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Cluster analysis of autoimmune rheumatic diseases based on autoantibodies. New insights for polyautoimmunity

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ABSTRACT

Autoimmune diseases (ADs) are a chronic and clinically heterogeneous group of diseases characterized by share common immunopathogenic mechanisms and risk factors (i.e., the autoimmune tautology), which explain the fact that one AD may coexist with others (i.e., polyautoimmunity - PolyA). In the present exploratory study, a mixed-cluster analysis of the most common autoimmune rheumatic diseases (ARDs) was done. A total of 187 consecutive women with established systemic lupus erythematosus (n = 70), rheumatoid arthritis (n = 51), systemic sclerosis (n = 35) and Sjögren's syndrome (n = 31) were included. A comprehensive clinical, autoantibody and cytokine assessment was simultaneously done. Total PolyA was registered in 142 (75.9%) patients. Six clusters were obtained, built mainly on autoantibodies: PolyA-I to -VI. The PolyA-III cluster showed the highest frequency of overt PolyA (p = 0.01), and the PolyA-I, -III, and -IV clusters exhibited the highest positivity for IL-12/23p40 (p = 0.015). These results provide new insights into the pathophysiology of PolyA and warrant prospective validation to enable development of a more accurate taxonomy of ARDs.

1. Introduction

Autoimmune diseases (ADs) are a chronic and clinically heterogeneous group of diseases affecting around 5% of the world population [1], with a progressive increase in their incidence and prevalence [2]. These conditions are characterized by share common immunopathogenic mechanisms and risk factors (i.e., the autoimmune tautology) [3], which explain the fact that one AD may coexist with others (i.e., polyautoimmunity - PolyA) [4].

In systemic lupus erythematosus (SLE), the coexistence of rheumatoid arthritis (RA), denoted as rhupus, is less than 10%, although the rheumatoid factor (RF) and anti-citrullinated peptide antibodies (CCP) or anti-citrullinated protein antibodies are present in 42% and 6%, respectively [5]. Interestingly, up to 54% of patients with SLE may exhibit antiphospholipid antibodies; however, only 10% develop antiphospholipid syndrome (APS) [5]. In euthyroid patients with SLE, thyroperoxidase (TPO) and thyroglobulin (Tg) autoantibodies are observed in 21% and 10% of patients, respectively. However, confirmed autoimmune hypothyroidism is observed in 12% of SLE patients [6]. In RA, the prevalence of autoimmune thyroid disease (AITD) is 10%, with a frequency of positivity for TPO and Tg autoantibodies of 38% and 21%, respectively [7]. Furthermore, patients with Sjögren's syndrome (SS), exhibit a high frequency of PolyA given by the simultaneous existence of AITD (15%–30%), RA (4%–31%), SLE (9%–19%), and systemic sclerosis (SSc) (14%) [8]. Of these, AITD (23%) and SS (25%) are the most commonly observed in patients with SSc [9].

All these data, although cross-sectional, consistently support the commonalities of ADs [3], and reveal two types of PolyA: overt PolyA which correspond to the presence of more than one well-defined AD in a single patient [10], and latent PolyA which correspond to the presence of several autoantibodies not directly related to the underlying AD but with predictive value for an additional AD [11–13].

Biology-based indicators of disease (i.e., biomarkers) play a key role in disease prediction, diagnosis and monitoring [14]. As classical biomarkers, diverse serological expression of autoantibodies and cytokines across autoimmune rheumatic diseases (ARDs) has been reported. Cytokine production is pivotal in the pathophysiology of ADs and may influence the synthesis of autoantibodies [15]. However, most studies

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in ADs have focused on a single condition despite the similarities among them. Similar patterns of cytokines may affect diverse ADs [16–18]. Nonetheless, variations in the expression of these patterns (e.g., Th1, Th2, Th17, Th9) exist depending on the nature of disease (i.e., organ specific or systemic), the affected organ (s), the time of evolution of the disease and the genetic background of the patient [1,16–19]. Moreover, the pathological functions of these biomarkers (e.g., autoantibodies, cytokines) are not isolated since they emerge from the interactions among them and between cells and tissues (i.e., systems medicine) [20,21].

Since clustering is a useful tool to classify different subsets of patients with similar features, we carried out a cluster analysis of patients with the most frequent ARDs in order to attempt a new classification of these conditions.

2. Methods

2.1. Study population

A cross-sectional analytical study was done on 187 patients with RA (n = 51), SLE (n = 70), SSc (n = 35), and SS (n = 31). The subjects have been followed at the Center for Autoimmune Diseases Research (CREA) in Bogotá, Colombia. The patients fulfilled either the 1987 American College of Rheumatology (ACR) classification criteria for RA [22], the 1997 ACR criteria for SLE [23], the 2013 ACR/European League Against Rheumatism classification criteria for SSc [24], or the revised American-European Consensus Group for SS [25]. Data regarding age, age at onset, and duration of disease were obtained. The PolyA phenomena was classified as overt PolyA and latent PolyA. Total PolyA was defined as the sum of overt and latent PolyA. This study was done in compliance with the Act 008430/1993 of the Ministry of Health of the Republic of Colombia, which classified it as minimal-risk research. The institutional review board of the Universidad del Rosario approved the study design.

2.2. Laboratory measurements

Serum samples were obtained in a state of fasting. As described in detail previously [6], detection of IgM RF, IgG third generation CCP (CCP3), IgM and IgG anti-cardiolipin antibodies (ACA), IgM and IgG anti-β2glycoprotein-1 (β2GP1) antibodies, IgG anti-dsDNA antibodies, IgG anti-TPO and anti-Tg antibodies, anti-SSA/Ro, anti-SSB/La, anti-Ribonucleoprotein (RNP) and anti-Smith (Sm) antibodies was done by Enzyme-Linked-Immunosorbent Assay (ELISA). Further, 11 additional autoantibodies were evaluated by immunoblot assay (Nucleosomes, Histones, PCNA, P0, ACApB, Scl70, AMA M2, Jo-1, PM-Scl, Mi-2, Ku) (IMTEC ANA-LIA Maxx from Human diagnostics) from which only anti-centromere antibody subunit B (ACApB) was individually included in the cluster analysis.

Concentration of 15 human cytokines (IL-2, IL-10, IL-6, IL-8, IL-9, IL-13, IL-12/23p40, G-CSF, IFNγ, IFNα, IL-4, IL-1β, TNFα, IL-5, IL-17A) was assessed on serum samples from patients by Cytometric Bead Array (CBA, Becton Dickinson Biosciences, San Diego, CA, USA) as previously reported in detail [21]. Results were considered positive when the assay results were above a threshold value, and these were obtained from healthy individuals in whom evidence of acute or chronic disease including autoimmune, cardiovascular, or metabolic was not detected [21,26,27].

2.3. Statistical methods

In order to determine clusters of patients with similar characteristics, we used the mixed-cluster methodology proposed by Lebart et al. [28]. In short, this mixed methodology involves three steps: first, a multiple correspondence analysis is done to obtain a new representation of the original data based on the principal components and to reduce the dimension of the data by choosing those with the highest eigenvalues. Second, a hierarchical cluster analysis is done on the components retained in the previous step, and the number of clusters is determined. Finally, a consolidation step is done in order to improve the clusters achieved in the previous step by doing a k-means clustering using, as initial centroids, the centers of the clusters derived in the previous step. Thus, the clusters obtained by means of hierarchical cluster analysis is refined with a k-means step.

We tested three different scenarios to derive the final clusters of patients: the first one, involved antibodies for building clusters; the second involved the cytokines, and the third approach used both types of biomarkers: antibodies and cytokines. In each scenario, several cluster options were assessed, varying the number of dimensions retained and the number of clusters chosen in the hierarchical cluster step.

The number of dimensions retained for clustering was chosen using two methods: 1) all dimensions retained or 2) dimensions retained based on the results of parallel analysis [29]. The number of clusters in the hierarchical cluster step was determined using the majority rule based on the 30 indices using the R package NbClust [30].

Finally, to compare the different clusters obtained in the three different scenarios three indices were used: Dunn index, Average silhouette width, and Hopkins statistics for the principal components used in the analysis according to Kassambara et al. [31]. Cytokines and autoantibodies with frequencies under 4% were excluded from the cluster analysis since these variables with low frequencies tend to generate clusters that include only those atypical patients. Clustering was done taken all the patients together (N = 187), and included data from a previous reported group of SLE patients [21]. Associations between the final clusters and other variables were assessed by Chi square and Kruskall-Wallis tests. Statistical analyses were done using R version 3.3.2.

3. Results

3.1. Cohort

General characteristics of patients with ARDs including overt and latent PolyA distribution are shown in Table 1. As expected, a lower age and early age at onset of disease were observed in SLE patients, whereas late-onset disease was observed in SS. Patients with RA had a longer duration of disease. Frequency of overt, latent, and total PolyA was not different among the four ARDs studied (Table 1). Total PolyA was registered in 142 (75.9%) patients.

3.2. Autoantibodies and cytokines

Fig. 1 presents the prevalence of autoantibodies and cytokines for each disease. As expected, RF (84.3%) and CCP3 (76.5%) were the most frequent autoantibodies in patients with RA (Table 2). Furthermore, IL-6 (59.6%), IFNα (55.3%), and IL-12/23p40 (53.2%) were the most frequent positive cytokines in this subset of patients (Tables 3 and 4).

In SLE, the most frequent antibodies were ANAs (71.4%). Anti-dsDNA, anti-RNP, and anti-SSA/Ro had frequencies of 47.1%, 44.3% and 42.9%, respectively. In the case of cytokines, IL-12/23p40 (52.2%) and G-CSF (46.3%) exhibited the highest positivity frequencies in this group.

For SSc, almost all patients showed positivity for ANAs (97.1%), and 62.9% of the patients had ACApB. Other antibodies such as RF (65.7%), anti-SSA/Ro (40%), and anti-SSB/La (25.7%) had high frequency of positivity. Regarding cytokines, IL-6 (51.4%), IL-8 (48.6%), IL-12/23p40 (65.7%), IL-17A (48.6%), IFNα (51.4%), and TNFα (48.6%) were the most frequent in these patients.

Finally, with respect to SS, 96.8% of these patients had ANAs, 74.2% had anti-SSA/Ro, 64.5% had RF, and 51.6% had anti-SSB/La. In these patients, IL-12/23p40 (64.5%), IL-17A (45.2%), and IFNα

Table 1
General characteristics of women with autoimmune diseases.

Variable	RA (n: 51)	SLE (n: 70)	SSc (n: 35)	SS (n: 31)	*P-value*
Age (IQR)	58 (48.5–63)	50 (36–57.25)	58 (51–63)	64 (55–71)	< 0.001
Age at onset disease (IQR)	36 (26–49)	28 (20.5–40)	46 (36–53)	50 (40–58)	< 0.001
Duration of disease (IQR)	17 (10.5–26)	13 (9–21.75)	7 (4–13)	12 (9–17)	< 0.01
Overt PolyA (%)[a]	14 (27.5)	17 (24.3)	14 (40.0)	8 (25.8)	0.408
RA	–	4 (23.5)	3 (21.4)	1 (12.5)	
SLE	2 (14.3)	–	3 (21.4)	3 (37.5)	
SSc	0	2 (11.8)	–	1 (12.5)	
SS	7 (50.0)	7 (41.2)	1 (7.1)	–	
AITD	8 (57.1)	5 (29.4)	6 (42.9)	5 (62.5)	
APS	0	4 (23.5)	2 (14.3)	1 (12.5)	
MG	0	1 (5.9)	0	0	
AIH	0	0	1 (7.1)	0	
PBC	0	0	1 (7.1)	0	
DM	0	0	1 (7.1)	0	
AV	0	0	1 (7.1)	0	
Latent PolyA (%)[b]	20 (39.2)	34 (48.6)	17 (48.6)	18 (58.1)	0.228
SSA/Ro	4 (20.0)	20 (58.8)	9 (52.9)	N.A.	
SSB/La	2 (10.0)	4 (11.8)	7 (41.2)	N.A.	
Sm	0	N.A.	1 (5.9)	0	
RNP	2 (10.0)	N.A.	3 (17.7)	2 (11.1)	
ACA -IgG	1 (5.0)	N.A.	0	0	
ACA-IgM	3 (15.0)	N.A.	5 (29.4)	2 (11.1)	
β2GP1-IgG	0	N.A.	1 (5.9)	1 (5.6)	
β2GP1-IgM	0	N.A.	5 (29.4)	2 (11.1)	
RF	N.A.	16 (47.1)	13 (76.5)	14 (77.8)	
CCP3	N.A.	0	0	0	
dsDNA	1 (5.0)	N.A.	1 (5.9)	3 (16.7)	
TPO[c]	8 (40.0)	4 (11.8)	1 (5.9)	3 (16.7)	
Tg[c]	3 (15.0)	3 (8.8)	1 (5.9)	3 (16.7)	
ACApB	2 (10.0)	1 (2.9)	N.A.	0	
Total PolyA (%)[d]	34 (66.6)	51 (72.9)	31 (88.6)	26 (83.9)	0.149

RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; SS: Sjögren's syndrome; AITD: autoimmune thyroid disease; APS: antiphospholipid syndrome, MG: Myasthenia gravis; AIH: autoimmune hepatitis; PBC: primary biliary cholangitis; DM: dermatomyositis; AV: ANCA-associated vasculitis. Sm: anti-Smith antibodies; RNP: anti-ribonucleoprotein antibodies; ACA: anti-cardiolipin antibodies; β2GP1: anti-β2glycoprotein-1 antibodies; dsDNA: anti-double-strand DNA antibodies; RF: rheumatoid factor; N.A.: Not applicable; CCP3: third generation anti-citrullinated peptide antibodies; TPO: anti-Thyroperoxidase antibodies; Tg: anti-thyroglobulin antibodies; ACApB: anti-centromere antibody subunit B.

[a] Data correspond to number of patients (%) with over PolyA.

[b] Data correspond to the number of patients. The percentages were calculated based on the total number of patients with latent PolyA. Specific autoantibodies were omitted (i.e., CCP3 and RF in RA; dsDNA, Sm, RNP, ACA and β2GP1 in SLE; SSA/Ro and SSB/La in SS; and ACApB in SSc).

[c] Anti-thyroid antibodies in euthyroid patients.

[d] Total PolyA corresponds to the sum of overt and latent PolyA. For quantitative variables, median and interquartile rage (IQR) are presented, for categorical variables absolute and relative frequency is presented. P-values for quantitative variables are form Kruskall-Wallis test, for categorical variables Chi-square test was used.

(48.4%) were the most frequent positive cytokines. Fig. 1 shows that autoantibodies and cytokines have similar frequencies across the four ARDs, except for autoantibodies with high specificity such as CCP3 for RA, anti-SSA/Ro for SS, and ACApB for SSc, as expected and in accordance with previous reports.

3.3. Autoantibody and cytokine clusters

Table 5 shows indices for the different clustering options considered. One of the best options based on the indices was clustering using cytokines and retaining two principal components which resulted in two clusters. However, these clusters divided patients into those with high levels of almost all cytokines and those with very low levels of cytokines. When considering both cytokines and antibodies, similar results were obtained. It should be noted that using both types of data produced the highest scores for the Hopkins statistics, revealing that the data set had a poor clustering tendency. In conclusion, cytokines provided neither good nor interpretable results for clustering patients.

Regarding antibodies, the indices indicated that using all information (i.e. retaining all principal components) provides better results. Two options were found: one with 3 clusters and another with 6. The differences in the values of indices were small and the option with 3 clusters had a slightly better performance than the 6 clusters option. However, the option with 6 clusters was a more refined subdivision of the three-cluster option and both options came from the same hierarchical tree.

Finally, we retained the option of clustering with antibodies, maintaining all principal components, and generating six clusters. Although it is not the best clustering options from the perspective of the clustering indices, it is not very far from the optimal solutions these produced, and it provides interesting results and insights for patients, as described below.

PolyA-I cluster consisted of 79 patients, with ANAs, anti-SSA/Ro, and RF as the most common autoantibodies, and a high positivity for IL-12/23p40 (62.0%) (Fig. 2A) (Table 6). The four ARDs were homogeneously integrated in this cluster (Fig. 2B). This is in line with those findings in PolyA-III (n: 18) and PolyA-IV (n: 17) clusters that exhibited predominance of IL-12/23p40 positivity ($p = 0.015$) and a similar distribution of the ARDs among them. Interestingly, the PolyA-III cluster had the highest frequency of overt PolyA (61.1%, $p = 0.01$), followed by the PolyA-IV (35.3%) and PolyA-I (29.1%) clusters (Fig. 2C).

PolyA-II cluster, which corresponds mainly to the coexistence of SLE and RA (i.e., rhupus), included 44 patients of whom 59.1% had RF and 47.7% CCP3. In these patients, IFNα (41.0%) and IL-6 (41.0%) exhibited the highest frequencies of positivity, and most of the patients in

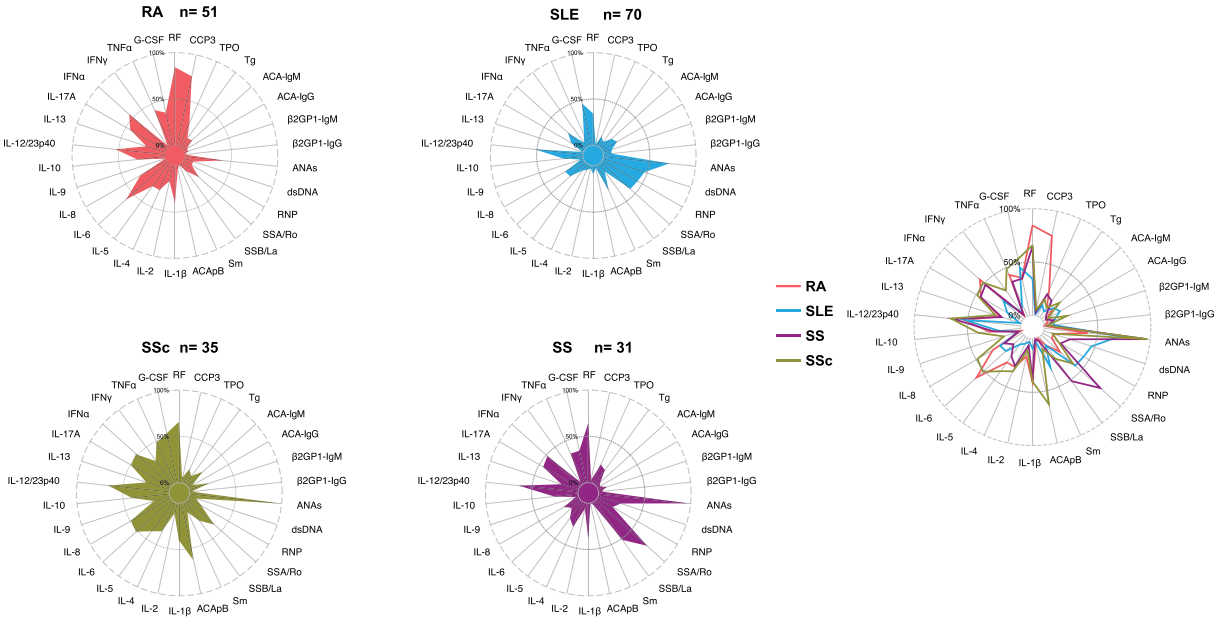

Fig. 1. Radar plots of antibody and cytokine prevalence in each disease. For a given circle each radius represents the percentage of patients with positivity for a given autoantibody or cytokine. Points at the center represent 0%, whereas points at the perimeter represent 100%.

Table 2
Autoantibodies in women with autoimmune rheumatic diseases.

Autoantibody (%)[a]	RA (n: 51)	SLE (n: 70)	SSc (n: 35)	SS (n: 31)	*P-value*
RF	43 (84.3)	24 (34.3)	23 (65.7)	20 (64.5)	< 0.0001
CCP3	39 (76.5)	1 (1.4)	2 (5.7)	1 (3.2)	< 0.0001
TPO	14 (27.5)	8 (11.4)	6 (17.1)	7 (22.6)	0.1471
Tg	6 (11.8)	5 (7.1)	4 (11.4)	6 (19.4)	0.3558
ACA-IgM	7 (13.7)	11 (15.7)	8 (22.8)	2 (6.5)	0.3119
ACA-IgG	2 (3.9)	13 (18.6)	2 (5.7)	1 (3.2)	0.015
β2GP1-IgM	2 (3.9)	7 (10.0)	8 (22.8)	3 (9.7)	0.05
β2GP1-IgG	0 (0.0)	6 (8.6)	1 (2.9)	1 (3.2)	0.1279
ANAs	21 (41.2)	50 (71.4)	34 (97.1)	30 (96.8)	< 0.0001
dsDNA	5 (9.8)	33 (47.1)	3 (8.6)	8 (25.8)	< 0.0001
RNP	5 (9.8)	31 (44.3)	6 (17.1)	6 (19.4)	< 0.0001
SSA/Ro	12 (23.5)	30 (42.9)	14 (40.0)	23 (74.2)	0.0001
SSB/La	5 (9.8)	7 (10.0)	9 (25.7)	16 (51.6)	< 0.0001
Sm	0 (0.0)	21 (30.0)	4 (11.4)	1 (3.2)	< 0.0001
ACApB	2 (3.9)	2 (2.9)	22 (62.9)	0 (0.0)	< 0.0001

RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; SS: Sjögren's syndrome; SD: standard deviation; ANAs: antinuclear antibodies; Sm: anti-Smith antibodies; RNP: anti-ribonucleoprotein antibodies; ACA; anti-cardiolipin antibodies; β2GP1: anti-β2glycoprotein-1 antibodies; dsDNA: anti-double-strand DNA antibodies; RF: rheumatoid factor; CCP3: third generation anti-citrullinated peptide antibodies; TPO: anti-thyroperoxidase antibodies; Tg: anti-thyroglobulin antibodies, ACApB: anticentromere antibody subunit B.

[a] Data correspond to number of patients (%).

this cluster fulfilled the criteria for RA (59.1%) or SLE (36.4%). This cluster exhibited the lowest frequency of overt PolyA (13.6%).

In the PolyA-V cluster (n: 21), all patients had ANAs and anti-Sm positivity. Similar to the PolyA-VI cluster, the G-CSF (52.6%) was the predominant cytokine. This cluster was composed mainly of patients with SLE (85.7%) and 23.8% showed overt PolyA.

In the PolyA-VI cluster (n: 8), characterized by the coexistence of SLE and APS, all patients showed anti-β2GP1 positivity and more than half of the patients showed reactivity to ANAs, anti-dsDNA, ACA-IgG, and RF antibodies. Furthermore, the G-CSF (62.5%) was the most common positive cytokine and 75.0% of the patients within this cluster had SLE. These patients had overt PolyA in 25.0% of the cases.

Last, a lack of association between clusters and cumulative overt clinical characteristics was observed (Table 7).

4. Discussion

In this exploratory study, 6 clusters were obtained from patients with the four most common ARDs. Three of them (PolyA-I, -III and -IV clusters) were associated with IL-12/23p40.

As previously revised [3], PolyA "represents the effect of a single genotype and similar environmental factors on diverse phenotypes, and is associated with female gender, familial autoimmunity, Amerindian ancestry and cigarette smoking".

In addition, our study highlights the importance of latent PolyA. In fact, clusters were built mainly on autoantibodies, which are recognized as helpful biomarkers not only for the diagnosis and the classification of ADs, but also for sub-grouping patients and for monitoring specific tissue/organ damage. Combinations of specific antibodies are also predictive for the eventual evolution of undifferentiated clinical variants at the beginning of their presentation [32]. Thus, it is likely that those patients with latent PolyA will develop overt PolyA in the future. The transit from latent to overt PolyA will depend upon several factors including the pathogenicity of autoantibodies (i.e., affinity, isotype switching, glycosylation, rise in the levels, epitope spreading) [33,34], the milieu (e.g., tertiary lymphoid structures) [35,36], environmental factors and both epigenetic and genetic characteristics of the patient [3,36,37].

The presence of either IgG or IgA anti-CCP antibodies preceded the appearance of RA by up to 14 years [38,39], and the frequency of RF also increased significantly over time [40]. Arbuckle et al. [41] found that ANAs, anti-SSB/La, anti-SSA/Ro, and antiphospholipid antibodies predated clinical symptoms of SLE by 3.4 years, followed by the appearance of anti-dsDNA antibodies (2.2 years), while RNP and Sm autoantibodies were present about 1.2 years before diagnosis. It was reported that any of the following: ANAs, anti-SSA/Ro, and anti-dsDNA antibodies were sensitive for prediction of SLE [42]. Furthermore, the levels of TPO and Tg autoantibodies rose gradually over time prior the clinical diagnosis of either Grave's disease (GD) or Hashimoto thyroiditis (HT) [43]. In fact, GD occurs fairly fast (i.e., 1 year) in euthyroid individuals after the appearance of autoantibodies, whereas in HT, this process may take some years [44].

Kallenberg et al. [45], after a 6-year follow-up study, found that

Table 3
Cytokine levels in women with autoimmune rheumatic diseases.

Cytokine[a]	RA[b] (n: 47)	SLE[c] (n: 67)	SSc (n: 35)	SS (n: 31)	*P-value*	Reference value[d]
IL-1β	6.43 (16.30)	0.97 (4.66)	5.80 (11.90)	5.80 (13.56)	0.0002	0 (0)
IL-2	5.50 (21.43)	0.39 (2.23)	1.18 (3.57)	1.40 (5.80)	0.06	0 (0)
IL-4	2.30 (8.03)	0.39 (2.01)	2.50 (5.20)	4.60 (9.00)	0.0012	0 (0)
IL-5	1.00 (2.70)	0.17 (0.77)	1.00 (2.22)	0.45 (1.10)	0.0026	0 (0)
IL-6	6.20 (8.95)	5.00 (28.1)	4.84 (7.10)	0.90 (3.40)	0.0001	0.11 (0.21)
IL-8	10.80 (8.40)	12.67 (25.13)	13.22 (7.60)	9.50 (12.50)	0.0041	11.71 (4.50)
IL-9	0.0 (0.0)	0.13 (0.75)	1.02 (3.12)	0.09 (0.50)	0.0027	0 (0)
IL-10	2.20 (5.61)	0.60 (1.79)	2.40 (4.60)	0.98 (1.98)	0.12	0 (0)
IL-12/23p40	37.40 (76.40)	27.10 (48.90)	46.00 (76.50)	52.50 (78.32)	0.24	16.13 (18.90)
IL-13	1.80 (5.20)	0.02 (0.20)	0.84 (3.0)	0.36 (0.94)	0.0019	0 (0)
IL-17A	31.30 (71.10)	7.40 (33.90)	34.70 (72.30)	36.90 (69.80)	0.0006	0 (0)
IFNα	13.20 (26.90)	3.72 (12.20)	14.80 (26.50)	17.40 (28.10)	0.0026	0 (0)
IFNγ	0.20 (1.38)	0.39 (2.10)	0.66 (1.40)	0.17 (0.95)	< 0.0001	0 (0)
TNFα	10.39 (21.71)	2.11 (9.34)	10.05 (20.60)	7.92 (18.73)	0.0005	0 (0)
G-CSF	3.37 (7.90)	2.20 (6.20)	6.72 (12.30)	3.80 (8.10)	0.32	0 (0)

RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; SS: Sjögren's syndrome; IL: interleukin; G-CSF: granulocyte colony-stimulating factor; IFN: interferon; TNF: tumor necrosis factor.

[a] Mean (standard deviation) in pg/mL.

[b] Results are based on 47 patients due to insufficient serum to test cytokines.

[c] Results are based on 67 patients due to insufficient serum to test cytokines.

[d] From Pacheco et al. [27].

Table 4
Cytokine positivity in women with autoimmune rheumatic diseases.

Cytokine[a]	RA[b] (n: 47)	SLE[c] (n: 67)	SSc (n: 35)	SS (n: 31)	*P-value*
IL-1β	19 (40.4)	6 (9.0)	14 (40.0)	12 (38.7)	0.001
IL-2	8 (17.0)	2 (3.0)	4 (11.4)	2 (6.5)	0.06
IL-4	14 (29.8)	4 (6.0)	12 (34.3)	9 (29.0)	0.0012
IL-5	14 (29.8)	6 (9.0)	14 (40.0)	7 (22.6)	0.0023
IL-6	28 (59.6)	15 (22.4)	18 (51.4)	4 (12.9)	< 0.001
IL-8	15 (31.9)	16 (23.9)	17 (48.6)	6 (19.4)	0.0034
IL-9	0 (0.0)	2 (3.0)	6 (17.1)	1 (3.2)	0.0028
IL-10	14 (29.8)	14 (20.9)	14 (40.0)	8 (25.8)	0.23
IL-12/23p40	25 (53.2)	35 (52.2)	23 (65.7)	20 (64.5)	0.4
IL-13	10 (21.3)	1 (1.5)	9 (25.7)	6 (19.4)	0.0018
IL-17A	21 (44.7)	11 (16.4)	17 (48.6)	14 (45.2)	< 0.001
IFNα	26 (55.3)	17 (25.4)	18 (51.4)	15 (48.4)	0.005
IFNγ	1 (2.1)	4 (6.0)	11 (31.4)	1 (3.2)	< 0.001
TNFα	20 (42.6)	9 (13.4)	17 (48.6)	11 (35.5)	< 0.001
G-CSF	17 (36.2)	31 (46.3)	19 (54.3)	11 (35.5)	0.3

RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; SS: Sjögren's syndrome; IL: interleukin; G-CSF: granulocyte colony-stimulating factor; IFN: interferon; TNF: tumor necrosis factor.

[a] Data correspond to those patients with positive values as compared to healthy controls (above the threshold) [21,26,27].

[b] Relative frequencies were calculated with 47 patients since 4 patients had insufficient serum to test cytokines.

[c] Relative frequencies were calculated with 67 patients since 3 patients had insufficient serum to test cytokines.

Table 5
Clustering options considered for taxonomy in autoimmune rheumatic diseases.

Data[a]	Number of principal components[b]	Number of clusters[c]	Dunn index	ASW	Hopkins statistic
Antibodies	All	6	0.236	0.19	0.2365086
Antibodies	All	3	0.255	0.21	0.2365086
Antibodies	4	2	0.213	0.19	0.3564948
Antibodies	4	5	0.227	0.15	0.3564948
Cytokines	All	2	0.274	0.42	0.283621
Cytokines	All	3	0.235	0.35	0.283621
Cytokines	3	2	0.274	0.42	0.2349624
Cytokines	3	3	0.26	0.36	0.2349624
Both	All	2	0.323	0.23	0.3335995
Both	All	3	0.245	0.14	0.3335995
Both	2	2	0.323	0.23	0.4060049
Both	2	3	0.245	0.12	0.4060049

ASW: Average silhouette width.

[a] Data used to build clusters.

[b] Number of principal components used in cluster analysis.

[c] Number of cluster obtained after analysis.

anti-ACApB antibodies were useful biomarkers for prediction of CREST (calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly and telangiectasia) syndrome, and anti-topoisomerase I antibodies (ATAs) exhibited a similar performance in predicting disease. Similar findings demonstrated that patients who experienced the simultaneous appearance of Raynaud's phenomenon with anti-ACApB, ATAs, or anti-RNA polymerase III antibodies were prone to develop overt SSc [46]. On the other hand, in the case of SS, up to 66% patients shows autoantibody positivity for ANAs, RF, anti-SSA/Ro, and anti-SSB/La antibodies before the onset of disease [47].

All above-mentioned data emphasize the role of autoantibodies in prediction of ARDs. However, as shown before, several autoantibodies share different specificities across ARDs. For example, anti-SSA/Ro and anti-SSB/La are considered the two most typical antibodies in SS [48] and nearly 63% of patients show positivity to anti-SSA/Ro [49]. Nevertheless, this autoantibody also predicts the development of SLE [42]. Anti-SSA/Ro in the presence of anti-SSB/La tends to identify patients with SS. It was found that 29 of 35 patients with both anti-Ro/SSA and anti-La/SSB antibodies had SS, whereas of 53 with only anti-Ro/SSA, 23 had SS, 25 had SLE, and 13 had another disease [50]. This suggests that the combination of some autoantibodies in the diagnostic approach of ARDs may improve the sensitivity and specificity of these tests. In this scenario, the cluster analysis allowed to group patients with similar serological characteristics based on autoantibodies. In this line, cluster analysis based on autoantibodies was clinical useful to define 4 major subgroups of patients with idiopathic inflammatory myopathies [51].

High levels of IL-12/23p40 was common in the clusters I, III and IV. These cytokines belong to the group of the IL-12 family that includes IL-12, IL-23, IL-27, and IL-35. Although they share structural features, these cytokines mediate diverse functional effects [52]. In the case of IL-12 and IL-23, they are considered pro-inflammatory and pro-stimulatory cytokines with key roles in the development of Th1 and Th17 subsets of T helper cells [53,54], whereas IL-27 and IL-35 exhibit regulatory functions [52,55]. The p40 chain of these cytokines can pair with p35 or p19 to form IL-12 or IL-23 respectively [56]. The IL-12 family is thought to have a greater effect on shaping immune responses

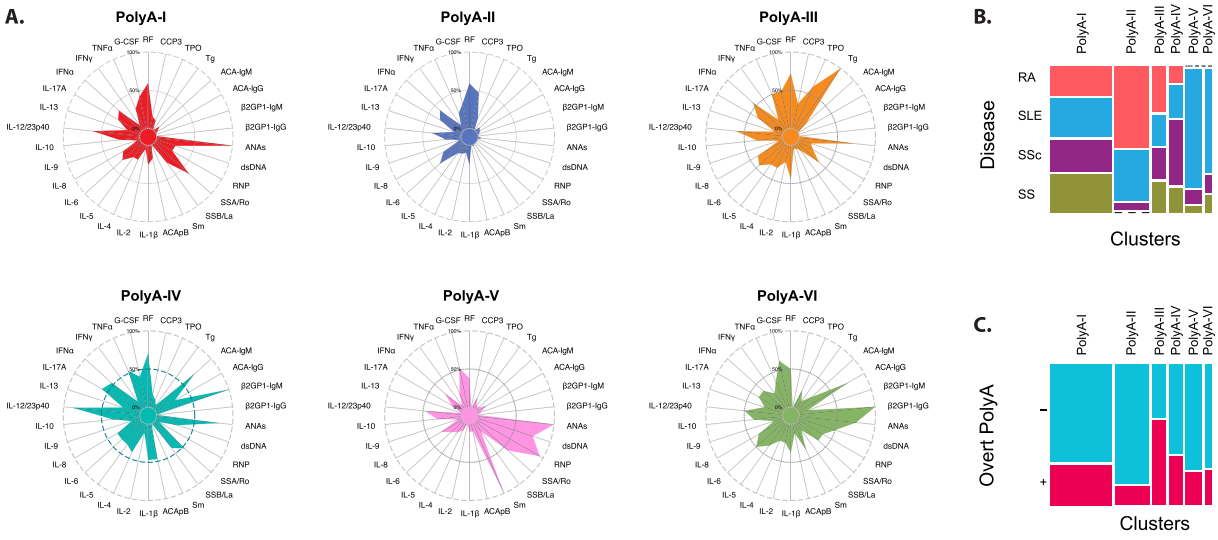

Fig. 2. A) Radar plots of antibody and cytokine seropositivity in each of the six clusters obtained. PolyA –I, -III and -IV clusters exhibited high positivity of IL-12/23p40 (p = 0.015). B) Mosaic plot showing the distribution of ARDs in clusters. The area of the tiles (i.e., the bin size), corresponds to the number of observations within each category (e.g., there were 79 (42%) patients in PolyA-I cluster - see Table 6-), as follows, PolyA-I: RA (21.5%), SLE (27.8%), SSc (22.8%) and SS (27.8%); PolyA-II: RA (59.0%), SLE (36.4%) and SSc (4.5%); PolyA-III: RA (33.3%), SLE (22.2%), SSc (22.2%) and SS (22.2%); PolyA-IV: RA (11.8%), SLE (23.5%), SSc (47.1%) and SS (17.6%); PolyA-V: SLE (85.7%), SSc (9.5%) and SS (4.8%); PolyA-VI: SLE (75.0%), SSc (12.5%) and SS (12.5%); C) Mosaic plot showing the distribution of overt PolyA among the clusters. Patients within PolyA-III cluster exhibited the highest frequency of overt PolyA (p = 0.01). ARDs: autoimmune rheumatic diseases, PolyA: polyautoimmunity, RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; SS: Sjögren's syndrome.

Table 6
Distributions of cytokines and autoantibodies among clusters.

Variable[a]	PolyA-I (n: 79)	PolyA-II (n: 44; n:39[b])	PolyA-III (n: 18)	PolyA-IV (n: 17)	PolyA-V (n: 21; n: 19[c])	PolyA-VI (n: 8)	*P-value*
RF	47 (59.5)	26 (59.1)	13 (72.2)	12 (70.6)	8 (38.1)	4 (50.0)	0.2777
CCP3	13 (16.5)	21 (47.7)	6 (33.3)	3 (17.6)	0 (0.0)	0 (0.0)	< 0.001
TPO	10 (12.7)	7 (15.9)	11 (61.1)	2 (11.8)	3 (14.3)	2 (25.0)	< 0.0002
Tg	0 (0.0)	(0.0)	18 (100)	1 (5.9)	1 (4.8)	1 (12.5)	< 0.0001
ACA-IgM	7 (8.9)	3 (6.8)	3 (16.7)	12 (70.6)	1 (4.8)	2 (25.0)	< 0.0001
ACA-IgG	2 (2.5)	2 (4.5)	0 (0.0)	5 (29.4)	3 (14.3)	6 (75.0)	< 0.0001
β2GP1-IgM	0 (0.0)	1 (2.3)	0 (0.0)	17 (100)	1 (4.8)	1 (12.5)	< 0.0001
β2GP1-IgG	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	8 (100)	< 0.0001
ANAs	79 (100)	2 (4.5)	13 (72.2)	14 (82.4)	21 (100)	6 (75.0)	< 0.0001
dsDNA	21 (26.6)	0 (0.0)	3 (16.7)	5 (29.4)	16 (76.2)	4 (50.0)	< 0.001
RNP	20 (25.3)	0 (0.0)	4 (22.2)	1 (5.9)	20 (95.2)	3 (37.5)	< 0.0001
SSA/Ro	48 (60.8)	0 (0.0)	7 (38.9)	9 (52.9)	12 (57.1)	3 (37.5)	< 0.0001
SSB/La	24 (30.4)	0 (0.0)	2 (11.1)	7 (41.2)	3 (14.3)	1 (12.5)	0.0004
Sm	0 (0.0)	1 (2.3)	1 (5.6)	0 (0.0)	21 (100)	3 (37.5)	< 0.0001
ACApB	14 (17.7)	0 (0.0)	1 (5.6)	8 (47.1)	2 (9.5)	1 (12.5)	0.0001
IL-1β	20 (25.3)	10 (25.6)	8 (44.4)	8 (47.1)	2 (10.5)	3 (37.5)	0.1080
IL-2	4 (5.1)	4 (10.3)	4 (22.2)	2 (11.8)	0 (0.0)	2 (25.0)	0.0735
IL-4	17 (21.5)	5 (12.8)	6 (33.3)	7 (41.2)	2 (10.5)	2 (25.0)	0.1309
IL-5	17 (21.5)	6 (15.4)	6 (33.3)	6 (35.3)	3 (15.8)	3 (37.5)	0.3634
IL-6	27 (34.2)	16 (41.0)	8 (44.4)	6 (35.3)	4 (21.1)	4 (50.0)	0.6054
IL-8	21 (26.6)	11 (28.2)	7 (38.9)	6 (35.3)	6 (31.6)	3 (37.5)	0.8963
IL-9	3 (3.8)	1 (2.6)	1 (5.6)	2 (11.8)	1 (5.3)	1 (12.5)	0.6497
IL-10	21 (26.6)	8 (20.5)	6 (33.3)	6 (35.3)	6 (31.6)	3 (37.5)	0.7986
IL-12/23p40	49 (62.0)	15 (38.5)	11 (61.1)	15 (88.2)	9 (47.4)	4 (50.0)	0.0152
IL-13	14 (17.7)	4 (10.3)	2 (11.1)	4 (23.5)	0 (0.0)	2 (25.0)	0.2634
IL-17A	27 (34.2)	13 (33.3)	7 (38.9)	10 (58.8)	3 (15.8)	3 (37.5)	0.1837
IFNα	32 (40.5)	16 (41.0)	10 (55.6)	9 (52.9)	6 (31.6)	3 (37.5)	0.6713
IFNγ	6 (7.6)	3 (7.7)	3 (16.7)	2 (11.8)	1 (5.3)	2 (25.0)	0.5053
TNFα	23 (29.1)	9 (23.1)	9 (50.0)	9 (52.9)	4 (21.1)	3 (37.5)	0.1077
G-CSF	34 (43.0)	13 (33.3)	9 (50.0)	7 (41.2)	10 (52.6)	5 (62.5)	0.5764

RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; SS: Sjögren's syndrome; ANAs: antinuclear antibodies; Sm: anti-Smith antibodies; RNP: anti-Ribonucleoprotein antibodies; ACA: anti-cardiolipin antibodies; β2GP1: anti-β2glycoprotein-1 antibodies; dsDNA: anti-double-strand DNA antibodies; RF: rheumatoid factor; CCP3: third generation anti-citrullinated peptide antibodies; TPO: anti-thyroperoxidase antibodies; Tg: anti-thyroglobulin antibodies, ACApB: anti-centromere antibody subunit B. IL: interleukin; G-CSF: granulocyte colony-stimulating factor; IFN: interferon; TNF: tumor necrosis factor.

[a] Data correspond to number of patients (%).

[b] For this cluster, there were 5 patients without serum measurement of cytokines.

[c] For this cluster, there were 2 patients without serum measurement of cytokines.

Table 7
Clinical manifestations across PolyA clusters.

ARD	Clinical manifestation[a]	PolyA-I (n: 79)	PolyA-II[b] (n: 44)	PolyA-III (n: 18)	PolyA-IV (n: 17)	PolyA-V[b] (n: 21)	PolyA-VI[b] (n: 8)	Total	*P-value*
RA (n: 51)		17	26	6	2	0	0		
	Digital vasculitis	0 (0.0)	1 (3.8)	0 (0.0)	0 (0.0)	–	–	1 (2.0)	0.8059
	Skin ulcers	1 (5.9)	1 (3.8)	0 (0.0)	0 (0.0)	–	–	2 (3.9)	0.9188
	Skin nodulosis	6 (35.3)	3 (11.5)	2 (33.33)	1 (50.0)	–	–	12 (23.5)	0.2137
	Pulmonary nodulosis	0 (0.0)	1 (3.8)	1 (16.7)	0 (0.0)	–	–	2 (3.9)	0.3390
	Peripheral nerve involvement	2 (11.8)	3 (11.5)	1 (16.7)	0 (0.0)	–	–	6 (11.8)	0.9388
	Pleural effusion	0 (0.0)	3 (11.5)	0 (0.0)	0 (0.0)	–	–	3 (5.9)	0.3817
	PAH	0 (0.0)	2/23 (8.7)	0/5 (0.0)	0 (0.0)	–	–	2/47 (4.3)	0.5360
	Pulmonary embolism	0 (0.0)	1 (3.9)	0 (0.0)	0 (0.0)	–	–	1 (2.0)	0.8059
SLE (n: 70)		22	16	4	4	18	6		
	Arthritis	15 (68.2)	16 (100)	3 (75.0)	3 (75.0)	16 (88.9)	4 (66.7)	57 (81.4)	0.1591
	Myalgia	10 (45.5)	12 (75.0)	2 (50.0)	1 (25.0)	11 (61.1)	5 (83.3)	41 (58.6)	0.2295
	Malar rash	12 (54.5)	11 (68.8)	0 (0.0)	2 (50.0)	15 (83.3)	5 (83.3)	45 (64.3)	0.0297
	Photosensibility	15 (68.2)	14 (87.5)	2 (50.0)	3 (75.0)	14 (77.8)	4 (66.7)	52 (74.3)	0.6348
	Alopecia	14 (63.6)	13 (81.3)	1 (25.0)	3 (75.0)	13 (72.2)	4 (66.7)	48 (68.6)	0.3962
	Oral ulcers	11 (50.0)	11 (68.8)	3 (75.0)	3 (75.0)	14 (77.8)	3 (50.0)	45 (64.3)	0.4859
	Discoid lupus	5 (22.7)	3 (18.8)	0 (0.0)	0 (0.0)	5 (27.8)	0 (0.0)	13 (18.6)	0.4861
	Raynaud's phenomenon	10 (45.5)	12 (75.0)	2 (50.0)	3 (75.0)	15 (83.3)	4 (66.7)	46 (65.7)	0.1739
	Vasculitis	2 (9.1)	5 (31.3)	0 (0.0)	1 (25.0)	5 (27.8)	2 (33.3)	15 (21.4)	0.4204
	Seizures	2 (9.1)	3 (18.8)	0 (0.0)	0 (0.0)	2 (11.1)	2 (33.3)	9 (12.9)	0.5141
	Psychosis	0 (0.0)	2 (12.5)	1 (25.0)	0 (0.0)	3 (16.7)	0 (0.0)	6 (8.6)	0.2874
	Serositis	7 (31.8)	3 (18.8)	2 (50.0)	2 (50.0)	4 (22.2)	3 (50.0)	21 (30.0)	0.5232
	Renal compromise	12 (54.6)	10 (62.5)	2 (50.0)	3 (75.0)	9 (50.0)	4 (66.7)	40 (57.1)	0.9188
	Haematological compromise	16 (72.7)	9 (56.3)	3 (75.0)	3 (75.0)	15 (83.3)	5 (83.3)	51 (72.9)	0.6110
SSc (n: 35)		18	2	4	8	2	1		
	Skin Thickening MCP joints[c]	6 (33.3)	1 (50.0)	3 (75.0)	3 (37.5)	0 (0.0)	0 (0.0)	13 (37.1)	0.4823
	Skin thickening[d]	14 (77.8)	1 (50.0)	2 (50.0)	7 (87.5)	2 (100)	0 (0.0)	26 (74.3)	0.2799
	Puffy fingers	4 (22.2)	0 (0.0)	0 (0.0)	3 (37.5)	0 (0.0)	0 (0.0)	7 (20.0)	0.5731
	Sclerodactyly	11 (61.1)	1 (50.0)	4 (100)	4 (50.0)	2 (100)	0 (0.0)	22 (62.9)	0.3092
	Digital tip ulcers	1 (5.6)	0 (0.0)	1 (25.0)	4 (50.0)	0 (0.0)	0 (0.0)	6 (17.1)	0.1095
	Pitting scars	2 (11.1)	0 (0.0)	0 (0.0)	2 (25.0)	0 (0.0)	1 (100)	5 (14.3)	0.1439
	Telangiectasia	16 (88.9)	1 (50.0)	3 (75.0)	6 (75.0)	2 (100)	1 (100)	29 (82.9)	0.6810
	Abnormal capillaroscopy	4 (22.2)	1 (50.0)	1 (25.0)	1 (12.5)	0 (0.0)	1 (100)	8 (22.9)	0.3799
	PAH	5 (27.8)	1 (50.0)	1 (25.0)	4 (50.0)	0 (0.0)	0 (0.0)	11 (31.4)	0.6748
	Interstitial lung disease	1 (5.6)	1 (50.0)	1 (25.0)	0 (0.0)	1 (50.0)	0 (0.0)	4 (11.4)	0.1364
	Raynaud's phenomenon	17 (94.4)	2 (100)	4 (100)	8 (100)	2 (100)	1 (100)	34 (97.1)	0.9648
SS (n: 31)		22	0	4	3	1	1		
	Arthralgia	19 (86.4)	–	3/3 (100)	3 (100)	1 (100)	1 (100)	27/30 (90.0)	0.8761
	Xerophthalmia	22 (100)	–	4 (100)	3 (100)	1 (100)	1 (100)	31 (100)	–
	Xerostomia	22 (100)	–	4 (100)	3 (100)	1 (100)	1 (100)	31 (100)	–
	Arthritis	10 (45.5)	–	2/3 (66.7)	2 (66.7)	0 (0.0)	0 (0.0)	14/30 (46.7)	0.6045
	Lymphadenopathy	3 (13.6)	–	1/3 (33.3)	1 (33.3)	0 (0.0)	0 (0.0)	5/30 (16.7)	0.7824
	Parotid enlargement	9 (40.9)	–	0/3 (0.0)	1 (33.3)	0 (0.0)	0 (0.0)	10/30 (33.3)	0.5465
	Urticaria	2 (9.1)	–	1/3 (33.3)	2 (66.7)	0 (0.0)	0 (0.0)	5/30 (16.7)	0.1204
	Photosensibility	7 (31.8)	–	2/3 (66.7)	3 (100)	1 (100)	0 (0.0)	13/30 (43,3)	0.0973
	Raynaud's phenomenon	6 (27.3)	–	1/3 (33.3)	2 (66.7)	0 (0.0)	0 (0.0)	9/30 (30,0)	0.5795
	Vasculitis	2 (9.1)	–	0/3 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2/30 (6.7)	0.9412
	Xerotrachea	8 (36.4)	–	1/3 (33.3)	2 (66.7)	0 (0.0)	0 (0.0)	11/30 (36,7)	0.6743
	Dysphagia	7 (31.8)	–	2/3 (66.7)	2 (66.7)	0 (0.0)	0 (0.0)	11/30 (36,7)	0.4473

RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; SS: Sjögren's syndrome; PAH: Pulmonary arterial hypertension. P-values were obtained by X^2 independence test. Denominator is shown when there were missing data.

[a] Data correspond to the number of ever clinical manifestation (%) in each cluster.

[b] Cluster PolyA-II did not include patients with SS, and clusters PolyA-V and PolyA-VI did not include patients with RA.

[c] Skin sclerosis proximal to metacarpophalangeal joints (MCP).

[d] Skin sclerosis distal to MCP joints. ARD: Autoimmune rheumatic disease.

than any other cytokine family [52]. The role of the IL-12/23p40 in diverse ADs has been previously described [57–62]. Herein, its role in the development of PolyA is suggested. In fact, our results showed that those clusters with the highest frequency of overt PolyA had the highest positivity frequency of IL-12/23p40 (Fig. 2A and C).

The shortcomings of our study must be acknowledged. The main objective of this exploratory study was to develop a novel grouping of ARDs. Clusters were built mainly based on autoantibody signatures, which presence (not the titles) is thought to be constant along the course of the disease [63,64]. In addition, a temporal relationship between them and clinical manifestations was unexpected, explaining the lack of association between clusters and cumulative clinical characteristics (Table 7), which indicates similar clinical manifestations of clusters. Due to the cross-sectional nature of clinical data, only representative subphenotypes of each ARD were registered. Future studies should consider an expanded list of clinical variables. Likewise, the effects of treatment on the modulation of cytokine/autoantibody levels were not taken into account. Our results cannot provide a predictive approach since our study design was exploratory and cross-sectional. In fact, the cluster approach provided a PolyA snapshot of our cohort at one point in time. Another potential limitation of the present study is that the observed results may be due to the moderate sample size. However, such a possibility would be unlikely given the significant results seen as well as their consistent direction. Ascertainment bias is also recognized since we evaluated a clinic and not a community sampling. In addition, this study was performed in prevalent and not in incident cases. Herein we applied conventional methods, but new platform technologies for comprehensive autoantibody detection and

molecular analyses are becoming available allowing routine integration into clinical practice with improved diagnostic and therapeutic outcomes [65].

5. Conclusions

A grouping of ARDs is presented. The results provide new insights into the pathophysiology of PolyA and deserve to be validated prospectively and in diverse populations, enabling the development of a more accurate taxonomy of ARDs. Finally, our finding on the association of IL-12/23p40 with PolyA deserves further attention.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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