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New insights into the taxonomy of autoimmune diseases based on polyautoimmunity

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ABSTRACT

Objective: The clinical coexistence of two or more autoimmune diseases (ADs) fulfilling classification criteria is termed *"overt polyautoimmunity" (PolyA),* whereas the presence of autoantibodies unrelated to an index AD, without clinical criteria fulfillment, is known as *"latent PolyA"*. We aimed to explore a new taxonomy of ADs based on PolyA.

Methods: In a cross-sectional study of 292 subjects, we evaluated the presence of PolyA in 146, 45, 29, 17, and 17 patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), autoimmune thyroid disease (AITD) and systemic sclerosis (SSc), respectively, and 38 healthy controls. Clinical assessment, autoantibody profile (by autoantigen array chip), lymphocytes immunophenotype and cytokine profile (by flow cytometry) were evaluated simultaneously. A mixed cluster methodology was used to classify ADs.

Results: Latent PolyA was more frequent than overt PolyA, ranging from 69.9% in RA to 100% in SSc. Nevertheless, both latent and overt PolyA clustered together. Over-expressed IgG autoantibodies were found to be hallmarks for the identification of index ADs. The combination of autoantibodies allowed high accuracy in the classification of ADs. Three well-defined clusters based on PolyA were observed with distinctive clinical and immunological phenotypes.

Conclusions: This proof-of-concept study indicates that ADs can be classified according to PolyA. PolyA should be considered in all studies dealing with ADs, including epidemiological, genetic, and clinical trials.

1. Introduction

Autoimmune diseases (ADs) may be presented as a single entity or as a combination of them. The latter condition is known as polyautoimmunity (PolyA) [1]. The clinical coexistence of two or more ADs fulfilling classification criteria is termed *"Overt PolyA"*, whereas the presence of autoantibodies unrelated to an index AD, without clinical criteria fulfillment, is named *"Latent PolyA"* [2]. In addition, both conditions can coexist in a single patient [3]. Although the clinical and immunological relevance of PolyA have not been fully studied, recognition of differential immunological patterns on autoimmunity may allow the implementation of personalized strategies in the management of ADs [4].

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Abbreviations

ACAs	Anti-cardiolipin antibodies
ACR	American college of rheumatology
ADs	Autoimmune diseases
AH	Autoimmune hypothyroidism
AITD	Autoimmune thyroid disease
AUC	Area under the curve
CBA	Cytometric bead array
CCP3	Anti-cyclic citrullinated peptide third-generation
CRP	C reactive protein
DAS-28	Disease activity score 28
DMARDs	Disease-modifying antirheumatic drugs
dsDNA	Anti-double-stranded DNA
ESR	Erythrocyte sedimentation rate
ESSPRI	EULAR SS patient reported index
EULAR	European league against rheumatism
FDR	False discovery rate
IQR	Interquartile range
NFI	Normalized fluorescence intensity
NPV	Negative predictive value
PBST	Phosphate buffered saline with Tween
PolyA	Polyautoimmunity
PPV	Positive predictive value
RA	Rheumatoid arthritis
RF	Rheumatoid factor
SLAQ	Systemic lupus activity questionnaire
SLE	Systemic lupus erythematosus
SNR	Signal-to-noise ratio
SS	Sjögren's syndrome
SSc	Systemic sclerosis
SSPRO	Scleroderma skin patient-reported outcome
Tg	Anti-thyroglobulin antibodies
TPO	Anti-thyroperoxidase antibodies
β2GPI	Anti-β2glycoprotein antibodies

Recent studies in systemic lupus erythematosus (SLE) showed that autoantibody-based classification allowed the identification of subgroups associated with disease activity, and inflammatory cytokines [5]. In addition, an association between the levels of IL-12/23p40 and overt PolyA has been shown [3], suggesting a differential immunological pattern that can be used as a therapeutic target in patients with PolyA.

It is well recognized that the development of autoantibodies precedes clinical manifestations of ADs [6], and combinations of autoantibodies are predictive for disease evolution [7]. Thus, it is likely that patients with latent PolyA may develop overt PolyA in the future [2,3]. In this line, studies on rheumatoid arthritis (RA) showed that IgG or IgA citrullinated peptide antibodies and IgA rheumatoid factor precede the appearance of RA by several years [8–10]. A similar scenario occurs in SLE [11], autoimmune thyroid disease (AITD) [12–14], systemic sclerosis (SSc) [15,16], and Sjögren's syndrome (SS) [17].

Family-based studies showed that first-degree relatives of patients with SLE were more likely to present latent PolyA for RA, AITD, type 1 diabetes mellitus, and antiphospholipid syndrome (APS) [18]. Factors such as age and gender were associated with the development of this type of PolyA [18]. As previously revised [19], PolyA *"may represent the effect of a single genotype and similar environmental factors on diverse phenotypes, and it is associated with female gender, familial autoimmunity (i.e., coaggregation), Amerindian ancestry, and cigarette smoking"*. All the above-mentioned data indicate that PolyA is a prominent condition in which multiple etiological factors converge. Herein, a proof-of-concept study is reported in which a new classification of ADs was evaluated based on PolyA.

2. Patients and methods

2.1. Study design

A cross-sectional study was conducted from December 1st, 2018, to November 30th, 2019, in three rheumatology outpatient clinics: the Center for Autoimmune Diseases Research (CREA) at the Clínica del Occidente, and the Dermatology and Rheumatology Foundation (FUNINDERMA), in Bogota, and the "Centro de Referencia en Osteoporosis, Reumatología & Dermatología", in Cali, Colombia.

Two hundred and eighty-one consecutive patients with the following index conditions were initially included: RA, SLE, SS, AITD, and SSc. The patients fulfilled either the 1987 American College of Rheumatology (ACR) classification criteria for RA [20], the 1997 ACR criteria for SLE [21], the 2013 ACR/European League Against Rheumatism (EULAR) classification criteria for SSc [22], or the revised American-European Consensus Group for SS [23]. For AITD, patients with autoimmune hypothyroidism (AH) were classified as follows: 1) confirmed AH (i.e., thyroid dysfunction, thyroid-stimulating hormone (TSH) $>$ 4.1 μIU/mL or levothyroxine treatment, and the presence of anti-thyroperoxidase (TPO) or anti-thyroglobulin (Tg) antibodies), 2) euthyroid patients with positive anti-TPO or anti-Tg antibodies, 3) non-autoimmune hypothyroidism (thyroid dysfunction and absence of anti-TPO or anti-Tg antibodies) [24].

Foreign patients (n: 2), patients with prior history of neoplasia (n: 4), and unfulfillment of classification criteria (n: 21) were excluded. A final sample size of 254 patients was included in the analyses as follows: RA (n: 146), SLE (n: 45), SS (n: 29), AITD (n: 17) and SSc (n: 17). In addition, a group of 38 healthy volunteers (i.e., subjects without overt autoimmunity nor familial autoimmunity) were included as a control group (Fig. 1A).

This study was done in compliance with act 008430/1993 of the Ministry of Health of the Republic of Colombia, which classified it as minimal-risk research. All the patients were asked for their consent and were informed about the Colombian data protection law (1581 of 2012). The institutional review board of the Universidad del Rosario approved the study design.

2.2. Patient monitoring and clinical evaluation

The patients' demographic and cumulative clinical data were simultaneously obtained by a previously standardized report form, physical examination, and chart review by trained physicians. Data included age, age at onset of disease, familial autoimmunity (i.e., presence of different autoimmune diseases in a nuclear family, coaggregation), and familial autoimmune disease (i.e., presence of one specific autoimmune disease in various members of a nuclear family - aggregation) [25], comorbidities (reported in clinical history or by the patients), and treatment on inclusion. All patients were evaluated for rheumatological or associated autoimmune clinical manifestations (Table 1).

In the presence of autoantibodies not related to the index condition, patients were assessed to confirm overt PolyA. In case of SS or AITD suspicion a Schirmer, and unstimulated saliva flow rate test were performed, and TSH measurement done, respectively. Patients with positivity for anti-phospholipid antibodies were tested within 12 weeks to confirm the classification criteria for APS.

The severity of symptoms was assessed by either disease activity score 28 (DAS-28) for RA [26], systemic lupus activity questionnaire (SLAQ) for SLE [27], scleroderma skin patient-reported outcome

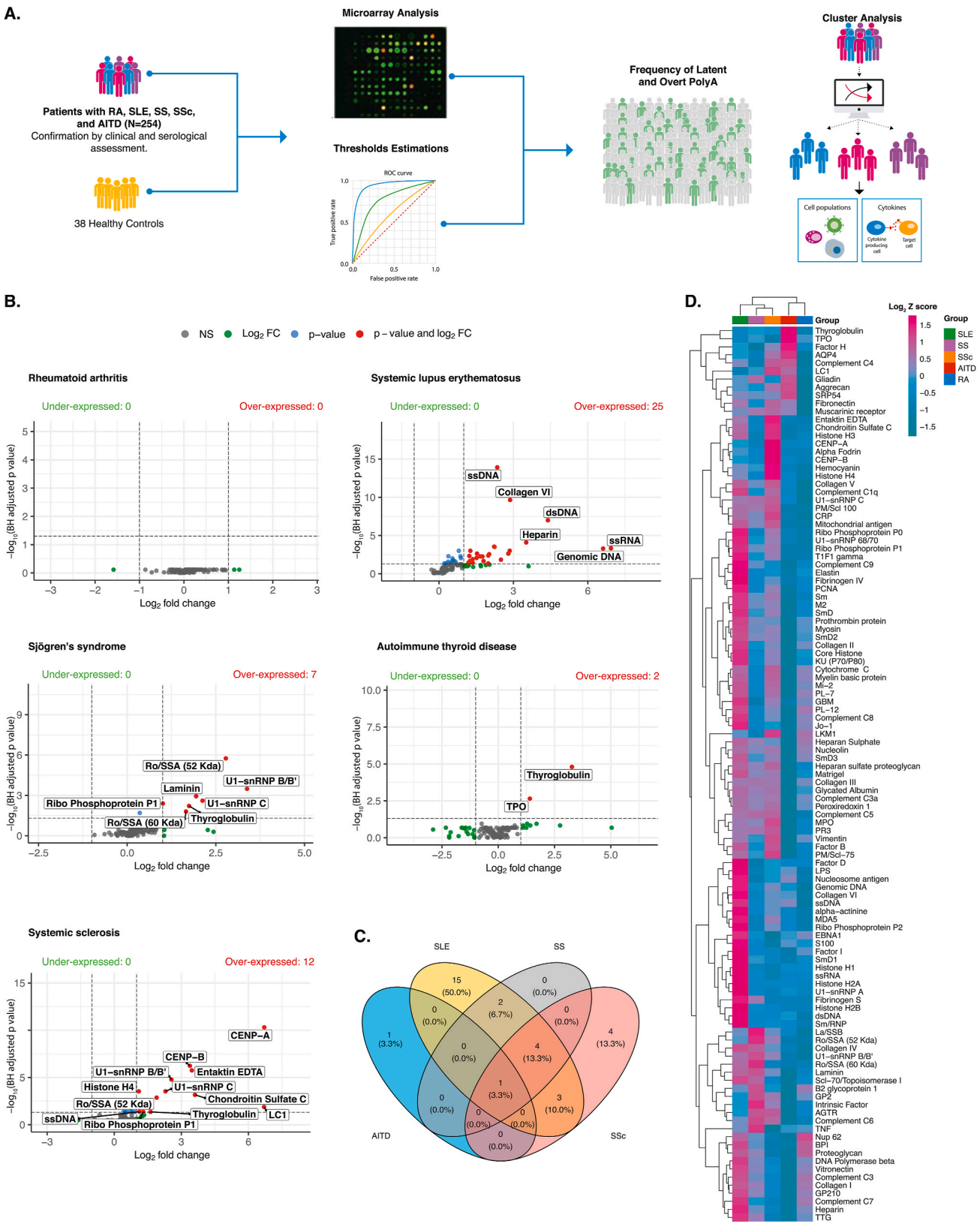

Fig. 1. IgG microarray analysis. (A) Study design. **(B)** Volcano plots for IgG autoantibodies in each condition. Red dots represent those autoantibodies with Log_2 fold change ≥ 1, and p-value FDR <0.05. Analysis included 146 patients with RA, 45 with SLE, 29 with SS, 17 with AITD, and 17 with SSc. **(C)** Venn diagram for over-expressed IgG autoantibodies shared among diseases. **(D)** Heatmap for 111 IgG autoantibodies. The color of the heatmap varies from blue, which indicates under-expression, to purple, which indicates over-expression. Clustering was performed using the ward agglomeration method. NS: Not significant; FC: Fold change; FDR: False discovery rate; BH: Benjamín-Hochberg; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; SS: Sjögren's syndrome; AITD: Autoimmune thyroid disease; SSc: Systemic sclerosis.

Table 1
General characteristics of patients with autoimmune diseases.

Variables (%)	RA (n: 146)	SLE (n: 45)	SS (n: 29)	AITD (n: 17)	SSc (n: 17)	P value[a]
Sociodemographic						
Age (Median – IQR)	55 (44.3–62)	39 (32–49)	55 (45–61)	57 (42–60)	54 (47–62)	<0.0001
Sex						0.1424
Male	21 (14.4%)	7 (15.6%)	0 (0.0%)	2 (11.8%)	1 (5.9%)	
Female	125 (85.6%)	38 (84.4%)	29 (100.0%)	15 (88.2%)	16 (94.1%)	
Body mass index (Median – IQR)	24.9 (22.33–28.5)	26 (23.73–27.65)	25.71 (24.1–26)	26.22 (21.65–27.39)	20.1 (18.58–21.53)	0.5447
Age at onset (Median – IQR)	41 (34–51)	29 (24–44)	46 (35.5–57)	35 (27–40)	46 (42–49)	<0.0001
Familial autoimmunity[b]	37 (25.3%)	10 (22.2%)	8/28 (28.6%)	4 (23.5%)	2 (11.8%)	0.7846
Familial autoimmune disease[b]	23 (15.8%)	5 (11.1%)	2/28 (7.1%)	1 (5.9%)	1 (5.9%)	0.6422
Overt polyautoimmunity	37 (25.3%)	18 (40.0%)	5 (17.2%)	14 (82.4%)	3 (17.6%)	0.0005
Comorbidities [c]						
Diabetes	8 (5.5%)	2 (4.4%)	1/28 (3.6%)	1 (5.9%)	0 (0.0%)	1.0000
Dyslipidemias	15 (10.3%)	5 (11.1%)	4/28 (14.3%)	3 (17.6%)	2 (11.8%)	0.7981
Gastrointestinal ulcers	2 (1.4%)	2 (4.4%)	0/28 (0.0%)	0 (0.0%)	0 (0.0%)	0.5747
Epilepsy	1 (0.7%)	3 (6.7%)	0/28 (0.0%)	0 (0.0%)	0 (0.0%)	0.1084
COPD	2 (1.4%)	0 (0.0%)	0/28 (0.0%)	0 (0.0%)	0 (0.0%)	1.0000
Hypertension	28 (19.2%)	11 (24.4%)	3/28 (10.7%)	3 (17.6%)	1 (5.9%)	0.4313
Thromboembolic disease	3 (2.1%)	6 (13.3%)	1/28 (3.6%)	1 (5.9%)	0 (0.0%)	0.0295
Coronary artery disease	4 (2.7%)	0 (0.0%)	0/28 (0.0%)	0 (0.0%)	0 (0.0%)	0.8571
Stroke	0 (0.0%)	2 (4.4%)	2/28 (7.1%)	0 (0.0%)	0 (0.0%)	0.0275
Severity of symptoms (Median – IQR)[d]	2.9 (2.28–4)	8.5 (4–17)	14 (10–19.3)	–	53.5 (45–74.8)	–
Treatment						
Corticosteroids	65 (44.5%)	30 (66.7%)	4 (13.8%)	3 (17.6%)	3 (17.6%)	0.0005
DMARDs	100 (68.5%)	36 (80.0%)	3 (10.3%)	8 (47.1%)	6 (35.3%)	0.0005
Antimalarials	32 (21.9%)	40 (88.9%)	5 (17.2%)	8 (47.1%)	1 (5.9%)	0.0005
Biologics	24 (16.4%)	3 (6.7%)	1 (3.4%)	0 (0.0%)	0 (0.0%)	0.0370
Clinical manifestations						
Photophobia	13/142 (9.2%)	9/43 (20.9%)	6/16 (37.5%)	3 (17.6%)	0/9 (0.0%)	0.0110
Malar rash	2/142 (1.4%)	9/43 (20.9%)	0/16 (0.0%)	0 (0.0%)	0/9 (0.0%)	0.0010[f]
Raynaud	10/142 (7.0%)	15/43 (34.9%)	3/16 (18.8%)	2 (11.8%)	8/9 (88.9%)	0.0005[f]
Arthritis	124/142 (87.3%)	15/43 (34.9%)	2/16 (12.5%)	8 (47.1%)	4/9 (44.4%)	0.0005[f]
Arthralgias	137/142 (96.5%)	30/43 (69.8%)	13/16 (81.2%)	15 (88.2%)	5/9 (55.6%)	0.0005[f]
Xeropthalmia	72/142 (50.7%)	20/43 (46.5%)	15/16 (93.8%)	13 (76.5%)	4/9 (44.4%)	0.0045
Xerostomia	66/142 (46.5%)	19/43 (44.2%)	14/16 (87.5%)	8 (47.1%)	4/9 (44.4%)	0.0230
Chronic kidney disease	2/142 (1.4%)	7/43 (16.3%)	0/16 (0.0%)	0 (0.0%)	0/9 (0.0%)	0.0045
Oral ulcers	4/142 (2.8%)	7/43 (16.3%)	2/16 (12.5%)	1 (5.9%)	2/9 (22.2%)	0.0030
Periodontal disease	6/142 (4.2%)	1/43 (2.3%)	4/16 (25.0%)	1 (5.9%)	0/9 (0.0%)	0.0340
Skin ulcers	0/142 (0.0%)	1/43 (2.3%)	0/16 (0.0%)	0 (0.0%)	2/9 (22.2%)	0.0020
Anemia	16/142 (11.3%)	15/43 (34.9%)	5/16 (31.2%)	2 (11.8%)	1/9 (11.1%)	0.0045
Telangiectasias	5/142 (3.5%)	0/43 (0.0%)	1/16 (6.2%)	1 (5.9%)	8/9 (88.9%)	0.0005[f]
Pleural effusion	1/142 (0.7%)	7/43 (16.3%)	1/16 (6.2%)	0 (0.0%)	0/9 (0.0%)	0.0015
Pulmonary embolism	0/142 (0.0%)	2/43 (4.7%)	1/16 (6.2%)	0 (0.0%)	0/9 (0.0%)	0.0500
Pericarditis	0/142 (0.0%)	1/43 (2.3%)	0/16 (0.0%)	0 (0.0%)	0/9 (0.0%)	0.3608
Seizures	1/142 (0.7%)	0/43 (0.0%)	0/16 (0.0%)	1 (5.9%)	0/9 (0.0%)	0.3843
Psychosis	0/142 (0.0%)	0/43 (0.0%)	0/16 (0.0%)	0 (0.0%)	0/9 (0.0%)	–
Vasculitis	0/142 (0.0%)	0/43 (0.0%)	0/16 (0.0%)	0 (0.0%)	0/9 (0.0%)	–
CNS compromise	0/142 (0.0%)	3/43 (7.0%)	1/16 (6.2%)	0 (0.0%)	0/9 (0.0%)	0.0210
PNS compromise	0/142 (0.0%)	1/43 (2.3%)	0/16 (0.0%)	0 (0.0%)	0/9 (0.0%)	0.3663
Myalgia	26/142 (18.3%)	9/43 (20.9%)	3/16 (18.8%)	1 (5.9%)	1/9 (11.1%)	0.7571
Calcinosis	1/142 (0.7%)	0/43 (0.0%)	0/16 (0.0%)	0 (0.0%)	0/9 (0.0%)	1.0000
Urticaria	2/142 (1.4%)	1/43 (2.3%)	2/16 (12.5%)	1 (5.9%)	0/9 (0.0%)	0.0865
Alopecia	12/142 (8.5%)	11/43 (25.6%)	1/16 (6.2%)	4 (23.5%)	1/9 (11.1%)	0.0255
Dysphagia	4/142 (2.8%)	1/43 (2.3%)	3/16 (18.8%)	3 (17.6%)	4/9 (44.4%)	0.0005[f]
Gastritis	20/142 (14.1%)	5/43 (11.6%)	8/16 (50.0%)	3 (17.6%)	1/9 (11.1%)	0.0105
Weight loss	4/142 (2.8%)	3/43 (7.0%)	3/16 (18.8%)	0 (0.0%)	1/9 (11.1%)	0.0415
Infertility	1/142 (0.7%)	1/43 (2.3%)	0/16 (0.0%)	1 (5.9%)	0/9 (0.0%)	0.2974
Periorbital edema	1/142 (0.7%)	1/42 (2.4%)	1/16 (6.2%)	1 (5.9%)	0/9 (0.0%)	0.1479
Sclerodactyly	1/142 (0.7%)	0/43 (0.0%)	0/16 (0.0%)	1 (5.9%)	8/9 (88.9%)	0.0005[f]
Miscarriage	19/123 (15.4%)	8/36 (22.2%)	3/16 (18.8%)	4/15 (26.7%)	1/8 (12.5%)	0.6822
Preeclampsia	8/123 (6.5%)	2/36 (5.6%)	0/16 (0.0%)	2/15 (13.3%)	0/8 (0.0%)	0.6682
Preterm delivery	9/123 (7.3%)	3/36 (8.3%)	1/16 (6.2%)	0/15 (0.0%)	0/8 (0.0%)	0.9245
Vascular thrombosis	2/142 (1.4%)	5/43 (11.6%)	1/16 (6.2%)	1 (5.9%)	1/9 (11.1%)	0.0140
Episcleritis	1/142 (0.7%)	0/43 (0.0%)	0/15 (0.0%)	0 (0.0%)	0/9 (0.0%)	1.0000
Uveitis	0/142 (0.0%)	0/43 (0.0%)	0/16 (0.0%)	0 (0.0%)	0/9 (0.0%)	–
Skin nodules	4/142 (2.8%)	1/43 (2.3%)	0/16 (0.0%)	1 (5.9%)	0/9 (0.0%)	0.8136
Pulmonary nodules	0/142 (0.0%)	0/43 (0.0%)	0/16 (0.0%)	0 (0.0%)	1/9 (11.1%)	0.0455
Goiter	4/142 (2.8%)	0/43 (0.0%)	0/16 (0.0%)	4 (23.5%)	0/9 (0.0%)	0.0070
Menstrual disorders	33/123 (26.8%)	13/36 (36.1%)	5/16 (31.2%)	5/16 (31.2%)	1/8 (12.5%)	0.7141
Morphea	0/142 (0.0%)	0/43 (0.0%)	0/16 (0.0%)	0 (0.0%)	0/9 (0.0%)	–
Nail dystrophy	0/142 (0.0%)	1/43 (2.3%)	0/16 (0.0%)	0 (0.0%)	0/9 (0.0%)	0.3688
Microstomia	0/142 (0.0%)	0/43 (0.0%)	0/16 (0.0%)	0 (0.0%)	1/9 (11.1%)	0.0395
Heliotrope rash	0/122 (0.0%)	0/33 (0.0%)	0/16 (0.0%)	0/16 (0.0%)	0/7 (0.0%)	–
Gottron papules	0/122 (0.0%)	0/32 (0.0%)	0/16 (0.0%)	0/16 (0.0%)	0/6 (0.0%)	–
Hand edema	3/121 (2.5%)	0/32 (0.0%)	0/16 (0.0%)	1/16 (6.2%)	2/6 (33.3%)	0.0235

(*continued on next page*)

Table 1 (*continued*)

Variables (%)	RA (n: 146)	SLE (n: 45)	SS (n: 29)	AITD (n: 17)	SSc (n: 17)	P value[a]
Clinical tests [e]						
ESR mm/hr (Median – IQR)	25 (14–36)	21 (14–36.5)	24 (13.5–36.5)	21 (11–37)	26 (19–35)	0.9436
CRP mg/dL (Median – IQR)	0.6 (0.3–1.5)	0.5 (0.3–1.6)	0.5 (0.3–1.2)	0.4 (0.3–0.7)	0.5 (0.4–0.8)	0.7438
Fibrinogen mg/dL (Median – IQR)	352 (284–479)	382 (283.5–467.5)	314 (261.5–442.3)	286 (257–338)	363.5 (277–476.3)	0.2501
TSH μIU/mL (Median – IQR)	2.2 (1.4–4.0)	3.0 (1.6–3.43)	2.3 (1.4–4.0)	3.3 (1.8–4.3)	4.6 (4.5–5.0)	0.3143
Leukocytes (Median – IQR)	7050 (5773–8025)	4470 (3508–6475)	6100 (4450–6750)	5250 (4078–6425)	6440 (5720–6520)	<0.0001
Lymphocytes (Median – IQR)	2000 (1600–2500)	1200 (900–1570)	1700 (1400–2250)	1380 (1130–1786.3)	1600 (1565–1600)	<0.0001
Hemoglobin g/dL (Median – IQR)	13.7 (12.7–14.5)	12.50 (11.2–13.3)	13.3 (12.0–14.8)	13.5 (12.2–14.5)	13.7 (11.4–13.7)	0.0219

Abbreviations: RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; SS: Sjögren's syndrome; AITD: Autoimmune thyroid disease; SSc: Systemic sclerosis; DAS-28: Disease activity score 28; SLAQ: Systemic lupus activity questionnaire; ESSPRI: EULAR Sjogren's syndrome patient reported index; SSPRO: Scleroderma skin patient reported outcome; DMARDs: Disease-modifying antirheumatic drugs; ESR: Erythrocyte sedimentation rate; CRP: C reactive protein; TSH: Thyroid stimulating hormone; CNS: Central nervous system; PNS: Peripheral nervous system; COPD: Chronic obstructive pulmonary disease; IQR: Interquartile range; NA: Not applicable/Available.

[a] Quantitative variables were analyzed by Kruskal-Wallis test, whereas qualitative variables were analyzed by Fisher's exact test.

[b] Familial autoimmunity corresponds to the presence of different autoimmune diseases in a nuclear family. On the other hand, familial autoimmune disease is defined as the presence of one specific autoimmune disease in various members of a nuclear family (i.e., coaggregation) [25].

[c] Reported in clinical history or by the patients.

[d] Severity of symptoms was evaluated as follows: DAS-28 in RA, SLAQ in SLE, ESSPRI in SS and SSPRO in SSc.

[e] For ESR and CRP: 143 patients for RA; 17 AITD; 9 SSc; 43 SLE; 16 SS. For fibrinogen: 141 RA; 17 AITD; 8 SSc; 43 SLE; 16 SS. For TSH: 79 for RA; 9 for AITD; 3 for SSc; 18 for SLE; 12 for SS. For leukocytes, lymphocytes, and hemoglobin: 80 for RA; 14 for AITD; 3 for SSc; 42 for SLE; 11 for SS.

[f] Statistically significant after Bonferroni correction. P value threshold for clinical manifestations: $0.05/41 = 0.0012$.

(SSPRO) for SSc [28,29], or EULAR SS patient-reported index (ESSPRI) for SS [29–31]. Assessment of severity of symptoms did not intent to include/exclude the patients, on the other hand, it helped to confirm the homogeneity of included patients regarding their clinical status. All data were collected in an electronic and secure database as described elsewhere [29].

2.3. Autoantibodies for classification of autoimmune diseases

Detection of IgM rheumatoid factor (RF), IgG anti-cyclic citrullinated peptide third-generation (CCP3), IgM and IgG anti-cardiolipin antibodies (ACAs), IgM and IgG anti-β2glycoprotein (β2GPI) antibodies, IgG anti-double-stranded DNA (dsDNA) antibodies, IgG anti-Tg antibodies, and anti-TPO antibodies were quantified as previously reported [24]. In addition, anti-nuclear antibodies were detected by IMTEC-ANA-LIA Maxx (Human Diagnostics Magdeburg, Germany) [32].

2.4. Microarray autoantibody profiling

For the purpose of this study, a microarray autoantibody platform was used to evaluate latent PolyA. Samples were analyzed by an autoantigen array chip containing 128 antigens and controls at the Microarray and Immune Phenotyping Core Facility, UT Southwestern Medical Center. Briefly, the autoantigens and control proteins are printed in duplicates onto nitrocellulose film slides. Serum samples were pretreated with DNAse-I and diluted in phosphate-buffered saline with Tween (PBST) for autoantibody profiling. The diluted serum samples were incubated with the autoantigen arrays, and autoantibodies binding with antigens on arrays were measured with cy3-conjugated anti-human IgG (Jackson ImmunoResearch Laboratories) and cy5-conjugated anti-human IgM (Jackson ImmunoResearch Laboratories), using a Genepix 4200 A scanner (Molecular Device). The resulting images were analyzed with Genepix Pro 7.0 software (Molecular Devices). The median of the signal intensity for each spot was calculated and subtracted from the local background around the spot, and data obtained from duplicated spots were averaged. The background-subtracted signal intensity of each antigen was normalized to the average intensity of the human IgG or IgM controls, which were spotted on the array as internal controls. Finally, the normalized fluorescence intensity (NFI) was generated as a quantitative measurement of the binding capacity of each antibody with the corresponding autoantigen. Signal-to-noise ratio (SNR) is another quantitative measurement of the true signal above background noise. SNR values equal to or greater than 3 were considered significantly higher than the background, and therefore as true signals. The autoantibody which has the SNR value of less than 3 in more than 90% of the samples was considered negative and excluded from further analysis.

2.5. Cytokine assay and lymphocytes immunophenotype

Serum of patients was collected in fasting state and spite of the treatment status. The concentration of 19 cytokines (IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IFN-α, TNF-α, G-CSF, GM-CSF, RANTES, MCP-1, IL-12p70, IL-13, IFN-γ) in serum samples from patients were assessed by Cytometric Bead Array (CBA, Becton Dickinson Biosciences, San Diego, CA, USA). The test was done according to the manufacturer's protocols. The concentration of the cytokines was calculated using the FCAP Array™ Software (BD Bioscience) as reported elsewhere [5]. For a detailed analysis of the cell phenotype, peripheral blood mononuclear cells were stained with fluorescent antibodies. A minimum of 100,000 lymphocytes per sample was acquired on a FACSCanto II™ flow cytometer (BD Biosciences™). Twenty-eight cell subsets (Supplementary Appendix) were analyzed with FlowJo software version 9 (BD Biosciences™) as reported elsewhere [33].

2.6. Statistical analysis

Univariate descriptive statistics were performed. Categorical variables were analyzed using frequencies, and quantitative continuous variables were expressed as the median and interquartile range (IQR). The Kruskal-Wallis or Fisher's exact tests were used based on the results. Bonferroni correction was used for multiple testing in clinical manifestations.

Array results were used to evaluate the over-expressed autoantibodies compared with healthy controls. Thus, data from the autoantigen array was standardized by a robust linear model as previously described [34,35]. The p-value was determined by unpaired *t*-test with a Benjamini and Bonferroni-Hochberg False Discovery Rate post-hoc correction (FDR). For all autoantibodies, the only selected were those that fulfilled: 1) p-value with a Benjamini-Hochberg FDR <0.05, and 2) Log2 fold change ≥ 1.

A logistic regression model was fitted to estimate the effect size of significant autoantibodies on ADs classification (i.e., Log2 fold change ≥ 1, and FDR <0.05). The dependent variable of the logistic model was the natural log of the odds of the index AD. The independent variables of the logistic model were selected through a backward selection procedure as previously described [36].

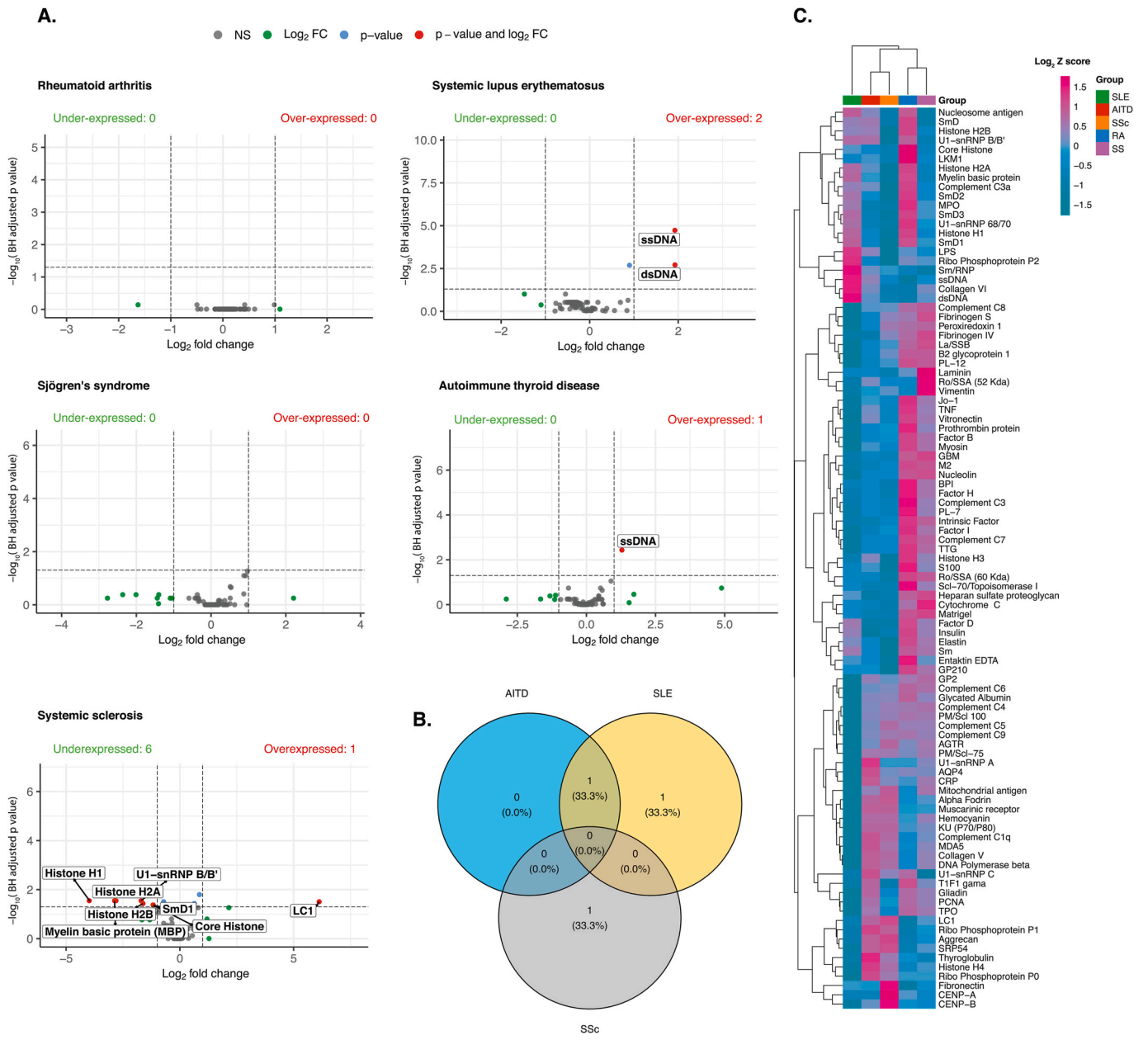

Fig. 2. IgM microarray analysis. (A) Volcano plots for IgM autoantibodies in each condition. Red dots represent those autoantibodies with Log_2 fold change ≥ 1, and p-value FDR <0.05. Analysis included 146 patients with RA, 45 with SLE, 29 with SS, 17 with AITD, and 17 with SSc. **(B)** Venn diagram for over-expressed IgM autoantibodies shared among diseases. **(C)** Heatmap for 97 IgM autoantibodies. The color of the heatmap varies from blue, which indicates under-expression, to purple, which indicates over-expression. Clustering was performed using the ward agglomeration method. NS: Not significant; FC: Fold change; FDR: False discovery rate; BH: Benjamín-Hochberg; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; SS: Sjögren's syndrome; AITD: Autoimmune thyroid disease; SSc: Systemic sclerosis.

From the selected autoantibodies, thresholds for positivity were obtained by maximizing the sum of sensitivity and specificity functions comparing it with healthy volunteers. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under the curve (AUC) were estimated for each threshold (https://github.com/thie1e/cutpointr).

Next, we aimed to develop a new ADs classification based on those autoantibodies selected in the previous step. We used the mixed-cluster methodology proposed by Lebart et al. [37]. First, principal component analysis of the data was conducted. Next, the number of clusters by a hierarchical cluster analysis was determined, and finally, a consolidation step by k-means clustering was performed.

After identification of those autoantibody-based subgroups, immunological characteristics were evaluated for each group (Fig. 1A). Cytokine concentrations were analyzed after log transformation. Linear regression models were fitted to estimate the differences in cytokines and lymphocyte populations among clusters. All models were adjusted by age and sex. Post-hoc comparison of means was based on both adjusted Bonferroni p-values and Fisher's protected least significant differences procedure using t statistics based on Satterwhaite's approximation. The significance level of the study was set to 0.05. Statistical analyses were done using R software version 4.0.2.

Table 2
Diagnostic accuracy of autoantibodies selected by multivariate analysis.

Diseases and autoantibodies[a,b]	Threshold[c]	Sensitivity	Specificity	PPV	NPV	AUC
Systemic lupus erythematosus						
dsDNA	3893.0	92.6%	94.7%	92.6%	94.7%	96.4%
Sm/RNP	767.9	96.3%	78.9%	76.5%	96.8%	93.1%
SsRNA	154.2	92.6%	92.1%	89.3%	94.6%	91.6%
Histone H2A	1647.4	77.8%	78.9%	72.4%	83.3%	77.7%
SLE model	NA	NA	NA	NA	NA	100.0%
Sjögren's syndrome						
U1−snRNP B/B′	787.3	87.5%	73.7%	67.7%	90.3%	85.9%
Ro/SSA (52 kDa)	4738.1	54.2%	94.7%	86.7%	76.6%	78.5%
Thyroglobulin	2923.9	83.3%	68.4%	62.5%	86.7%	78.4%
Laminin	1475	58.3%	92.1%	82.4%	77.8%	71.3%
SS model	NA	NA	NA	NA	NA	96.7%
Autoimmune thyroid disease						
Thyroglobulin	4954.5	94.1%	89.5%	80%	97.1%	94.6%
Systemic sclerosis						
U1−snRNP B/B′	1423.8	71.4%	97.4%	90.9%	90.2%	90.6%
CENP-A	910.5	78.6%	97.4%	91.7%	92.5%	88.0%
Ribo Phosphoprotein P1	547.7	50%	94.7%	77.7%	83.7%	75.8%
Thyroglobulin	3448	57.1%	76.3%	47.1%	82.9%	65.8%
SSc model	NA	NA	NA	NA	NA	100.0%

Abbreviations: RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; SS: Sjögren's syndrome; AITD: Autoimmune thyroid disease; SSc: Systemic sclerosis; PPV: Positive predictive value, NPV: Negative predictive value; AUC: Area under the curve; NA: Not applicable/Available; PolyA: Polyautoimmunity.

[a] Patients with overt PolyA were excluded from this analysis. The final sample size for each group included: 27 patients with SLE, 24 patients with SS, and 14 patients with SSc. In AITD, only 3 patients did not exhibit overt PolyA. Thus, hindering the analysis. In this scenario, all the 17 patients with AITD were included in the final model despite the confirmed overt PolyA. All analyses included 38 healthy controls.

[b] Logistic regression models were obtained by backward selection. All autoantibodies in each disease with Log2 Fold change $\geq$1 and *p-value* FDR $<$0.05 were included in the analysis.

[c] Thresholds were obtained by maximize the sum of sensitivity and specificity functions.

3. Results

3.1. Clinical manifestations are shared among autoimmune diseases due to polyautoimmunity

The general characteristics of patients are shown in Table 1. Most patients included were women. Patients with SLE were younger and exhibited an earlier age at onset. Familial autoimmunity was equally distributed among diseases. Patients with SLE disclosed the highest rates of management with corticosteroids, disease-modifying antirheumatic drugs (DMARDs), and antimalarials, whereas patients with RA reported the highest rates of management with biologics.

Patients with RA presented a low activity of the disease, according to DAS-28. On the contrary, most patients with SLE showed moderate clinical reported activity (i.e., SLAQ score $\geq$3). Raynaud, telangiectasias, dysphagia, and sclerodactyly were most common in SSc. Arthritis and arthralgias were most frequently presented in RA, whereas malar rash was distinctive of SLE (Table 1). Interestingly, some clinical manifestations were equally distributed across diseases. Clinical inflammatory biomarkers (i.e., ESR, CRP, and fibrinogen) and thyroid function did not differ among diseases. Patients with SLE disclosed lower levels of total leukocytes, lymphocytes, and hemoglobin (Table 1).

3.2. Patients with autoimmune diseases share expression of IgG autoantibodies

Initially, we evaluated the expression of autoantibodies compared with 38 healthy volunteers. After quality control filtering (i.e., SNR $>$3), 111 IgG and 97 IgM autoantibodies were included in the final analysis. It was found that 25 IgG autoantibodies in SLE, 7 in SS, 2 in AITD, and 12 in SSc were over-expressed (i.e., Log2 fold change $\geq$1, and p-value FDR $<$0.05) (Supplementary Material). There were no over-expressed nor under-expressed IgG autoantibodies in patients with RA (Fig. 1B). This finding was related to the lack of RF and citrullinated antigens included in the microarray. Patients with different index ADs shared several IgG autoantibodies (i.e., PolyA) (Fig. 1C).

Patients with SLE showed over-expression of IgG autoantibodies against nuclear, thyroid, complement, and collagen-associated antigens (Fig. 1D). This was similar for SS, in which autoantibodies for ribonuclear proteins and thyroid antigens were over-expressed. Concerning AITD, only IgG autoantibodies against thyroid antigens were significantly over-expressed. Patients with SSc showed over-expression of autoantibodies against nuclear, cytoplasmatic, and thyroid antigens (Fig. 1D).

Few IgM autoantibodies were over-expressed in ADs (Supplementary Appendix) (Fig. 2A) and sharing of IgM autoantibodies was less likely (Fig. 2B). IgM autoantibodies against nuclear antigens in AITD and SLE were over-expressed. Interestingly, patients with SSc showed under-expression of autoantibodies for nuclear and myelinic antigens, but over-expression of liver-associated autoantibodies (i.e., LC1) (Fig. 2C). There were no under-expressed nor over-expressed IgM autoantibodies in patients with RA and SS.

3.3. IgG autoantibodies yield high accuracy for classification of autoimmune diseases

We focused on the evaluation of over-expressed IgG autoantibodies (i.e., Log2 fold change $\geq$1, and p-value FDR $<$0.05) as hallmarks for the identification of index AD. To avoid the bias of patients with overt PolyA, this analysis only included those patients without such conditions. Multivariate logistic regression yielded that IgG autoantibodies against nuclear antigens disclosed the best performance in SLE (Table 2). In AITD, IgG against Tg was the only associated autoantibody. Tg was associated with the classification of SS and SSc. In addition, autoantibodies against ribonucleoproteins and centromere were also associated with the classification of SS and SSc, respectively (Fig. 3A). Although CCP3 and RF were not included in the microarray, all the patients with RA were assessed for CCP3 and RF (by ELISA) and were positive in 86.1% and 91.8%, respectively.

3.4. Latent polyautoimmunity surpasses overt polyautoimmunity

Given the thresholds of the IgG autoantibodies obtained (Table 2), we evaluated their positivity in all included diseases to estimate the

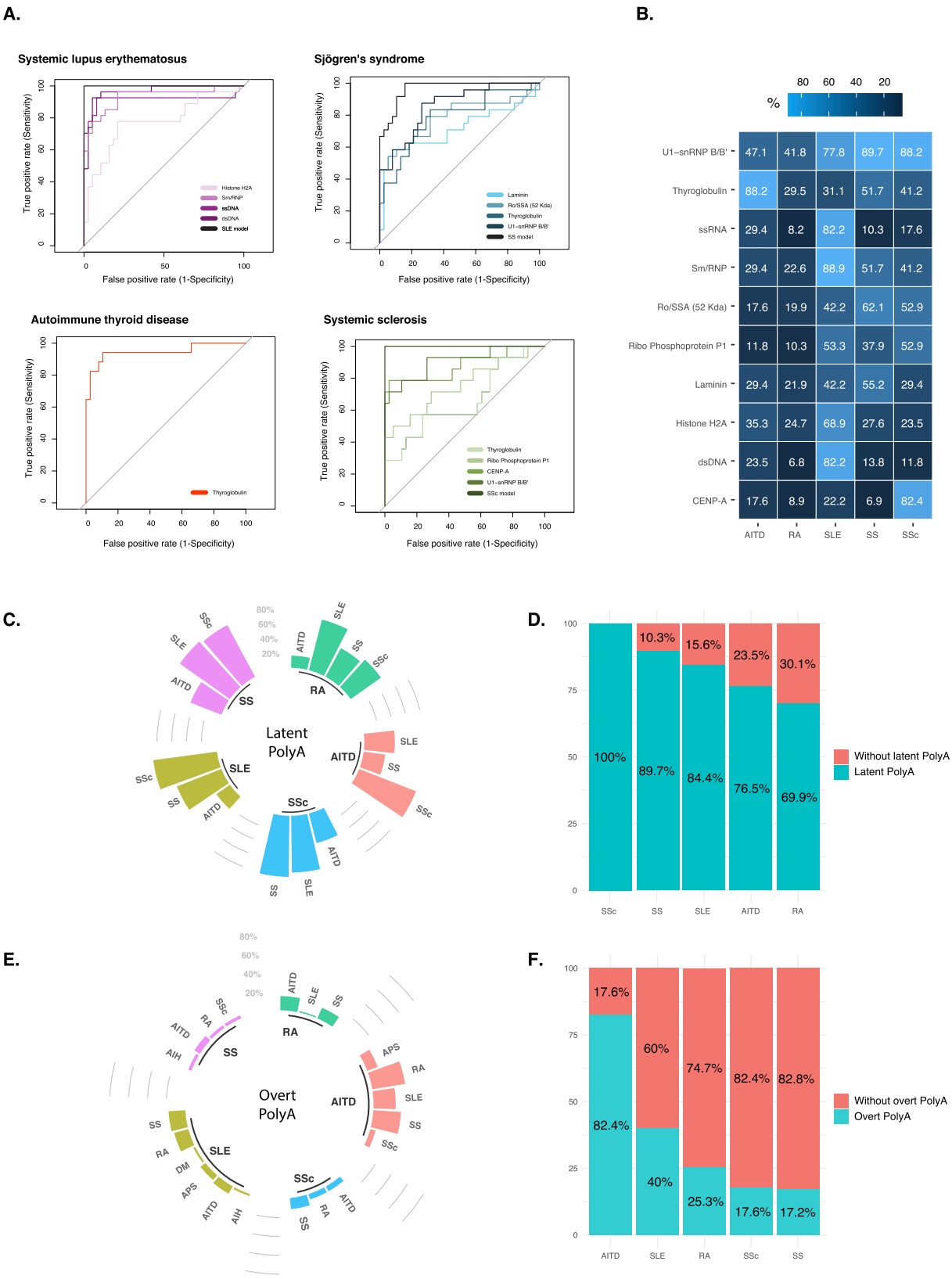

Fig. 3. Prevalence of latent and overt PolyA. (A) ROC curves for selected IgG autoantibodies from multivariate logistic regression analysis. **(B)** Heat map for positivity of selected IgG autoantibodies. **(C)** Circular bar plot for sources of latent PolyA. **(D)** Bar plot for overall prevalence of latent PolyA. **(E)** Circular bar plot for sources of overt PolyA. **(F)** Bar plot for overall prevalence of overt PolyA. ROC: Receiver operating characteristic; PolyA: Polyautoimmunity; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; SS: Sjögren's syndrome; AITD: Autoimmune thyroid disease; SSc: Systemic sclerosis; AIH: Autoimmune hepatitis; APS: Antiphospholipid syndrome; DM: Type 1 diabetes mellitus.

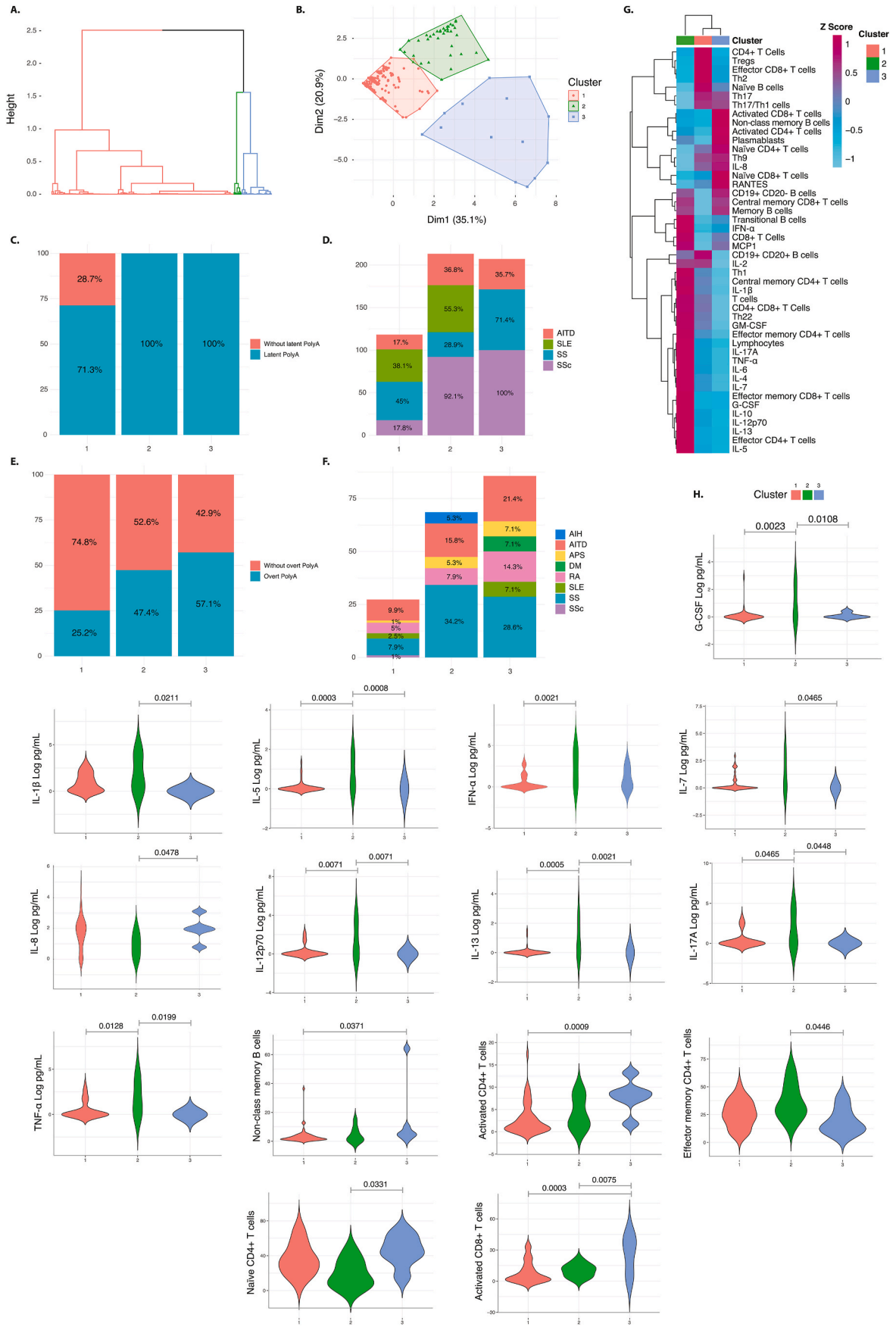

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Fig. 4. PolyA-based classification of ADs. **(A)** Cluster dendrogram for classification of ADs based on selected autoantibodies. **(B)** Factor map for obtained clusters. **(C)** Bar plot for overall prevalence of latent PolyA by cluster. **(D)** Bar plot for sources of latent PolyA by cluster. **(E)** Bar plot for overall prevalence of overt PolyA by cluster. **(F)** Bar plot for sources of overt PolyA by cluster. **(G)** Heatmap for cytokines (n: 61) and lymphocytes immunophenotype (n: 67). The color of the heatmap varies from blue, which indicates under-expression, to purple, which indicates over-expression. Clustering was performed using the ward agglomeration method. The log-transformed cytokine concentration was used to construct the heatmap. **(H)** Representative violin plots of cytokines and lymphocytes phenotypes. Statistical analysis was performed using linear models that were adjusted for age, and sex. G-CSF: Granulocyte colony-stimulating factor; IL: Interleukin; TNF-α: Tumor necrosis factor-alpha; IFN-α: Interferon-alpha; RANTES: Regulated on activation, normal T cell expressed and secreted; MCP-1: Monocyte chemotactic protein-1; PolyA: Polyautoimmunity; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; SS: Sjögren's syndrome; AITD: Autoimmune thyroid disease; SSc: Systemic sclerosis; AIH: Autoimmune hepatitis; APS: Antiphospholipid syndrome; DM: Type 1 diabetes mellitus.

frequency of latent PolyA. Since Tg and U1−snRNP B/B′ IgG autoantibodies were shared in several conditions, only those estimated thresholds for AITD and SS were used, respectively.

Positivity for included autoantibodies in all patients is shown in Fig. 3B. Although IgG autoantibodies obtained by multivariate analysis exhibited high frequency in specific index conditions, patients showed positivity for other autoantibodies (i.e., PolyA). The overall frequency of these autoantibodies was low in RA, whereas SLE, SS, and SSc showed higher positivity rates (Fig. 3B).

Then, we looked for those autoantibodies that were not associated with overt ADs to estimate the occurrence of latent PolyA in each condition (Fig. 3C). Latent SLE (i.e., dsDNA, Sm/RNP, SsRNA, or Histone H2A) was the most common in patients with RA and SS. On the other hand, latency for SSc (i.e., CENP-A, or Ribo Phosphoprotein P1) was the most common in patients with AITD and SLE, whereas latency for AITD (i.e., Thyroglobulin) was the most common in SSc. This analysis yielded that more than 70% of patients presented at least 1 type of latency for SLE, SS, AITD, or SS (Fig. 3D).

Based on classification criteria, the frequency of overt PolyA was estimated. AITD was the most common overt PolyA in SS and RA (Fig. 3E). Conversely, RA was the most common overt PolyA in AITD. In patients with SLE and SSc, overt PolyA was predominantly defined by SS. In contrast to latent PolyA, overt PolyA was less frequent, and AITD presented the highest rates (Fig. 3F).

3.5. Polyautoimmunity influences clinical and immunological phenotypes

After estimation of the occurrence of overt and latent PolyA, we developed a classification of ADs based on autoantibodies. Three main clusters were obtained (Fig. 4A–B). Their general characteristics are shown in Table 3. Clusters 2 and 3 exhibited the highest frequency of latent (Fisher's exact test, $P = 0.0005$) (Fig. 4C), and overt PolyA (Fisher's exact test, $P = 0.0020$) (Fig. 4E). SSc and SS were the most common cause of latent and overt PolyA in both clusters, respectively (Fig. 4D–F).

Interestingly, cluster 3 was most likely to receive treatment with corticosteroids, DMARDs, and antimalarials (Fisher's exact test, $P < 0.0105$), and presented higher frequency of malar rash, Raynaud, oral ulcers, and central nervous system compromise (Fisher's exact test, $P < 0.0500$). In addition, patients with SLE belonging to cluster 3 exhibited a high severity of symptoms. On the other hand, cluster 2 was characterized by xeropthalmia, xerostomia, periodontal disease, and weight loss (Fisher's exact test, $P < 0.0500$).

Cytokine and lymphocyte profiles were different among clusters (Fig. 4G). Cluster 2 exhibited a dysregulated immunological profile given by high levels of G-CSF, IL-5, INF-α, IL-7, IL-12p70, IL-13, IL-17A, TNF- α, and effector memory CD4+ T cells, whereas naïve CD4+ T cells were decreased (Fig. 4H). On the other hand, Cluster 3, showed high levels of IL-8, activated CD4+ and CD8+ T cells (Fig. 4H).

4. Discussion

In this proof-of-concept study, it is confirmed that latent PolyA allows a new taxonomy of ADs. Clusters of PolyA were well-differentiated and characterized by a unique immune signature (i.e., cytokine response and cellular subphenotypes) (Fig. 4). Latent PolyA was more frequent than overt PolyA [2,3,5,38]. Both latent and overt PolyA cluster together.

Several autoantibodies share different specificities across ADs. For example, anti-SSA/Ro and anti-SSB/La are considered the two most classic autoantibodies in SS [6], and nearly 63% of patients show positivity to anti-SSA/Ro [39]. However, this autoantibody is also associated with the development of SLE [40]. Anti-SSA/Ro in the presence of anti-SSB/La tends to identify patients with SS. It was found that 29 out of 35 patients with both anti-Ro/SSA and anti-La/SSB had SS, whereas of 53 with only anti-Ro/SSA, 23 had SS, 25 had SLE, and 13 had another disease [41]. This suggests that the combination of some autoantibodies in the diagnostic approach of ADs may improve the sensitivity and specificity of these tests [3].

The microarray analysis allowed the identification of mixtures of IgG autoantibodies that helped to develop high accuracy models for the classification of ADs. Thus, confirming the usefulness of antibody combination in the diagnosis of disease. Tg and U1−snRNP B/B′ autoantibodies were shared as diagnostic biomarkers for AITD, SS, and SSc. This may suggest that some autoantibodies yielded similar specificities across diseases, or a phenomenon of latent PolyA, in which these patients may develop overt PolyA in the future.

Regarding IgM autoantibodies, we did not find any clinical significance of this isotype in the differentiation of ADs from healthy controls. Paradoxically, for SSc, IgM autoantibodies were under-expressed when compared with healthy controls. In a study on lupus-prone lymphoproliferative (lpr) mice. Compared with regular lpr mice, lpr mice lacking secreted IgM developed elevated levels of IgG autoantibodies to ds-DNA and histones. Similarly, the absence of secreted IgM also resulted in the accelerated development of IgG autoantibodies in normal mice [42]. In this line, Ricci et al. [43] demonstrated that IgM Tg autoantibodies seemed to prevent overt AITD favoring the clearance of Tg in subacute thyroiditis. Thus, in some ADs IgM autoantibodies may have a protective role but limited value for classifying overt ADs.

The frequency of latent and overt PolyA matches with prior studies in which different sources of PolyA were described [1–3]. In this study, we found combinations of diverse ADs conforming the spectrum of PolyA [44]. Latent PolyA (69.9% in RA to 100% in SSc) was more frequent than overt PolyA (17.2% in SS to 82.4% in AITD). This may suggest that most patients with an index condition present autoantibody positivity for other ADs, and thus inferring that primary or secondary labels of ADs are inaccurate. This may have implications for follow-up and treatment (i.e., primary prevention). As mentioned, positivity for autoantibodies predate the appearance of overt ADs [6]. Although the effect of PolyA on the index disease may vary across ADs, it has been suggested that PolyA may influence deleterious outcomes such as pulmonary fibrosis and mortality in patients with SSc [45,46].

A recent study on megakaryocyte expansion in peripheral blood showed that patients with RA, SLE, and SS shared similar transcriptional profiles and common gene expression signatures associated with nucleosome assembly and hemostasis [47]. A similar study found that clustering of CXCL10, IL-6, IL-2, and TNF-α was associated with worse clinical profile in patients with SLE, SS, RA, and SSc [48]. In a similar study, four groups of patients with similar genetic, clinical, serological, and cellular features were found by cluster analysis [49]. Longitudinal analysis of these groups showed no variation over time. Altogether, data confirm that ADs share common pathophysiology (i.e., autoimmune

Table 3
General characteristics of patients with autoimmune diseases by cluster.

Variables (%)	Cluster 1 (n: 202)	Cluster 2 (n: 38)	Cluster 3 (n: 14)	P value[a]
Sociodemographic				
Age (Median – IQR)	55 (43.3–61)	46.5 (38–59.75)	35.5 (29–39.75)	<0.0001
Sex				0.1029
Male	28 (13.9%)	1 (2.6%)	2 (14.3%)	
Female	174 (86.1%)	37 (97.4%)	12 (85.7%)	
Body mass index (Median – IQR)	25.39 (22.3–28.2)	23 (22.2–23.8)	25.6 (24.6–30.1)	0.1509
Age at onset (Median – IQR)	43 (34–52)	41 (34–52)	25.5 (24.3–29)	<0.0001
Familial autoimmunity[b]	50/201 (24.9%)	9 (23.7%)	2 (14.3%)	0.7901
Familial autoimmune disease[b]	28/201 (13.9%)	2 (5.3%)	2 (14.3%)	0.3103
Overt polyautoimmunity	51 (25.2%)	18 (47.4%)	8 (57.1%)	0.0020
Comorbidities [c]				
Diabetes	10/201 (5.0%)	1 (2.6%)	1 (7.1%)	0.7041
Dyslipidemias	25/201 (12.4%)	2 (5.3%)	2 (14.3%)	0.4148
Gastrointestinal ulcers	3/201 (1.5%)	1 (2.6%)	0 (0.0%)	0.6117
Epilepsy	2/201 (1.0%)	0 (0.0%)	2 (14.3%)	0.0265
COPD	1/201 (0.5%)	1 (2.6%)	0 (0.0%)	0.3778
Hypertension	34/201 (16.9%)	6 (15.8%)	6 (42.9%)	0.0665
Thromboembolic disease	8/201 (4.0%)	2 (5.3%)	1 (7.1%)	0.5427
Coronary artery disease	4 (2.0%)	0 (0.0%)	0 (0.0%)	1.0000
Stroke	3 (1.5%)	0 (0.0%)	1 (7.1%)	0.3148
Severity of symptoms (Median – IQR) [d]				
DAS-28	3.1 (2.17–4.02)	2.64 (2.41–3.03)	2.06 (1.59–2.64)	0.3105
ESSPRI	15 (11.5–17.8)	12.5 (8.75–20)	17 (11–21)	0.5992
SSPRO	52.5 (36.5–74.75)	49 (49–49)	–	–
SLAQ	5 (3–7)	5 (2–17.25)	17 (12–26.25)	<0.0001
Treatment				
Corticosteroids	81 (40.1%)	13 (34.2%)	11 (78.6%)	0.0105
DMARDs	124 (61.4%)	16 (42.1%)	13 (92.9%)	0.0020
Antimalarials	61 (30.2%)	13 (34.2%)	12 (85.7%)	0.0005
Biologics	22 (10.9%)	4 (10.5%)	2 (14.3%)	0.7931
Clinical manifestations				
Photophobia	23/183 (12.6%)	5/30 (16.7%)	3 (21.4%)	0.4718
Malar rash	5/183 (2.7%)	1/30 (3.3%)	5 (35.7%)	0.0005[f]
Raynaud	23/183 (12.6%)	9/30 (30.0%)	6 (42.9%)	0.0025
Arthritis	136/183 (74.3%)	10/30 (33.3%)	7 (50.0%)	0.0005[f]
Arthralgias	165/183 (90.2%)	24/30 (80.0%)	11 (78.6%)	0.0825
Xeropthalmia	92/183 (50.3%)	24/30 (80.0%)	8 (57.1%)	0.0070
Xerostomia	83/183 (45.4%)	21/30 (70.0%)	7 (50.0%)	0.0445
Chronic kidney disease	6/183 (3.3%)	1/30 (3.3%)	2 (14.3%)	0.1449
Oral ulcers	8/183 (4.4%)	5/30 (16.7%)	3 (21.4%)	0.0055
Periodontal disease	6/183 (3.3%)	6/30 (20.0%)	0 (0.0%)	0.0070
Skin ulcers	2/183 (1.1%)	1/30 (3.3%)	0 (0.0%)	0.4963
Anemia	24/183 (13.1%)	12/30 (40.0%)	3 (21.4%)	0.0030
Telangiectasias	14/183 (7.7%)	1/30 (3.3%)	0 (0.0%)	0.6782
Pleural effusion	6/183 (3.3%)	2/30 (6.7%)	1 (7.1%)	0.2729
Pulmonary embolism	2/183 (1.1%)	0/30 (0.0%)	1 (7.1%)	0.2204
Pericarditis	1/183 (0.5%)	0/30 (0.0%)	0 (0.0%)	1.0000
Seizures	2/183 (1.1%)	0/30 (0.0%)	0 (0.0%)	1.0000
Psychosis	0/183 (0.0%)	0/30 (0.0%)	0 (0.0%)	–
Vasculitis	0/183 (0.0%)	0/30 (0.0%)	0 (0.0%)	–
CNS compromise	0/183 (0.0%)	1/30 (3.3%)	3 (21.4%)	0.0005
PNS compromise	0/183 (0.0%)	0/30 (0.0%)	1 (7.1%)	0.0540
Myalgia	34/183 (18.6%)	3/30 (10.0%)	3 (21.4%)	0.4518
Calcinosis	1/183 (0.5%)	0/30 (0.0%)	0 (0.0%)	1.0000
Urticaria	3/183 (1.6%)	2/30 (6.7%)	1 (7.1%)	0.1154
Alopecia	21/183 (11.5%)	3/30 (10.0%)	5 (35.7%)	0.0455
Dysphagia	13/183 (7.1%)	1/30 (3.3%)	1 (7.1%)	0.7481
Gastritis	30/183 (16.4%)	6/30 (20.0%)	1 (7.1%)	0.6052
Weight loss	4/183 (2.2%)	5/30 (16.7%)	2 (14.3%)	0.0025
Infertility	2/183 (1.1%)	0/30 (0.0%)	1 (7.1%)	0.2214
Periorbital edema	2/183 (1.1%)	1/30 (3.3%)	1 (7.7%)	0.0910
Sclerodactyly	10/183 (5.5%)	0/30 (0.0%)	0 (0.0%)	0.5312
Miscarriage	29/157 (18.5%)	4/29 (13.8%)	2/12 (16.7%)	0.9315
Preeclampsia	10/157 (6.4%)	1/29 (3.4%)	1/12 (8.3%)	0.7301
Preterm delivery	9/157 (5.7%)	2/29 (6.9%)	2/12 (16.7%)	0.2109
Vascular thrombosis	6/183 (3.3%)	3/30 (10.0%)	1 (7.1%)	0.1489
Episcleritis	1/173 (0.6%)	0/30 (0.0%)	0/13 (0.0%)	1.0000
Uveitis	0/183 (0.0%)	0/30 (0.0%)	0 (0.0%)	–
Skin nodules	6/183 (3.3%)	0/30 (0.0%)	0 (0.0%)	0.7256
Pulmonary nodules	1/183 (0.5%)	0/30 (0.0%)	0 (0.0%)	1.0000
Goiter	7/183 (3.8%)	1/30 (3.3%)	0 (0.0%)	1.0000
Menstrual disorders	42/158 (26.6%)	11/29 (37.9%)	4/12 (33.3%)	0.4353
Morphea	0/183 (0.0%)	0/30 (0.0%)	0 (0.0%)	–
Nail dystrophy	0/183 (0.0%)	0/30 (0.0%)	1 (7.1%)	0.0600

(*continued on next page*)

Table 3 (*continued*)

Variables (%)	Cluster 1 (n: 202)	Cluster 2 (n: 38)	Cluster 3 (n: 14)	P value[a]
Microstomia	1/182 (0.5%)	0/30 (0.0%)	0/10 (0.0%)	1.0000
Heliotrope rash	0/157 (0.0%)	0/27 (0.0%)	0/10 (0.0%)	–
Gottron papules	0/155 (0.0%)	0/27 (0.0%)	0/10 (0.0%)	–
Hand edema	5/154 (3.2%)	1/27 (3.7%)	0 (0.0%)	1.0000
Clinical tests [e]				
ESR mm/hr (Median – IQR)	24 (14–35)	27.5 (17.25–41.3)	21 (14.5–36)	0.5258
CRP mg/dL (Median – IQR)	0.5 (0.3–1.3)	0.49 (0.29–2.66)	0.9 (0.4–2.49)	0.3285
Fibrinogen mg/dL (Median – IQR)	338 (278.5–457.75)	426 (268–550)	369 (269.75–428.5)	0.5602
TSH μIU/mL (Median – IQR)	2.32 (1.48–4.05)	2.05 (1.41–3.22)	3.27 (2.71–5.18)	0.2649
Leukocytes (Median – IQR)	6500 (5277.5–7900)	4750 (4250–6350)	4400 (3425–5030)	0.0006
Lymphocytes (Median – IQR)	1775 (1400–2300)	1400 (1175–2000)	1100 (825–1375)	0.0005
Hemoglobin g/dL (Median – IQR)	13.55 (12.5–14.5)	12.2 (11.3–13.13)	12.95 (11.7–13.35)	0.0098

Abbreviations: RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; SS: Sjögren's syndrome; AITD: Autoimmune thyroid disease; SSc: Systemic sclerosis; DAS-28: Disease activity score 28; SLAQ: Systemic lupus activity questionnaire; ESSPRI: EULAR Sjogren's syndrome patient reported index; SSPRO: Scleroderma skin patient reported outcome; DMARDs: Disease-modifying antirheumatic drugs; ESR: Erythrocyte sedimentation rate; CRP: C reactive protein; TSH: Thyroid stimulating hormone; CNS: Central nervous system; PNS: Peripheral nervous system; COPD: Chronic obstructive pulmonary disease; IQR: Interquartile range; NA: Not applicable/Available.

[a] Quantitative variables were analyzed by Kruskal-Wallis test, whereas qualitative variables were analyzed by Fisher's exact test.

[b] Familial autoimmunity corresponds to the presence of different autoimmune diseases in a nuclear family. On the other hand, familial autoimmune disease is defined as the presence of one specific autoimmune disease in various members of a nuclear family (i.e., coaggregation) [25].

[c] Reported in clinical history or by the patients.

[d] Severity of symptoms was evaluated as follows: DAS-28 in RA, SLAQ in SLE, ESSPRI in SS and SSPRO in SSc.

[e] For ESR and CRP: 184 patients for cluster 1; 30 for cluster 2; 14 for cluster 3. For fibrinogen: 182 for cluster 1; 29 for cluster 2; 14 for cluster 3. For TSH: 98 for cluster 1; 15 for cluster 2; 8 for cluster 3. For leukocytes, lymphocytes, and hemoglobin: 116 for cluster 1; 20 for cluster 2; 14 for cluster 3.

[f] Statistically significant after Bonferroni correction. P value threshold for clinical manifestations: $0.05/41 = 0.0012$.

tautology) [19].

Our cluster analysis yielded that patients exhibit differential immunological profiles based on PolyA, thus suggesting that despite similarities among ADs, PolyA is a critical factor for the classification and treatment of these conditions. Those clusters gathering most of overt and latent PolyA showed dysregulation of multiple inflammatory and anti-inflammatory cytokines such as IL-12p70 and IL-17A. In a similar study, patients with PolyA presented high levels of IL-12p70 [3], and IL-17A has been associated with the inflammatory response across multiple ADs [50–54]. In addition, patients from these clusters showed overactivity in $CD4^+$ and $CD8^+$ T cells, and one of them disclosed low levels of Treg cells. Cluster 2 showed high levels of effector T cells correlating with pro-inflammatory cytokines, whereas cluster 3 disclosed an activated phenotype (becoming specialized effector T cells). This may suggest that patients with PolyA require a differential approach in their management, and further clinical trials including evaluation of these targets are warranted.

The shortcomings of our study are acknowledged. This cross-sectional analysis reflects the frequency of PolyA in patients with the most common ADs attending our rheumatology clinics. Other non-rheumatic autoimmune and inflammatory disorders should be evaluated. Analysis of latent autoimmunity was restricted to the autoantigen array chip. Inclusion of other antigens as well as IgA autoantibodies may improve clustering of PolyA and therefore the classification of ADs.

In conclusion, this proof-of-concept study indicates that ADs can be classified according to PolyA. Latent and overt PolyA cluster together. Differential clinical and immunological characteristics were observed among clusters. Our results also add further evidence on the commonalities among ADs. PolyA should be considered in all studies dealing with ADs, including epidemiological, genetic, and clinical trials.

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Author contributions

Conceptualization: JMA; Acquisition of data: MRJ, EZ, ANP, ASA, CP, WZR, MAV, MR, JMA; Methodology: YAA, DMM, LJRS, QZL, MR, CRS, JMA; Statistical Analysis: MR, CZ; Funding acquisition: CRS, JMA; Project administration: CRS, JMA; Supervision: JMA; Writing – review & editing: MR, CRA, YAA, DMM, JMA. All authors read and approved the final version of this manuscript.

Patient and public involvement

Patients or the public were not involved in the design, or conduct, or reporting, or dissemination plans of our research.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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