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The interaction between anti-Ro/SSA and anti-La/SSB autoantibodies and anti-infectious antibodies in a wide spectrum of auto-immune diseases: another angle of the autoimmune mosaic

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Abstract

Objective

The presence of anti-Ro/SSA and anti-La/SSB antibodies has been linked with autoimmunity in general and with several autoimmune diseases (AID) in particular. In the current study we evaluated these antibodies in a wide spectrum of AID as well as the links between them and anti-infectious antibodies

Methods

We examined 2082 sera from patients with 16 different AID compared to 524 sera from geographically-matched healthy controls, for the presence and titres of anti-Ro/SSA and anti-La/SSB. All samples were also tested for a variety of anti-infectious agents' antibodies using the BioPlex 2200-immunoassay (Bio-Rad, USA).

Results

Anti-Ro/SSA was more prevalent, with significantly higher titre in 5 autoimmune diseases namely Sjögren's syndrome (SS), systemic lupus erythematosus (SLE), antiphospholipid syndrome (APS) both primary and APS linked to SLE, systemic sclerosis (SSc) and primary biliary cirrhosis (PBC). Anti-La/SSB was more prevalent with higher titers in SS, SLE, APS linked to SLE and PBC. Prevalence, but not titers, of both antibodies were higher also in polymyositis (PM). Additionally, we found a correlation between anti-Ro/SSA antibodies and antibodies of the IgM and IgG subtypes directed at cytomegalovirus as well as IgG-antibodies directed at Epstein-Barr virus (EBV) and toxoplasma ($p<0.001$). Anti-La/SSB antibodies correlated with the presence of IgG antibodies against EBV early antigen ($p<0.001$).

Conclusion

In a large cohort of patients with autoimmune diseases we found an association between anti-Ro/SSA and anti-La/SSB antibodies and 6 autoimmune diseases, amongst which primary APS and PM. Additionally, we observed linkages between these autoantibodies and anti-infectious antibodies directed at Epstein-Barr virus, toxoplasma and cytomegalovirus. Our findings support the concept of interplay between infectious agents and autoimmunity, such as the plausibility of an infectious agent that trigger the immune system to produce specific antibodies which will later result in a unique group of AID.

Key words

anti-Ro/SSA antibody, anti-La/SSB antibody, autoimmunity, Sjögren's syndrome, antiphospholipid syndrome, systemic lupus erythematosus

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Introduction

Anti-Ro/SSA and anti-La/SSB autoantibodies have been traditionally described as the serological hallmark of Sjögren's syndrome (SS) and a few other autoimmune diseases (AID). Moreover their presence is an integral part of the diagnosis of SS according to the older American-European Consensus Group diagnostic criteria for SS (1, 2) and the newer American College of Rheumatology (ACR) diagnostic criteria (3). The association of Anti-Ro/SSA with systemic lupus erythematosus (SLE), sub-acute cutaneous lupus, neonatal lupus syndrome (4) and less often systemic sclerosis (SSc) and myositis (5) were previously described.

The primary target antigen Ro/SSA was identified as protein components of the cytoplasmic ribonucleoprotein complexes (hY-RNA complexes) the Ro60 kDa antigen was identified in 1988 by Deutscher *et al*. (6) and shortly afterwards the Ro52kDa antigen by Ben Chetrit *et al*. (7). But only in 1991, was it confirmed by Chan *et al*. (8) that both Ro52 and Ro60 (SS-A) antigens indeed consisted of two different proteins coded by different complementary DNAs (cDNA) and both serve as the targets of anti-Ro/SSA antibodies. The La protein, a transcription termination factor of the RNA polymerase III transcripts, is the target of anti La/SSB antibody. The coexistence of both Anti-Ro/SSA and anti-La/SSB has been explained in the context of epitope spreading between components of the Ro and La ribonucleoprotein, finding that was also supported in animal models (9).

Anti-Ro/SSA and anti-La/SSB have been related to specific clinical symptoms such as the 'Sicca syndrome' and photosensitive rash (10, 11). Moreover, in the seminal study by Arbuckle MR *et al*. (12) the presence of anti-Ro/SSA and anti-La/SSB preceded the development of clinical systemic lupus erythematosus (SLE) by many years and was considered an early marker of autoimmunity. The appearance of those antibodies antedating other autoantibodies and clinical disease was also reported in patients with SS (13-17).

Anti-Ro/SSA and anti-La/SSB antibodies were linked to exposure to infectious agents *via* several mechanisms such as molecular mimicry to Epstein-Barr virus (EBV), Hepatitis A and C and adenovirus (15, 18-22). Another study provided evidence that cytomegalovirus (CMV) infection induced 60 KD/Ro antigen expression on the surface of human keratinocytes (23), while both EBV and CMV were suggested as triggers of SLE and SS diseases (24-26). Hence, in the current study, we evaluated the prevalence of anti-SSA/Ro and anti-SSB/La among a large cohort of patients with various AID in comparison to healthy controls. In addition, we analysed in this cohort the correlation between the presence of anti-SSA/Ro and anti-SSB/La to the presence of a variety of anti-infectious-agents antibodies.

Methods

Sera collection

In this cross-sectional study we analysed 2606 serum samples from 2082 patients with autoimmune conditions and 524 healthy controls. Patients were followed and classified according to the ACR classifications and by their local physicians either in Europe or in South-America. 812 patients were from South American including 199 patients with SLE, 82 patients with SS, 152 patients with rheumatoid arthritis (RA), 180 patients with type 1 diabetes mellitus (DM), 199 patients with multiple sclerosis (MS). While 1270 European patients consisted of 101 with polymyositis (PM), 98 with primary antiphospholipid syndrome (APS), 63 with antiphospholipid syndrome associated with SLE (APS+SLE), 91 with SLE, 78 Hepatitis C virus associated cryoglobulinaemic vasculitis (HCV+Cryo), 80 with SSc, 35 with RA, 69 with primary biliary cirrhosis (PBC), 119 with inflammatory bowel disease (IBD), 197 with autoimmune thyroid disease (Graves' disease and Hashimoto's thyroiditis), 173 with ANCA associated vasculitis (AAV), 35 with giant cell arteritis (GCA), 41 with AID associated mixed cryoglobulinaemic vasculitis (MIXC) (27) and 90 with coeliac diseases. Of the 524 matched controls, 277 were from South American and 247 were European. Controls were healthy individuals with no known inflamma-

tory or AID, or a history of a chronic infectious disease, including tuberculosis and human immunodeficiency virus. Patients in the HCV+Cryo tested positive for Hepatitis C virus (HCV) infection while patients in the MIXC tested negative for HCV. The study protocol was approved by the Ethical Review Board of our institution and procedures were in accordance with the ethical standards laid down in Helsinki Declaration, as revised in 2000.

Autoantibody testing

The Bio-Rad BioPlex 2200 (Bio-Rad Laboratories, Hercules CA, USA) system is an automated analyser that uses multiplex bead technology to simultaneously detect antibodies to several antigens in a single tube. This device uses 25 different 8-µm magnetic beads, which are dyed with two fluorophores for classification purposes. Each bead is coated with specific proteins, according to the different assay being tested, thus representing a different target antigen. The amount of antibody bound to the bead was determined by fluorescence analysis; raw data were subsequently converted to the fluorescence ratio using a pre-dyed internal normaliser the detector signal. Elevated titres were determined as above the cut-off of 2 standard deviations from the normal control. The technology applied in this work had already been published and evaluated prior to this study in previous publications by our group as well as in by others (28-30). The BioPlex 2200 ANA Screen is intended for the qualitative screening of ANA, the quantitative detection of antibody to dsDNA, and the semi-quantitative detection of separate antibodies (Chromatin, Ribosomal P, Ro/SSA and La/SSB, Sm, Sm/RNP, RNP, Scl-70, Jo-1, and Centromere B).

Anti-infectious antibody testing

Anti-infectious agents were tested by two methods:

1. *Multiplexed assay*: Screening for IgG and IgM antibodies against Epstein-Barr virus (EBV), Toxoplasma gondii, and cytomegalovirus (CMV), Rubella, and Treponema were performed using the Bio-Rad BioPlex 2200. The EBV kit uses three different populations of beads screening for Abs directed against EBV nuclear antigen (EBVNA), EBV viral capsid antigen (EBVCA), and EBV early antigen diffuse (EBVEA). For syphilis the beads screen for Abs against different epitopes of the TPr protein from Treponema. Finally, ToRC packs screen for antibodies against Toxoplasma gondii, Rubella and CMV.

2. *Enzyme immunoassay*: Anti-HCV (recombinant hepatitis C virus antigen: c22-3, c200 and NS5) was tested using the HCV encoded antigen ORTHO HCV v. 3.0 ELISA test system (Johnson & Johnson, Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Briefly, sera were added at dilution of 1:21 to hepatitis C recombinant c22-3, c200 and NS5 antigen-coated ELISA plates and the binding was detected by anti-human-IgG peroxidase and appropriate substrate. Positive results were calculated according to the manufacturer's equations for cut-off value determination. Antibodies to hepatitis B virus core protein (recombinant HBc antigen) and *Helicobacter pylori* (HP) were tested using MONOLISA anti-HBc plus commercial kit and MONOLISA pylori - IgG commercial kit (Bio-Rad, Hercules, CA), respectively, according to the manufacturer's instructions.

Of note, in the analysis of anti-infectious agents' antibodies we considered differences between groups, only according to titres above the manufacturer protocol, similarly to clinical practice; In other words different titres below positivity cut-off may not be of clinical significant.

Table I. The prevalence of Anti-Ro/SSA and anti-La/SSB antibodies in different autoimmune diseases.

Autoimmune disease	Anti-Ro/SSA	Anti-La/SSB	*p*-value	
			Anti Ro/SSA *vs*. Control*	Anti La/SSB *vs*. Control**
European subjects				
Controls n=247	0.8%	0.8%		
APS n=98	**12.2%**	1%	**<0.000001**	NS
APS associated with SLE n=63	**28.5%**	**9.5%**	**<0.000001**	**0.001>**
SLE n=91	**49.4%**	**18.7%**	**<0.000001**	**<0.000001**
SSc n=80	**11.2%**	3.8%	**0.0001>**	NS
RA n=35	2.9%	0%	NS	NS
HCV+ Cryoglobulinaemia n=78	**12.8%**	**6.4%**	**0.00001>**	**0.01>**
MIX. Cryoglobulinaemia n=41	2.4%	2.4%	NS	NS
Polymyositis n=101	**35.6%**	**14.8%**	**<0.000001**	**<0.000001**
PBC n=69	**7.2%**	**7.2%**	**<0.01**	**<0.01**
IBD n=119	0%	0%	NS	NS
AITD n=197	1.5%	1.5%	NS	NS
AAV n=173	1.7%	0.6%	NS	NS
GCA n=35	0%	0%	NS	NS
Coeliac n=90	0%	2.2%	NS	NS
South-American subjects				
Controls n=277	3.30%	2.20%		
SLE n=199	**32.2%**	**9.5%**	**<0.000001**	**0.001>**
SS n=82	**62.2%**	**37.8%**	**<0.000001**	**<0.000001**
RA n=152	**9.9%**	3.9%	**0.01>**	NS
DM1 n=180	1.1%	0.6%	NS	NS
MS n=199	2%	2%	NS	NS

SLE: systemic lupus erythematosus; SS: Sjögren's syndrome; RA: rheumatoid arthritis, DM1: diabetes mellitus type 1; MS: multiple sclerosis; APS: antiphospholipid syndrome; SSc: systemic sclerosis; HCV+ cryoglobulinaemia: hepatitis C virus associated cryoglobulinaemic vasculitis; PBC: primary biliary cirrhosis; IBD: inflammatory bowel disease; AITD: autoimmune thyroid disorders, GCA: giant cell arteritis; MIXC: mixed cryoglobulinaemia; NS: not significant; AAV: ANCA-associated vasculitis includes the following granulomatosis with polyangiitis (Wegener's) (GPA), microscopic polyangiitis (MPAN) and eosinophilic granulomatosis with polyangiitis (Churg-Strauss syndrome).

*p-value for the comparison between the prevalence of anti Ro/SSA antibodies in autoimmune disease patients compare with healthy control.

**p-value for the comparison between the prevalence of anti La/SSB antibodies in autoimmune disease patients compare with healthy control.

Statistical analysis

Data was analysed using SPSS version 22.0 software. Mean and standard deviation (SD) were calculated for quantitative variables. The percentage and titres of each autoantibody was compared between groups. Statistical tests used were student's *t*-test, Pearson chi-squared, Fisher's exact test, and spearman correlation test as appropriate. Tests were considered significant when *p*-value was <0.05.

Results

In the current study we analysed sera from 2082 patients with 16 different AID compared to 524 geographically matched healthy controls, for the presence and titres of anti-Ro/SSA and anti-La/SSB and their correlation to a profile of anti-infections antibodies.

Anti-Ro/SSA and anti-La/SSB prevalence in different autoimmune diseases

Out of 16 AID, prevalence and titers of anti-Ro/SSA were significantly higher among 5 autoimmune diseases ($p<0.001$) namely Sjögren's syndrome, SLE, APS both primary disease and APS linked to SLE, systemic sclerosis (SSc), and primary biliary cirrhosis (PBC). Prevalence and titers of anti-La/SSB were significantly higher ($p<0.001$) in Sjögren's syndrome, SLE, APS linked to SLE and PBC as described in Table I. It is of note, that in polymyositis (PM) as well as in HCV+Cryo the prevalence but not titres of both autoantibodies were found to be higher among European patients.

In contrast, both anti-Ro/SSA and anti-La/SSB were not found to be more prevalent among patients with IBD, AITD, AAV, coeliac, GCA, and MS and type 1 DM.

Notably, the prevalence of anti-Ro/SSA and anti-La/SSB differ significantly amid the two control groups with higher prevalence among controls from South America compare to Europeans (Table I). This observation was previously reported by our group (31, 32).

In addition, we analysed the association of anti-Ro/SSA and anti-La/SSB among the different patient populations and compared them to controls. Notably as specified in Table I, other than the conditions reported earlier in the results section, we tested all sera for distinctive positivity to anti-Ro52, anti-Ro60 and total anti-Ro/SSA. The data is not specified as specific serology to Ro52/Ro60 autoantibodies was in agreement with the positivity found to the total Anti-Ro/SSA. Not surprisingly, we noticed as others before us (8, 33), that some patients were seropositive to a single epitope (-Ro52 or -Ro60) while others were seropositive to both.

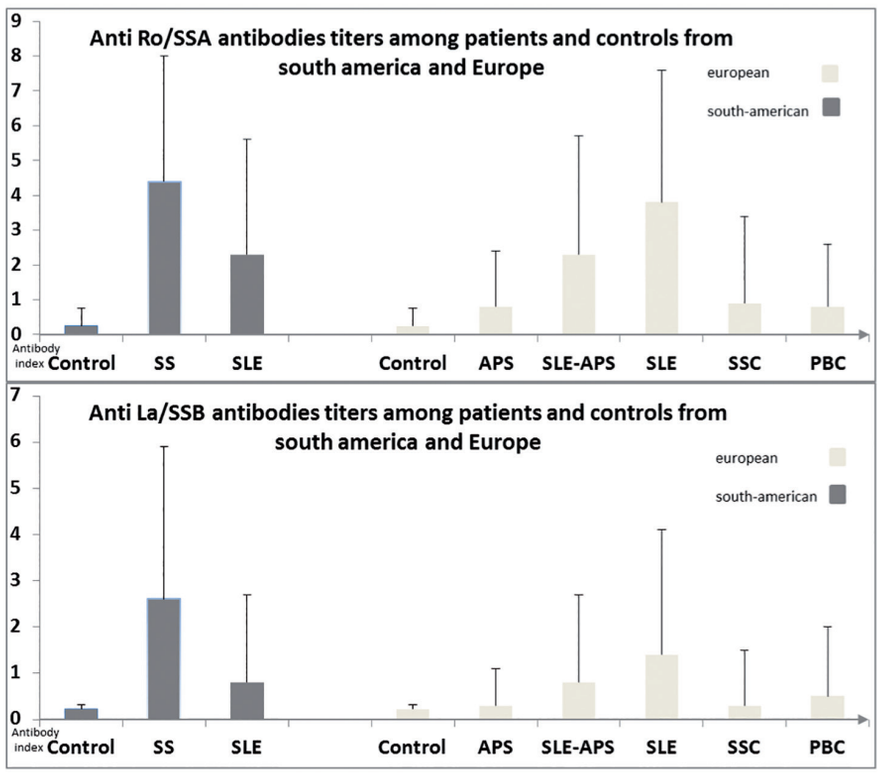

Fig. 1. Titre of anti Ro/SSA and anti La/SSB in various autoimmune diseases.
SLE: systemic lupus erythematosus; SS: Sjögren's syndrome; APS: antiphospholipid syndrome; SSc: systemic sclerosis; PBC: primary biliary cirrhosis.

Titre of anti Ro/SSA and anti La/SSB in various autoimmune diseases

Significant differences between titres of anti-Ro/SSA and anti-La/SSB in several conditions are described in Figure 1. Titres for both antibodies reached statistical significance in patients with SLE, SS, APS+SLE, PBC and SSc. Additionally we found high titres of anti-Ro/SSA only in primary APS. Generally high antibody titres were found in the same conditions that exhibited high antibody prevalence. Similarly, in accordance with their prevalence, also the titres of the autoantibodies were greater in South American controls compared to European controls.

Interactions between anti Ro/SSA and anti La/SSB autoantibodies and anti-infectious agents

We analysed the associations between a wide profile of anti-infectious antibodies and anti-Ro/SSA and anti-La/SSB autoantibodies (Table II). We found positive correlation between anti-Ro/SSA and antibodies of the IgM-subtype directed at cytomegalovirus as well as of the IgG-subtype directed towards toxoplasma, cytomegalovirus and Epstein-Barr virus. Anti-La/SSB antibodies correlated only with the presence of IgG antibodies against EBV early antigen. Interestingly, an inverse correlation was found between anti-Ro/SSA and anti-HCV antibodies. Similar observation was noted for anti -La/SSB, although for these autoantibodies the effect did not reach statistical significance.

Discussion

The links between autoimmunity (*e.g.* the presence of autoantibodies), auto-

Table II. Association between the presence of anti-infectious antibodies and the presence of anti-Ro/SSA and anti-La/SSB.

Anti-infectious antibodies (IgM or IgG) (+) seropositive (-) seronegative		Prevalence of Anti-Ro/SSA	*p*-value* Anti-Ro/SSA	Prevalence of Anti-La/SSB	*p*-value** Anti-La/SSB
Rub IgM(+)	n=470	15.0%	NS	6.4%	NS
Rub IgM(-)	n=1095	12.8%		5.4%	
Rub IgG(+)	n=2774	9.9%	NS	4.7%	NS
Rub IgG(-)	n=269	8.9%		5.2%	
CMV IgM(+)	n=440	**18.6%**	**<0.001**	7.3%	NS
CMV IgM(-)	n=1130	**11.6%**		5 %	
CMV IgG(+)	n=2358	**11.0%**	**<0.001**	4.9%	NS
CMV IgG(-)	n=685	**6.0%**		4.2%	
EBVNA(+)	n=2687	**10.5%**	**<0.001**	4.8%	NS
EBVNA(-)	n=344	**5.2%**		4.6%	
EBVCA(+)	n=2696	**10.7%**	**<0.01**	5.0%	NS
EBVCA(-)	n=335	**3.9%**		3.3%	
EBVEA(+)	n=828	**20.8%**	**<0.001**	**9.5%**	**<0.001**
EBVEA(-)	n=2202	**5.9%**		**3.0%**	
HBV Core IgG (+)	n=210	11.4%	NS	3.3%	NS
HBV Core IgG (-)	n=1771	12%		5.4%	
HCV IgG (+)	n=116	**8.6%**	**NS**	2.6%	NS
HCV IgG (-)	n=1447	**13.4%**		5.7%	
HP IgG (+)	n=823	10.8%	NS	6.1%	NS
HP IgG (-)	n=613	9.1%		3.9%	
Toxo IgM(+)	n=58	17.2%	NS	5.2%	NS
Toxo IgM(-)	n=1512	13.4%		5.7%	
Toxo IgG(+)	n=1212	**12.5%**	**<0.001**	5.6%	NS
Toxo IgG(-)	n=1831	**8.1%**		4.2%	
Syph(+)	n=152	7.2%	NS	6.6%	NS
Syph(-)	n=2888	10.0%		4.7%	

*p-value for the comparison between the prevalence of anti Ro/SSA antibodies in seropositive patients for the tested anti infectious antibody compare with seronegative patients.
**p-value for the comparison between the prevalence of anti La/SSB antibodies in seropositive patients for the tested anti infectious antibody compare with seronegative patients.
Rub: rubella; CMV: cytomegalovirus; EBVNA: Epstein-Barr virus IgG antibody to nuclear antigen; EBVCA: Epstein-Barr virus IgG antibody to viral capsid antigen; EBVEA: Epstein-Barr virus early antigen diffuse; HBV: hepatitis B virus; HCV: hepatitis C virus, HP-Helicobacter Pylori; Toxo: Toxoplasma Gondii; Syph: Treponema Pallidum antigen; NS: not significant.

immune diseases and infectious agents have been extensively studied (34-37). Herein we report associations of the same nature between anti-Ro/SSA and anti-La/SSB antibodies anti-infectious antibodies in a large cohort of patients with various autoimmune diseases.

We found an association between these antibodies and 6 autoimmune diseases namely SS, SLE, SSc, PBC, APS and PM. These associations are well established for 4 diseases (namely SS (1, 38-40), SLE (41, 42), PBC (43-45), SSc (5, 46)) that were further consolidated in this large cohort. In addition, new association between anti-Ro/SSA and primary APS as well as between anti-La/SSB and PM were described in this study for the first time to the best of our knowledge.

It is of interest that the 6 AID related to anti-Ro/SSA (SS, SLE, PBC, SSc, APS, PM) are interlinked and may overlap both clinically and serologically. One plausible explanation for having high anti-Ro/SSA in patients with no overt clinical manifestations of SS or SLE is that those patients are already suffering of mild SS overlap or are about to develop SS in the future but do not present the clinical features of SS. This explanation is consistent with the findings of Theander *et al*., (15) that the mere serological existences of anti-Ro/SSA and anti-La/SSB are predictive of later development of SS. Another potential explanation is that diseases with a high presence of anti-Ro/SSA, share a similar unique pathogenic background unlike other autoimmune diseases evaluated in this study, *e.g*. AAV, type 1 DM, IBD, coeliac, and GCA. This mutual pathogenic background leads later on to a spectrum of diseases sharing mutual clinical symptoms, *i.e*. Raynaud's phenomena and arthralgia, and also a greater tendency to clinically overlap, *i.e*. PBC-SS, SSc-PM, etc.

The data about Anti-La/SSB prevalence in different inflammatory diseases is less abundant. This antibody which is thought to be more specific to SS interact with a 47-kD protein, which shuttles between the nucleus and cytoplasm but which is predominantly found in the nucleus (47). Our finding of increased prevalence of anti-La/SSB not only in SS, SLE and PBC, but also in PM, is important and further supports the notion that PM is in overlap with other connective tissue diseases.

An additional interesting finding was the increased prevalence of anti-Ro/SSA in HCV+Cryo. This finding might be explained by secondary Sjögren's syndrome found in up to 20% of the populations with cryoglobulinaemia (48, 49). In this study we found an association between anti-infectious agents' antibodies and the existence of anti-Ro/SSA. This was especially noticeable among patients exposed to CMV and EBV. The link between SLE and SS to previous infection with EBV was reported both by our group and by others (50-53), and so was the link between these autoimmune diseases and CMV (23, 54). In line with this concept Poole *et al*. (55) showed that immunisation of rabbits with epitopes derived from EBV (EBNA-1 58-72 peptide) develop anti-ENBA as well as specific anti-RO autoantibodies followed by clinical manifestation of SLE-SS-like disease. Hence, one may suggest that a past exposure to those infectious agents, can lead to the presence of anti-Ro/SS, which serves as marker for early autoimmunity (13, 15)

and as a preliminary stage in the later development of an AID. Why would one patient develop a certain AID out of the 6 AID related to anti-Ro/SSA described earlier and not another? This might be related to exposure to other environmental factors and/or exposure to other infectious agents as well as different genetic background. Unlike Anti-Ro/SSA, anti-La/SSB, correlated only with EBV-early antigen (EBVEA). This direct correlation between anti-La/SSB and EBVEA is reported herein for the first time to the best of our knowledge. However a related finding of high levels of EBVEA in SS patients was recently published (53), Further supporting the theory that in SS patients a subclinical EBV reactivation may trigger or perpetuate articular involvement.

The current study evaluated specific autoantibodies in a large cohort including patients with different AID and controls in order to compare between these AID. However it is limited by its retrospective design, the absence of exact matching between patients' groups as well as association with clinical data. Notably, a more particular analysis regarding each group of patients matched with controls was analysed in former studies from our group which dealt more thoroughly with different sub-groups and specific clinical manifestations relevant to each entity (27, 56, 57).

To summarise, in this study done on a very large group of patients we consolidated the link between the presence of anti-Ro/SSA and anti-La/SSB antibodies and 6 autoimmune conditions, including the less reported links between anti-Ro/SSA and primary APS or anti-La/SSB and PM. We also reported of an association between exposure to certain infectious agents and a higher prevalence of anti-Ro/SSA and anti-La/SSB antibodies. Those findings led us to theorise that exposure to certain infectious agents can lead to the development of anti-Ro/SSA and anti-La/SSB antibodies in susceptible subjects which may further evolve into certain AID that share a common denominators and possibly common pathogenesis.

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