinant: $p=2.16 \cdot 10\text{-}6$; and KMO=0.915, $p<10\text{-}10$). All ISGs showed communalities higher than 0.9, with the exception of IFI27 (0.536). However, only one component was extracted, accounting for 85.9% of the total variance and with all ISGs having loadings >0.9 except for IFI27 (0.756). Then, after confirming the high collinearity of all ISGs analysed, the IFN score was computed. The composite score revealed quantitative differences across APS subsets, being elevated in aPL carriers and PAPS subsets compared to HCs (both $p<0.050$) and increasing in SAPS ($p<0.010$) and lupus patients ($p<0.001$) (Figure 1B).

An unsupervised cluster analysis built with the individual ISGs revealed the identification of three clusters (referred to as clusters I to III) (Figure 1C). Interestingly, the aPL carriers group clustered closer to SAPS, whereas PAPS did with HCs. SLE patients showed the highest differences with the rest of the groups entered in the analysis. Importantly, correspondence analyses demonstrated that cluster usage differed across APS subsets ($p<0.001$), thus correlating with different clinical status (Figure 1D). Again, aPL carriers localized closer to cluster II, although in a less divergent position (closer to the graph centre) compared to both PAPS and SAPS.

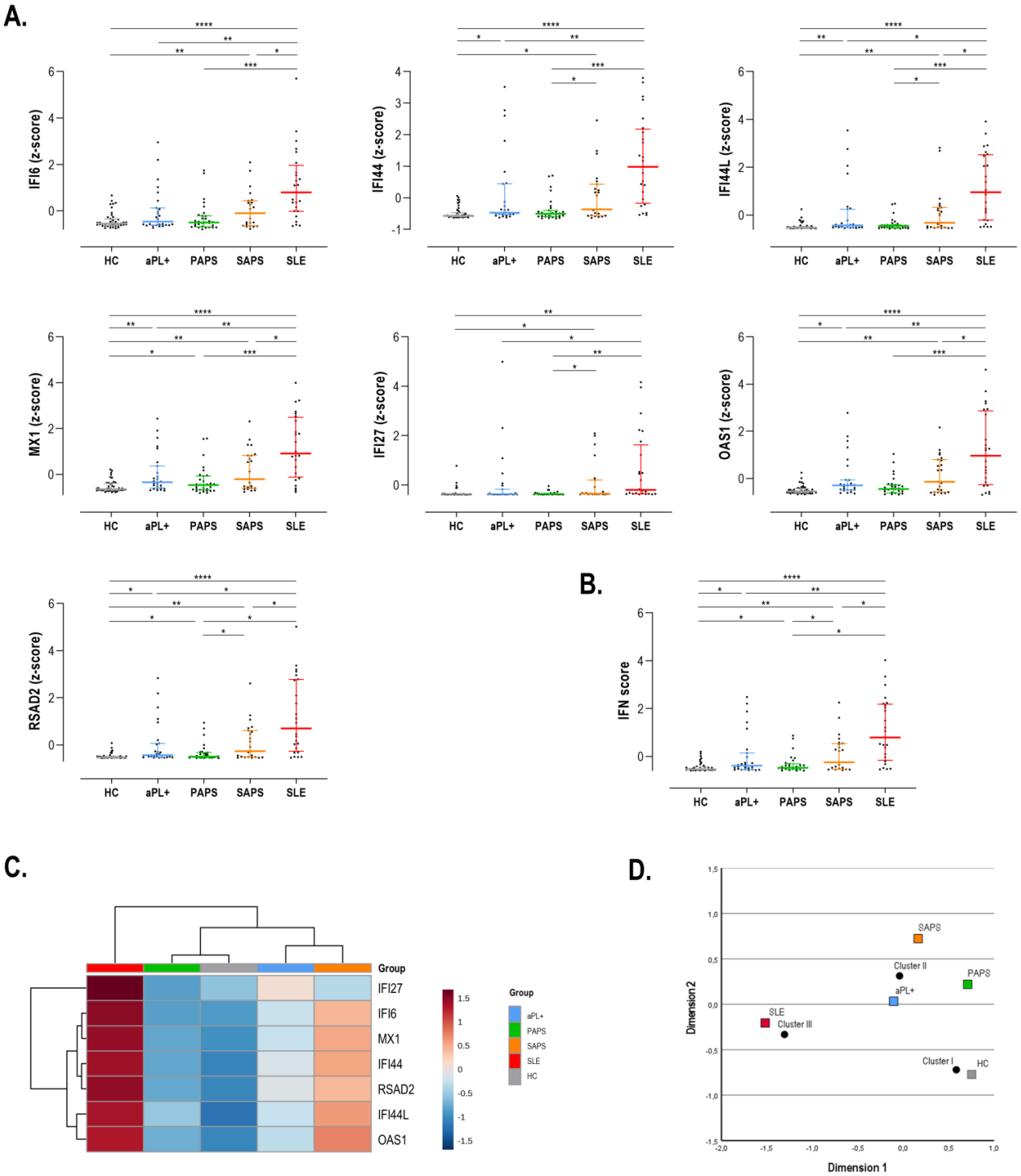
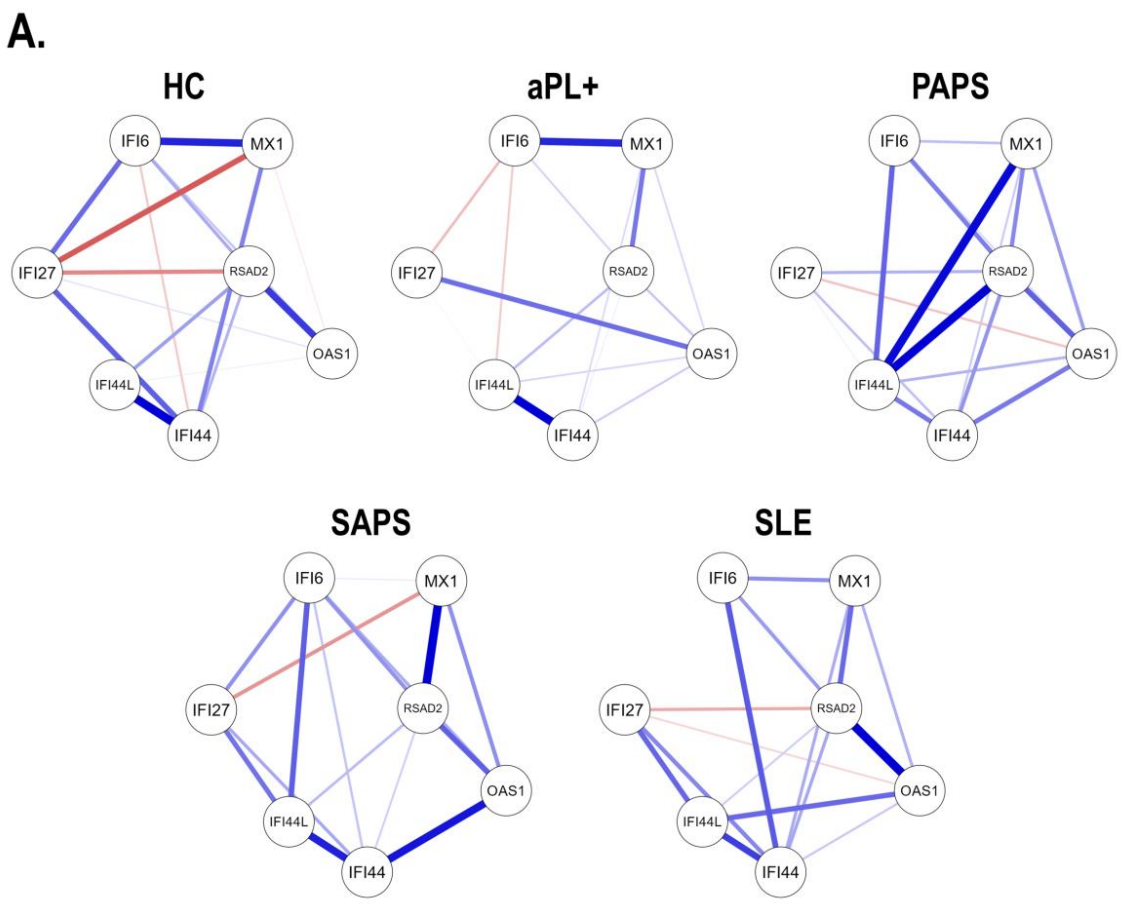

A.
IFI6 (z-score)
IFI44 (z-score)
IFI44L (z-score)
MX1 (z-score)
IFI27 (z-score)
OAS1 (z-score)
RSAD2 (z-score)
HC
aPL+
PAPS
SAPS
SLE
B.
IFN score
C.
Group
IFI27
IFI6
MX1
IFI44
RSAD2
IFI44L
OAS1
D.
SAPS
Cluster II
PAPS
aPL+
SLE
Cluster III
Cluster I
HC
Dimension 1
Dimension 2

Figure 1. IFN pathway activation across the APS spectrum

The IFN pathway activation measured as individual IRG (A) or as a composite score (B) was compared among APS subsets. Results are shown as scatter plots, where lines represent the 25th, 50th (median) and 75th percentiles, and each dot represents one individual. Differences were evaluated by Kruskal-Wallis with Dunn-Bonferroni tests for multiple comparisons. The p-values correspond to those obtained in the multiple comparisons tests and are indicated as follows: *p<0.050, ** p<0.010, *** p<0.001, and **** p<0.0001. (C) A group-averaged (columns) heatmap based on the expression of the IRG (rows). Top bar indicates the APS subsets, as per the group legend (right). Tile colors are based on gene expression levels, red and blue indicating low or high levels respectively, as per the column legend. Vertical and horizontal dendrograms show the clustering patterns among disease subsets and IRG, respectively. (D) Correspondence analysis showing the associations between disease subsets (colored squares) and the three clusters identified (black dots). Axes represent the dimensions derived from the analysis. *HCs means healthy controls; aPL, antiphospholipid antibodies; PAPS, primary antiphospholipid syndrome; SAPS, secondary antiphospholipid syndrome; SLE, systemic lupus erythematosus.*

Finally, network graphs were generated to evaluate the gene-gene interactions (Figure 2A). These analyses revealed that different pictures hallmarked the different subsets. HCs exhibited a uniform network, also showing negative correlations. On the contrary, APS subsets exhibited more heterogeneous networks, mostly composed by positive correlations. The sparsity and degree of the networks increased from aPL carriers (fuzzy pattern) to SLE (strong and high degree network), as the number, strength and edge locations did. These findings were supported by centrality measures, with higher differences across groups being found for IFI44, IFI44L, MX1 and OAS1 (Figure 2B). Centrality measures confirmed similar patterns for SAPS and SLE, especially for closeness and strength, whereas a highly heterogenous profile was observed for aPL carriers. PAPS lie in between these groups.

Taken together, all these results support an early and progressive IFN-I pathway activation across the APS spectrum, where quantitative and qualitative differences were observed. The expression of ISGs delineated certain clinically-relevant clusters which paralleled nosological status.


A.
HC
aPL+
PAPS
SAPS
SLE
IFI6
MX1
IFI27
RSAD2
OAS1
IFI44L
IFI44

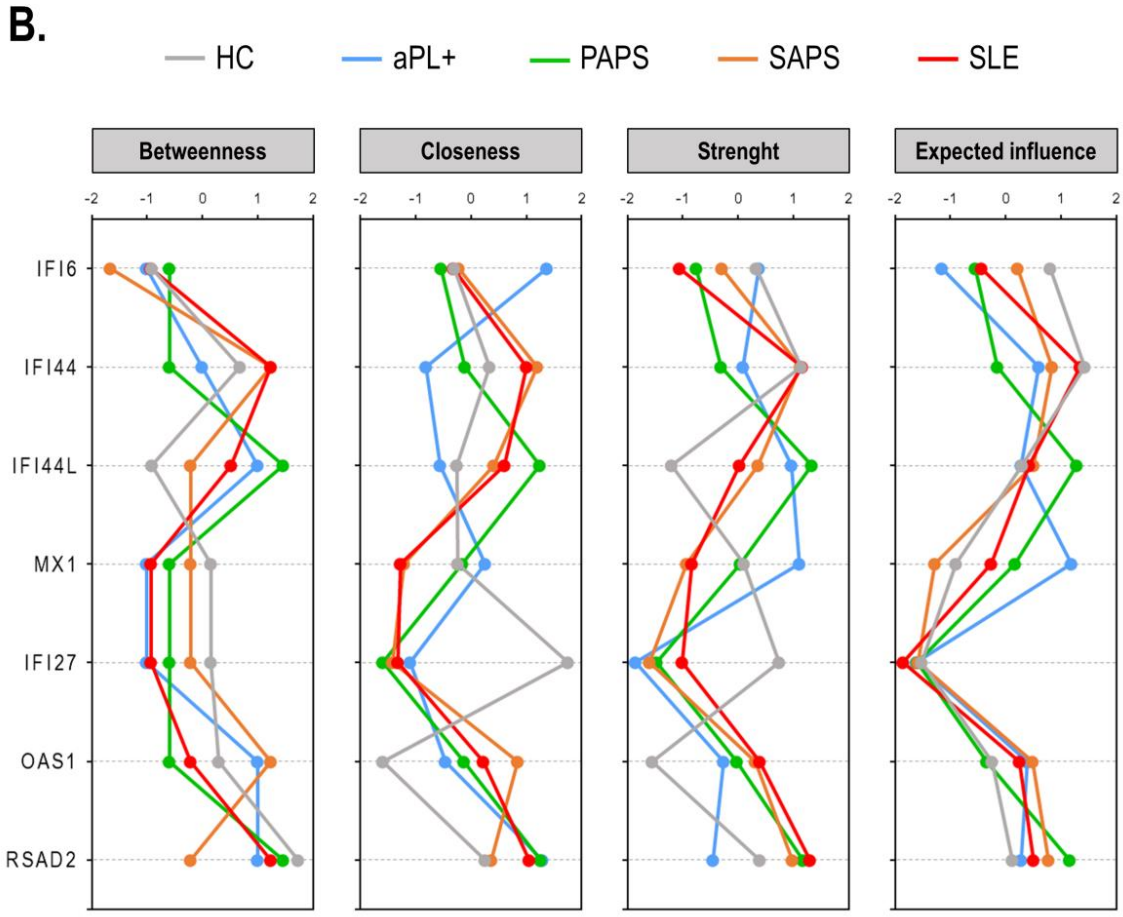

B.
HC
aPL+
PAPS
SAPS
SLE
Betweenness
Closeness
Strenght
Expected influence
-2
-1
0
1
2
IFI6
IFI44
IFI44L
MX1
IFI27
OAS1
RSAD2

Figure 2. Network analyses of IFN pathway activation patterns across the APS spectrum

(A) Network analyses depicted based on the gene-gene correlations among APS subsets. Each node corresponds to a single gene and the lines between nodes illustrate the strength (width) and type (blue: positive, red: negative) of the correlations between each pair of genes. (B) Centrality measures (betweenness, closeness, strength and expected influence) of the IRG network analyses. IRGs are indicated in the vertical axes and centrality measures are represented in the horizontal axes for each study group [lines coloured as per plot legend (top)]. *HCs means healthy controls; aPL, antiphospholipid antibodies; PAPS, primary antiphospholipid syndrome; SAPS, secondary antiphospholipid syndrome; SLE, systemic lupus erythematosus.*

IFN pathway activation and clinical features across APS subsets

Next, the associations between ISGs and IFN score with several clinical features were evaluated across APS subsets.

Thrombosis occurrence (arterial or venous) was unrelated to IFN-I pathway activation, either measured by individual IRG expression or as a composite score (Table 2). However, the presence and extent of recurrence of thrombosis were positively associated with the expression of IFI44, OAS1, RSAD2, as well as with the IFN score in patients with SAPS (Table 2). No effect was noted in the rest of the groups. Moreover, IFN score was unrelated to GAPSS across the APS spectrum (aPL: r=0.224, p=0.261; PAPS: r=0.028, p=0.880; SAPS: r=-0.026, p=0.907; and SLE: r=-0.276, p=0.214). Similarly, no associations with total white blood cell count were found (aPL: r=0.052, p=0.839; PAPS: r=0.048, p=0.818; SAPS: r=-0.299, p=0.229; and SLE: r=0.008, p=0.974). Equivalent findings were observed when ISGs were analysed individually (data now shown).

	IFI6	IFI44	IFI44L	MX1	IFI27	OAS1	RSAD2	IFN score
Arterial thrombosis								
aPL+	p=0.593	p=0.999	p=0.963	p=0.889	p=0.593	p=0.815	p=0.999	p=0.815
PAPS	p=0.312	p=0.258	p=0.594	p=0.921	p=0.767	p=0.567	p=0.650	p=0.489
SAPS	p=0.880	p=0.525	p=0.449	p=0.740	p=0.211	p=0.608	p=0.608	p=0.525
SLE	p=0.750	p=0.999	p=0.667	p=0.999	p=0.250	p=0.750	p=0.999	p=0.750
Venous thrombosis								
aPL+	p=0.091	p=0.410	p=0.365	p=0.365	p=0.462	p=0.239	p=0.462	p=0.205
PAPS	p=0.520	p=0.830	p=0.770	p=0.861	p=0.140	p=0.953	p=0.800	p=0.626
SAPS	p=0.534	p=0.389	p=0.376	p=0.615	p=0.501	p=0.397	p=0.640	p=0.441
SLE	p=0.145	p=0.043	p=0.087	p=0.464	p=0.217	p=0.181	p=0.145	p=0.181
Recurrences of thrombosis								
PAPS	p=0.856	p=0.999	p=0.897	p=0.448	p=0.548	p=0.696	p=0.735	p=0.938
SAPS	p=0.067	**p=0.037**	p=0.111	p=0.080	p=0.174	**p=0.046**	p=0.067	**p=0.050**
SLE	p=0.333	p=0.083	p=0.083	p=0.583	p=0.083	p=0.250	p=0.250	p=0.167
Number of recurrences of thrombosis								
PAPS	r=-0.034 p=0.856	r=0.003 p=0.987	r=-0.021 p=0.911	r=-0.137 p=0.462	r=0.132 p=0.478	r=-0.079 p=0.672	r=-0.053 p=0.776	r=-0.009 p=0.961
SAPS	**r=0.397** **p=0.050**	**r=0.445** **p=0.033**	r=0.350 p=0.102	r=0.381 p=0.053	r=0.302 p=0.161	**r=0.429** **p=0.041**	**r=0.397** **p=0.041**	**r=0.381** **p=0.045**
SLE	r=0.256 p=0.227	r=0.346 p=0.097	r=0.346 p=0.097	r=0.166 p=0.438	r=0.347 p=0.097	r=0.286 p=0.175	r=0.286 p=0.175	r=0.316 p=0.132

Table 2. Associations between IFN pathway activation and thrombotic outcomes across APS subsets

The associations between IFN pathway activation and thrombotic outcomes were evaluated by Mann-Withney U or Spearman's rank tests, as appropriate. Associations reaching statistical significance were highlighted in bold. *IFN means interferon; APS, antiphospholipid syndrome; aPL+, antiphospholipid antibodies carriers; PAPS, primary APS; SAPS, secondary APS; SLE, systemic lupus erythematosus.*

The presence of criteria aPL (LA, aCL, a□2GPI) was not found to be associated with the IFN-I pathway activation in any of the APS subsets (Supplementary Table S2), but when computed in terms of the aPL profile, triple aPL positivity was associated with enhanced IFN-I pathway activation only in aPL carriers (IFN score: p=0.050), although differences were found among ISGs (IFI6: p=0.033, IFI44: p=0.019, IFI44L: p=0.023, MX1: p=0.028, IFI27: p=0.257, OAS1: p=0.113, and RSAD2: p=0.086). However, no associations were observed in the rest of subsets (all p>0.050). A similar picture was found for ANA positivity (Table 3). Additionally, the levels of aPS/PT IgG antibodies strongly correlated with IFN-I pathway activation in aPL carriers, and to a lesser extent in PAPS patients (Table 3), with no effect in SAPS and SLE groups. Similar findings were retrieved with the IgM isotype.

	IFI6	IFI44	IFI44L	MX1	IFI27	OAS1	RSAD2	IFN score
aCL								
aPL+	p=0.458	p=0.999	p=0.999	p=0.905	p=0.756	p=0.867	p=0.583	p=0.943
PAPS	p=0.317	p=0.444	p=0.253	p=0.274	p=0.999	p=0.417	p=0.274	p=0.341
SAPS	p=0.083	p=0.250	p=0.201	p=0.734	p=0.224	p=0.250	p=0.688	p=0.250
LA								
aPL+	p=0.130	p=0.685	p=0.198	p=0.198	p=0.145	p=0.198	p=0.288	p=0.240
PAPS	p=0.502	p=0.764	p=0.764	p=0.945	p=0.999	p=0.182	p=0.872	p=0.945
SAPS	**p=0.012**	p=0.054	**p=0.035**	**p=0.009**	p=0.324	p=0.218	**p=0.021**	p=0.067
aβ2GPI								
aPL+	p=0.223	p=0.141	p=0.359	p=0.359	p=0.359	p=0.537	p=0.604	p=0.309
PAPS	p=0.370	p=0.663	p=0.546	p=0.519	p=0.053	p=0.999	p=0.787	p=0.441
SAPS	p=0.235	p=0.211	p=0.169	p=0.740	p=0.118	p=0.190	p=0.413	p=0.169
ANA								
aPL+	**p=0.002**	**p=0.013**	**p=0.009**	**p=0.023**	**p<0.001**	**p=0.001**	**p=0.011**	**p=0.004**
PAPS	p=0.151	p=0.247	p=0.247	p=0.202	p=0.572	p=0.446	p=0.495	p=0.188
SAPS	p=0.273	p=0.727	p=0.727	p=0.727	p=0.727	p=0.545	p=0.545	p=0.636
aPS/PT IgM								
aPL+	r=0.379 p=0.075	r=0.369 p=0.083	**r=0.422** **p=0.045**	**r=0.434** **p=0.045**	r=0.272 p=0.209	r=0.302 p=0.209	r=0.302 p=0.161	r=0.389 p=0.067
PAPS	r=0.173 p=0.387	r=0.218 p=0.275	r=0.228 p=0.252	r=0.189 p=0.345	r=0.332 p=0.091	r=-0.116 p=0.563	r=0.180 p=0.370	r=0.180 p=0.370
SAPS	r=0.230 p=0.374	r=0.113 p=0.667	r=0.088 p=0.736	r=0.228 p=0.379	r=0.306 p=0.232	r=0.078 p=0.765	r=0.147 p=0.573	r=0.157 p=0.548
SLE	r=0.020 p=0.929	r=0.156 p=0.477	r=0.171 p=0.435	r=0.174 p=0.427	r=0.278 p=0.200	r=0.189 p=0.388	r=0.194 p=0.376	r=0.186 p=0.396
aPS/PT IgG								
aPL+	**r=0.571** **p=0.006**	**r=0.621** **p=0.002**	**r=0.585** **p=0.004**	**r=0.586** **p=0.004**	r=0.150 p=0.506	**r=0.591** **p=0.004**	**r=0.576** **p=0.005**	**r=0.606** **p=0.003**
PAPS	**r=0.404** **p=0.037**	**r=0.390** **p=0.044**	r=0.310 p=0.116	r=0.241 p=0.226	r=0.372 p=0.056	r=-0.010 p=0.959	r=0.266 p=0.180	r=0.295 p=0.135
SAPS	r=-0.038 p=0.880	r=0.119 p=0.639	r=0.024 p=0.926	r=0.185 p=0.463	r=-0.232 p=0.354	r=0.220 p=0.381	r=0.168 p=0.505	r=0.063 p=0.804
SLE	r=-0.069 p=0.755	r=0.001 p=0.998	r=0.126 p=0.567	r=0.037 p=0.868	r=-0.085 p=0.701	r=0.094 p=0.668	r=0.121 p=0.582	r=0.059 p=0.788

Table 3. Associations between IFN pathway activation and autoantibody profiles across APS subsets

The associations between IFN pathway activation and autoantibody profiles were evaluated by Mann-Withney U or Spearman's rank tests, as appropriate. Associations reaching statistical significance were highlighted in bold. *IFN means interferon; APS, antiphospholipid syndrome; aPL+, antiphospholipid antibodies carriers; PAPS, primary APS; SAPS, secondary APS; SLE, systemic lupus erythematosus; aCL, anti-cardiolipin antibodies; LA, lupus anticoagulant; aβ2GPI, anti-β2 glycoprotein I; ANA, anti-nuclear antibodies; aPS/PT, anti-phosphatidylserine/prothrombin antibodies.*

Finally, the effect of medications on IFN-I pathway activation was assessed. Of note, no effects of treatments were registered across the APS spectrum (Table 4).

	IFI6	IFI44	IFI44L	MX1	IFI27	OAS1	RSAD2	IFN score
Prednisone or equivalent ≤ 5 mg/day								
aPL+	p=0.495	p=0.743	p=0.561	p=0.781	p=0.232	p=0.433	p=0.403	p=0.463
PAPS	p=0.827	p=0.789	p=0.865	p=0.906	p=0.999	p=0.715	p=0.751	p=0.981
SAPS	p=0.833	p=0.928	p=0.928	p=0.651	p=0.880	p=0.928	p=0.880	p=0.928
SLE	p=0.516	p=0.431	p=0.227	p=0.473	p=0.759	p=0.256	p=0.354	p=0.392
HCQ (200-400 mg/day)								
aPL+	p=0.232	p=0.668	p=0.495	p=0.348	p=0.322	p=0.275	p=0.561	p=0.375
PAPS	p=0.729	p=0.695	p=0.999	p=0.945	p=0.595	p=0.999	p=0.800	p=0.945
SAPS	p=0.769	p=0.579	p=0.452	p=0.376	p=0.413	p=0.413	p=0.278	p=0.341
SLE	p=0.608	p=0.347	p=0.566	p=0.316	p=0.413	p=0.413	p=0.379	p=0.487
LDA (100 mg/day)								
aPL+	p=0.202	p=0.616	p=0.458	p=0.867	p=0.094	p=0.325	p=0.519	p=0.402
PAPS	p=0.244	p=0.157	p=0.227	p=0.381	p=0.338	p=0.197	p=0.261	p=0.227
SAPS	p=0.999	p=0.877	p=0.926	p=0.734	p=0.829	p=0.877	p=0.781	p=0.877
SLE	p=0.505	p=0.907	p=0.725	p=0.845	p=0.725	p=0.999	p=0.907	p=0.725
Vitamin K antagonists								
aPL+	-	-	-	-	-	-	-	-
PAPS	p=0.826	p=0.617	p=0.795	p=0.459	p=0.704	p=0.287	p=0.589	p=0.734
SAPS	p=0.190	p=0.288	p=0.413	p=0.316	p=0.566	p=0.211	p=0.288	p=0.413
SLE	-	-	-	-	-	-	-	-
Other immunosuppressive treatment								
aPL+	-	-	-	-	-	-	-	-
PAPS	-	-	-	-	-	-	-	-
SAPS	p=0.140	p=0.161	p=0.161	p=0.121	p=0.860	p=0.140	p=0.104	p=0.140
SLE	p=0.060	p=0.070	p=0.148	p=0.057	p=0.131	p=0.131	p=0.148	p=0.098

Table 4. Associations between IFN-I pathway activation and treatments across APS subsets. The associations between IFN-I pathway activation and treatments were evaluated by Mann-Withney U tests. Associations reaching statistical significance were highlighted in bold. *IFN means interferon; APS, antiphospholipid syndrome.*

In conclusion, these findings suggest that although certain associations between IFN-I pathway activation and clinical features may be found, these are restricted to specific APS subsets, thereby pointing to certain heterogeneity in IFN-I pathway activation that may influence clinical value.

Characterizing systemic APS through IFN pathway activation

Next, IFN pathway activation was evaluated in systemic APS. A total of 9 patients from our cohort were considered as having systemic APS and were compared with those not having systemic APS (Table 5).

	Systemic APS		p-value
	No	Yes	
Total number of patients, n	**48**	**9**	
Demographic features			
Age, years, mean (±SD)	51.5 (14)	53 (14)	n.s.
Sex, females, n (%)	25 (52)	5 (55)	n.s.
Ethnicity, Caucasians, n (%)	47 (98)	9 (100)	n.s.
Clinical features			
Thrombosis (arterial and/or venous), n (%)	20 (42)	7 (78)	n.s.
Thrombotic recurrences, n (%)	3 (6)	2 (22)	n.s.
Obstetric complications (APS criteria*), n (%)	4 (8)	1 (11)	n.s.
Arthritis, n (%)	0	2 (22)	-
Serositis, n (%)	0	0	-
Mucocutaneous manifestations, n (%)	1 (2)	2 (22)	n.s.
Raynaud's Phenomenon, n (%)	0	1 (11)	-
Photosensitivity, n (%)	1 (2)	1 (11)	n.s.
Livedo reticularis, n (%)	0	1 (11)	-
Neuropsychiatric manifestations, n (%)	1 (2)	0	-
Serological features			
aPL positive, n (%)	48 (100)	12 (100)	n.s.
aCL positive (IgG/IgM), n (%)	22 (46)	7 (78)	n.s.
aβ2GPI positive (IgG/IgM), n (%)	17 (35)	7 (78)	.04
LA positive, n (%)	25 (52)	6 (67)	n.s.
aPS/PT positive (IgG/IgM), n (%)	13 (27)	4 (45)	n.s.
Hypocomplementemia (C3 and/or C4 fractions), n (%)	2 (4)	4 (45)	.002
Autoimmune hemolysis, n (%)	0	3 (33)	-
Leukopenia, n (%)	0	3 (33)	-
Thrombocytopenia, n (%)	2 (4)	7 (78)	<0.0001
ANA positive, n (%)	11 (23)	9 (100)	-
Anti-dsDNA positive, n (%)	0	0	-
ENA positive**, n (%)	2 (4)	2 (22)	n.s.
Traditional cardiovascular risk factors and GAPSS			
Arterial hypertension, n (%)	17 (35)	4 (45)	n.s.
Dyslipidemia, n (%)	10 (21)	6 (67)	.02
Diabetes mellitus, n (%)	3 (6)	2 (22)	n.s.
Smoking (ongoing), n (%)	9 (19)	0	-
GAPSS, value, mean (±SD)***	11 (5)	12 (4)	n.s.
Treatment (at the time of sample collection)			
Prednisone or equivalent ≤ 5 mg/day, n (%)	4 (8)	3 (33)	n.s.
Prednisone or equivalent > 5 mg/day, n (%)	0	1 (11)	-
HCQ (200-400 mg/day), n (%)	8 (17)	2 (22)	n.s.
LDA (100 mg/day), n (%)	16 (33)	7 (78)	.03
Vitamin K antagonists, n (%)	13 (27)	7 (78)	.01
DOACs, n (%)	3 (6)	0	n.s.
Other immunosuppressive treatment, n (%)	3 (6)	2 (22)	n.s.

Table 5. Characteristics of systemic APS

Patients were stratified according to their systemic APS status and their demographic, clinical and serological features were compared. Variables were summarized as median (interquartile range) or n(%), unless otherwise stated. Differences were evaluated by Mann-Withney U or chi-squared tests (using Yates correction), as appropriate. *APS means antiphospholipid syndrome; aCL, anti-cardiolipin antibodies; aβ2GPI, anti-β2-glycoprotein I antibodies; LA, lupus anticoagulant; aPS/PT, anti-phosphatidylserine/prothrombin antibodies; ANA, anti-nuclear antibodies; anti-dsDNA, anti-double stranded DNA antibodies; ENA, extractable nuclear antigens; GAPSS, Global Antiphospholipid Syndrome Score; HCQ, hydroxychloroquine; LDA, low dose aspirin; DOACs, direct oral anticoagulants; n.s. means not significant. * Miyakis S, et al. J Thromb Haemost, 2006. ** We excluded patients who tested positive for anti-Smith antibodies, based on their high specificity for SLE diagnosis. *** Sciascia S, et al. Rheumatology (Oxford), 2013.*

No differences in IRGs expression levels or IFN score in association with systemic APS status were retrieved (Table 6).

	Systemic APS		p-value
	No (n=48)	Yes (n=9)	
IFI6	-0.32 (1.11)	-0.24 (0.44)	0.587
IFI44	-0.26 (1.17)	-0.42 (0.38)	0.272
IFI44L	-0.40 (0.94)	-0.42 (0.21)	0.239
MX1	-0.25 (0.66)	-0.41 (1.14)	0.208
IFI27	-0.36 (0.25)	-0.35 (0.09)	0.755
OAS1	-0.27 (1.33)	-0.33 (0.42)	0.112
RSAD2	-0.32 (1.17)	-0.40 (0.23)	0.197
IFN score	-0.33 (1.07)	-0.37 (0.34)	0.295

Table 6. IFN pathway activation in systemic APS

The expression of individual IRGs (Z-scores) and the composite IFN score were evaluated according to systemic APS status by Mann-Withney U tests. Variables are summarized as median (Interquartile range). Associations reaching statistical significance were highlighted in bold. *IFN means interferon; APS, antiphospholipid syndrome.*

However, network analyses revealed noticeable differences in gene-gene interactions, as systemic APS patients showed a stronger and higher-degree network (Figure 3A). Nodes presenting with the higher correlations differed depending on systemic APS status. Centrality measures supported these findings (Figure 3B), with IFI44, IFI44L and MX1 showing the largest differences between groups, followed by OAS1 and RSAD2.

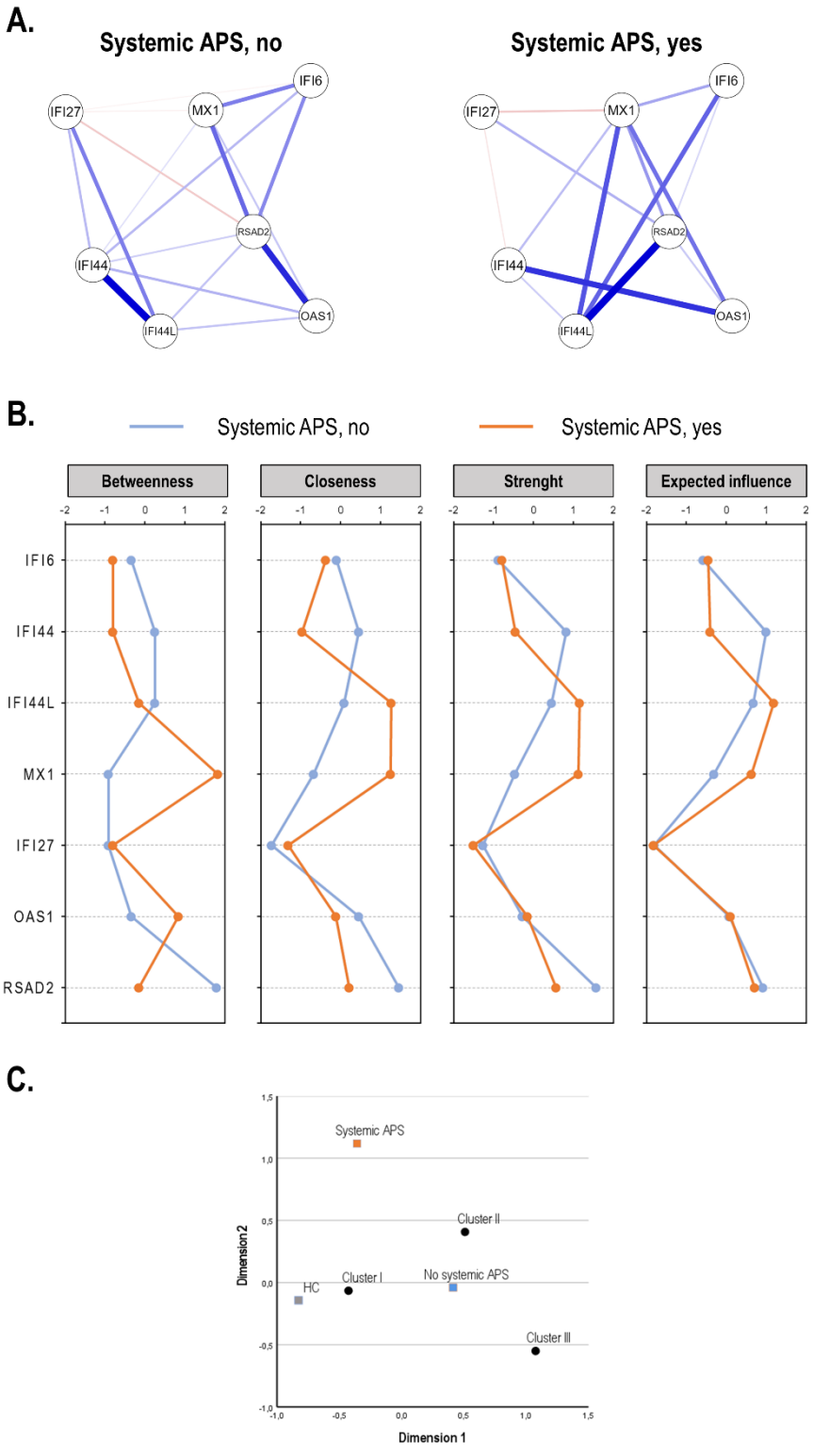


Figure 3. Analysis of the IFN pathway activation in the systemic APS subset

The IFN pathway activation according to systemic APS status (no vs yes) was evaluated by network analysis (A), centrality measures (B) and correspondence analysis (C). *APSmeans antiphospholipid syndrome.*

Finally, systemic APS related to a differential distribution of the clusters previously identified, being more likely to use clusters I and II, compared to those without systemic APS (p=0.003) (Figure 3C). Importantly, the usage of these clusters was different than that of conventional APS. Of note, when systemic APS diagnosis was added to the nosological/clinical groups, it segregated from PAPS and SAPS (Figure 4), thereby confirming their differential status. Finally, it must be noted that these findings were obtained using a stringent definition of systemic APS. However, with a less strict definition (excluding ANA positivity), a slightly higher number of patients were classified as having systemic APS (n=12), but equivalent findings for IFN-I pathway activation were obtained (data not shown).

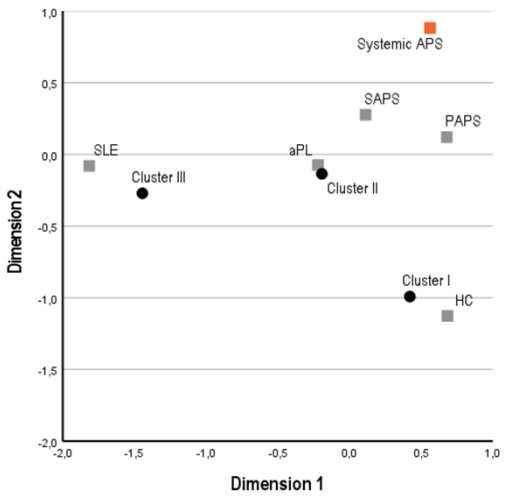


Figure 4. Correspondence analysis of systemic APS and APS subsets. The usage of the three clusters (black dots) depending of systemic APS status was evaluated by correspondence analysis. Patients presenting with systemic APS are grouped as systemic APS (orange square), whereas those without are grouped according to the conventional classification (PAPS, SAPS, SLE and HCs) (gray squares). *APS means antiphospholipid syndrome; PAPS, primary APS; SAPS, secondary APS; SLE, systemic lupus erythematosus; HCs, healthy controls.*

These findings suggest that the systemic APS subset is hallmarked by a distinct IFN-I pathway activation profile, which can be attributed to a distinct coordinated expression of certain ISGs rather than their absolute expression values.

DISCUSSION

Despite the well-established role of IFN-I in autoimmunity and the urgent need for reliable circulating biomarkers, an important knowledge gap is observed on the IFN-I pathway activation in the APS field. The findings herein presented confirm that an overall IFN-I pathway activation is a common hallmark across the entire APS spectrum, although differences were noted among genes and clinical subsets. To the best of our knowledge, this is the first study performing such a broad characterization as well as extending the analysis to alternative clinical phenotypes, such as the systemic APS subset, where a differential co-expression profile of ISGs was observed, thereby paving the way for the translational use of the IFN-I assays for patients' profiling and characterization in APS.

Our findings revealed a strong IFN-I pathway activation across the whole APS spectrum, even in individuals not fulfilling classification criteria or clinical manifestations of APS but persistently positive for aPL. Moreover, a progressive activation increases from those individuals towards patients with a more complex clinical phenotypes such as SAPS or SLE was observed. Although similar findings have been reported in isolated monocytes [108] these were not confirmed at the whole blood level. Furthermore, although some studies have addressed the analysis of IFN signatures or scores in PAPS and SAPS [109,110], evidence in aPL carriers subgroup is scarce and represents a major unmet need. The observation of an enhanced IFN-I pathway activation in those subjects only characterized by the persistent presence of aPL in the absence of clinical manifestations of the syndrome, represents an interesting tool for patients profiling, and it also suggest a potential role of IFN-I pathway activation in their monitoring. Indeed, prospective studies are needed to confirm this observation and to demonstrate the usefulness of IFN-I pathway in predicting disease evolution and stratifying patients according to the risk of developing clinical manifestations, as reported in other scenarios. Of note, a significant heterogeneity was observed within the aPL carriers subset in terms of genes and extent of activation, which may reflect its clinical within-group heterogeneity as it may be associated with different clinical trajectories. The findings from cluster and correspondence analyses supported this idea.

Regarding individual ISGs trends, whereas some genes were increased in all subsets compared to HCs (such as IFI44L, MX1 and RSAD2), other genes were found to be increased only SAPS and lupus patients (such as IFI6 or IFI27). These findings were paralleled, at least in part, by differences in influence in network analyses. Taken together, these results may inform different expression programs specific for each disease subset. Although this notion has been hypothesized in previous studies [111], suboptimal reporting practices and little evidence has limited its appraisal. Our findings align with the idea that differential, APS-specific components can be found within IFN-I fingerprints, probably in relation to distinct pathogenic substrates among related conditions. Gaining understanding towards these trends will not only shed light into disease taxonomy, but also provide a better understanding of the connections between clinically-relevant signatures and nosological entities in APS.

A remarkable breakthrough from our study was the assessment of gene-gene correlations. Network analyses reinforced that different gene expression programs could be distinguished across the APS spectrum, which cannot be captured solely by analyzing expression levels. Overall, our results unveil a significant heterogeneity among IFN-I pathway activation patterns within APS. This heterogeneity may be linked with different clinical value, hence explaining the diverging associations between IFN-I pathway and clinical and serologic features, such as thrombotic events or autoantibody profiles, among APS subsets. A similar scenario has been reported in rheumatoid arthritis and SLE populations by our group and others [105,112][113,114]. However, this phenomenon had not been explored in APS until date. Furthermore, this notion may explain the controversy observed in previous studies about the association between IFN-I pathway activation and clinical outcomes, especially serological features, such as the association with aβ2GPI antibodies [108,115]. Of note, our study unveiled an association between IFN-I pathway activation and the presence of aPS/PT antibodies in both aPL carriers and PAPS patients, which shed new light into the connections between IFN-I signalling and humoral response in APS. Previous evidence suggested that aPL can trigger IFNα production [116]. On the one hand, whether this applies to the aPS/PT antibodies requires further mechanistic research. On the other hand, these findings add to the emerging clinical relevance of the aPS/PT antibodies, as these may help to identify groups of patients with specific characteristics, including an elevated IFN-I pathway activation. In fact, while criteria aPL are still considered the main determinant for risk stratification, data supporting the additional role of scoring systems, such as the GAPSS and "extra-criteria" aPL, in specific subgroups of subjects, like those at high suspicion for APS diagnosis but tested negative for criteria aPL or when LA testing is not available or not reliable, are rapidly growing [117–119].

Interestingly, our study also focused on the recently described systemic APS subset. Although proof-of-concept, our analyses revealed that patients considered as having systemic APS are hallmarked by

specific gene-gene correlations and a differential usage of ISGs clusters, which segregate from those in APS (either aPL carriers, PAPS, or SAPS) and SLE populations. The involvement of IFN-I in this scenario aligns with the solid association of this mediator with the occurrence of autoantibodies and cytopenia in systemic conditions. Therefore, these data suggest that the IFN-I pathway may represent an innovative tool for identifying those patients who present with an intermediate clinical and serological phenotype between PAPS and SLE and who might benefit from therapeutic approaches not only limited to counterbalancing the procoagulant state, such as immunomodulant agents, and dedicated preventive strategies and monitoring to avoid organ damage and long-term disability.

We must acknowledge that suggesting the use of IFN-I as profiling tool while identifying an additional discrete subset, called systemic APS, might sound contradictory. Nevertheless, since the use of IFN-I pathway activation assays and molecular characterization for profiling purposes needs further investigation before overcoming the traditional categorization approach, classification criteria still represent a fundamental tool for practical clinical guidance and patients' management. Taken together, our results further confirm the usefulness of IFN-I pathway activation in aPL positive patients profiling, mirroring the role of this pathway in APS pathogenesis, and further demonstrate the existence of clinical phenotypes beyond traditional classification criteria.

Contemporary IFN research has been characterized by a large heterogeneity in terms of preclinical standardization, assay methodology and clinical validation, which may account for its lack of translation into the clinical setting. Recently, a EULAR taskforce has been published in order to guide future steps on measurement, reporting and application of IFN-I assays in clinical research and practice [120]. This study represents the first work following these recommendations in the setting of APS, including a separate description of the IFN score, empirical support for composite score calculation and uptake of consensus terminology. Moreover, the analysis of a less explored disease, such as APS, is compliant with the proposed research agenda. Therefore, this study paves the ground for future research ensuring comparability and enabling international collaborations.

Our study has certain limitations that should be acknowledged. First, the prevalence of obstetric manifestations in our cohort was low, thus limiting our ability to capture possible associations between the IFN-I pathway and pregnancy complications. In addition, the cross-sectional design of the study does not allow for the observation of variations and fluctuations in the IFN-I pathway activation over time, therefore preventing a correlation with disease activity, clinical manifestations, and disease evolution. Nevertheless, our results were derived from a well-characterized and monitored patients, reflecting real-world patient population and covering the whole spectrum of APS.

CONCLUSIONS

In conclusion, IFN-I pathway activation is a common hallmark across the APS spectrum, being found elevated even in those aPL positive subjects who did not fulfil classification criteria for the syndrome. Far from being a uniform expression program, different expression patterns could be distinguished, which may underlie the distinct clinical correlates among APS subsets. Finally, aPL positive patients who present with a higher rate of systemic features, named systemic APS, exhibited a characteristic IFN-I pathway activation profile. Further larger and prospective studies are needed to evaluate the potential role of IFN-I pathway activation to predict thrombotic events and recurrences as well as disease evolution in aPL positive patients. Furthermore, preclinical research has demonstrated beneficial effects of the abrogation of IFN-I signalling in APS, which adds to the successful results from phase III trials in SLE [121]. Although anticoagulation stays as the therapeutic mainstay in APS, in the era of IFN-targeted therapies it may be conceivable to evaluate the effects of IFN-I blockade in patients presenting with IFN-I pathway activation.

FINAL REMARKS

This PhD research project aimed to identify new tools for a more precise and tailored management of patients affected by CTDs, such as SLE and APS, especially those who are at higher risk for developing gestational complications, with the ultimate goal of improving patients' outcomes and the overall quality of life, taking into account the extreme inter-individual and intra-individual variability.

Future research will allow the development of risk stratification strategies and tools in the field of low prevalence and rare diseases such as CTDs and APS.

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