
α -enolase is an antigenic target in primary Sjögren's syndrome

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ABSTRACT

Objective. Although anti-cyclic citrullinated peptides antibodies are specific markers for rheumatoid arthritis (RA), they might also be present in other diseases. Our aim was to assess the native or citrullinated antigens recognised by patients with primary Sjögren's syndrome (pSS) and to evaluate their association with clinical and serological features.

Methods. In an initial screening, we assessed the serum reactivity of 12 patients with pSS against native or in-vitro citrullinated antigens of HEp-2 cells by immunoblotting. We identified a 47kDa band, which was preferentially recognised and corresponded to α -enolase. Thus, levels of IgA and IgG anti-native and citrullinated α -enolase antibodies were measured in 50 pSS patients, 20 RA patients and 20 healthy subjects (HS) by ELISA.

Results. We identified α -enolase as a predominant antigen recognised in pSS. These patients had higher levels of anticitrullinated α -enolase IgG antibodies compared with RA patients or HS ($p=0.003$ and $p<0.0001$, respectively). Furthermore, there was an increase of IgG anti-citrullinated α -enolase versus IgG anti-non-citrullinated α -enolase antibodies in pSS patients ($p=0.001$), by contrast no difference was found in RA. The presence of IgA and IgG anti-non-citrullinated and anti-citrullinated α -enolase antibodies were not associated with any clinical manifestation whatsoever, including non-erosive arthritis among pSS, but an association of IgA anti-citrullinated α -enolase with anti-Ro/SSA antibodies was found.

Conclusion. We characterised α -enolase as a dominant antigen in lysates of HEp-2 cells in pSS. Nevertheless, their precise role in pSS remains to be elucidated.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease associated with infiltration of salivary and lacrimal glands by mononuclear cells. Consequently, patients with pSS presented irreversible damage of salivary and lacrimal glands, resulting in oral and ocular dryness (1), and extra-glandular manifestations in among 60% of the patients (2). This entity is characterised by the presence of various serum antibodies such as anti-Ro/SSA, anti-La/SSB, anti-nuclear antibodies (ANA), rheumatoid factor (RF) and cryoglobulins (3). On the other hand, the presence of other antibodies is less frequent, including anti-citrullinated protein antibodies (ACPA) (2, 4).

ACPA are highly specific for rheumatoid arthritis (RA) (5-6) and correspond to a collection of antibodies with overlapping and non-overlapping reactivity, which are detectable in sera of patients with early RA even several years before clinical manifestations, and have been associated with a severe disease outcome (7-8). Diverse studies have described antibodies against different epitopes of citrullinated proteins and relationship with clinical features in RA (9-11). In this sense, radiographic progression has been associated with anti-citrullinated vimentin antibodies (12), anti-citrullinated fibrin antibodies as well as with anti-citrullinated α -enolase antibodies (13-14).

On the other hand, the prevalence of ACPA in pSS ranges from 3–22% (15-22). Some studies have found no differences between patients with pSS and the presence or absence of ACPA in terms of synovitis (15-17, 20), whereas others have found a positive association (18-19, 21-22). Nevertheless, the knowledge about the reactivity against intracellular citrullinated proteins in pSS has been scarcely studied. In this

sense, a citrullinated α -enolase peptide (CEP-1) was recently identified as a major antigenic target of ACPA in patients with pSS. (23).

Thus, the aim of this study was to identify the cellular citrullinated antigens recognised by pSS patients and to establish their association with clinical manifestations and laboratory data.

Patients and methods

Patients

Serum samples were obtained from 50 randomly selected patients with a diagnosis of pSS according the AECG criteria (3). Control serum samples were obtained from 20 patients with RA according the 2010 ACR criteria (24) and 20 healthy subjects. The study was approved by the Institutional Committee of Biomedical Research, all patients and controls signed an informed consent, and all procedures followed the Helsinki Declaration of 1975/83.

Rheumatoid factor, anti-Ro/SSA and anti-La/SSB antibodies were tested by ELISA (Orgentec, Mainz, Germany). ACPA were tested using anti-cyclic citrullinated peptide-3 (anti-CCP3) (QUANTA Lite Inova Diagnostics, San Diego, CA, USA) according to the manufacture's specifications.

Culture and homogenisation of HEp-2 cells

HEp-2 cells were cultured in RPMI (GIBCO, Life Technologies, Grand Island, NY, USA) with 10% foetal bovine serum (GIBCO, Life Technologies, Grand Island, NY, USA). Cells were lysed at 5.0×10^6 cells per 200 μ L of lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1.0 mM dithiothreitol (DTT) 1mM phenylmethylsulphonyl fluoride (PMSF). Next, the samples were frozen and thawed three times and then centrifuged at 4°C for 10 minutes. The supernatants were collected and its protein concentration was determined using bicinoninic acid method.

In-vitro citrullination of cell lysate

Citrullination was performed as described previously (25). Briefly, cell lysate was placed at a concentration of 0.86 mg protein/mL in peptidyl arginine deiminase (PAD) buffer (0.1M

Tris-HCl, pH 7.4, 10 mM $CaCl_2$, 5 mM DTT, 1 mM PMSF) and was citrullinated *in-vitro* with rabbit muscle PAD (0.5 U/mg of protein) (SIGMA, St Louis, MO, USA) for 3 hours at 55°C. The reaction was stopped by addition of 0.5 M EDTA to a final concentration of 20 mM. The same treatment was done for the non-citrullinated lysate except the addition of PAD. In addition α -enolase (ABNOVA, Walnut CA, USA) was citrullinated in the same conditions.

Immunoblotting

The samples were separated on 10% SDS-PAGE and then transferred to nitrocellulose membranes, blocked with 3% non-fat milk in PBS/0.2% Tween, and incubated with human serum at a 1:100 dilution. Membranes were washed three times with PBS/0.2% Tween and incubated with IgG anti-human or IgA anti-human conjugated with alkaline-phosphatase. After a further wash, membranes were developed with 2 mg/mL fast red (SIGMA, St Louis, MO, USA) and 1 mg/mL naphthol phosphate (SIGMA, St Louis, MO, USA). Citrullinated proteins were identified with an anti-citrulline modified (anti-CM) kit (Upstate Biotechnology, Temecula, CA) according to the manufacturer's instructions.

Two-dimensional electrophoresis (2DE)

The samples were desalted with protein desalting spin columns (Thermo Scientific, Meridian, CA) and dissolved in rehydration buffer (7 M urea, 2 M thio-urea, 4% CHAPS, 50 mM DTT, 2.0% carrier ampholyte [Bio-Rad, Hercules, CA, USA]). Then, samples were loaded in focusing tray (Bio-Rad, Hercules, CA, USA) and pH 4 to 7 IPG strips 7 cm long (Bio-Rad, Hercules, CA, USA) were gently placed gel side down onto samples. Rehydration was performed with PROTEAN IFE cell (Bio-Rad, Hercules, CA, USA) at 50 V for 16 hours. After rehydration was complete, wet papers wicks (Bio-Rad, Hercules, CA, USA) were placed between the IPG strip and the electrodes. Isoelectric focusing was performed in PROTEAN IFE cell (Bio-Rad, Hercules, CA, USA) at 250 V for 15 minutes and ramped 4000V

Table I. Clinical and serologic features of the SS patients.

Variable	Total
Females, n (%)	49 (98)
Age in years (mean $\pm$ SD)	56 $\pm$ 11.2
Disease duration in years, (mean $\pm$ SD)	12.6 $\pm$ 11
Smoking, n (%)	4 (8)
Non-erosive arthritis, n (%)	27 (54)
Raynaud's phenomenon, n (%)	9 (18)
Lung involvement, n (%)	15 (30)
Skin vasculitis, n (%)	5 (10)
Neurological involvement, n (%)	14 (26)
Lymphadenopathies, n (%)	17 (34)
Parotid enlargement, n (%)	28 (56)
Renal involvement, n (%)	2 (4)
Lymphoma, n (%)	2 (4)
Antinuclear antibodies, n (%)	44 (88)
Rheumatoid factor, n (%)	18 (36)
Median, cumulative ESSDAI score	9 (2-29)
Anti-Ro/SSA antibody, n (%)	37 (74)
Anti-La/SSB antibody, n (%)	13 (26)
Anti-CCP IgG, n (%)	2 (4)

in a linear fashion and maintained two hours. When the electrophoresis was completed, IPG strips were placed in an equilibration tray (Bio-Rad, Hercules, CA, USA). Prior to the two dimensional electrophoresis, IPG strips were equilibrated with two SDS-containing buffers, equilibration buffer I (Bio-Rad, Hercules, CA, USA) with DTT which reduces sulfhydryl groups, and equilibration buffer II with iodoacetamide which alkylates the reduced sulfhydryl groups. The IPG strips were transferred to a 12% SDS-PAGE and run at 200 V for 40 minutes. Gels were transferred to nitrocellulose membranes and incubated with human serum as described above. Citrullinated proteins were identified with quimioluminescence. Further gels were stained with Coomassie blue.

Mass spectrometry

Samples were reduced with DTT, alkylated with iodoacetamide and digested in gel with trypsin. Obtained peptide were applied in LC-MS with nanoflow pump EASY -nLC II (Thermo-Fisher Co. San Jose, CA, USA) system, coupled to a mass spectrometer LTQ-Orbitrap Velos (Thermo-Fisher Co. San Jose, CA, USA) with ionisation system type nano-electrospray.

The spectrometric data were subjected to search in NCBI database through the Protein Prospector programme, and

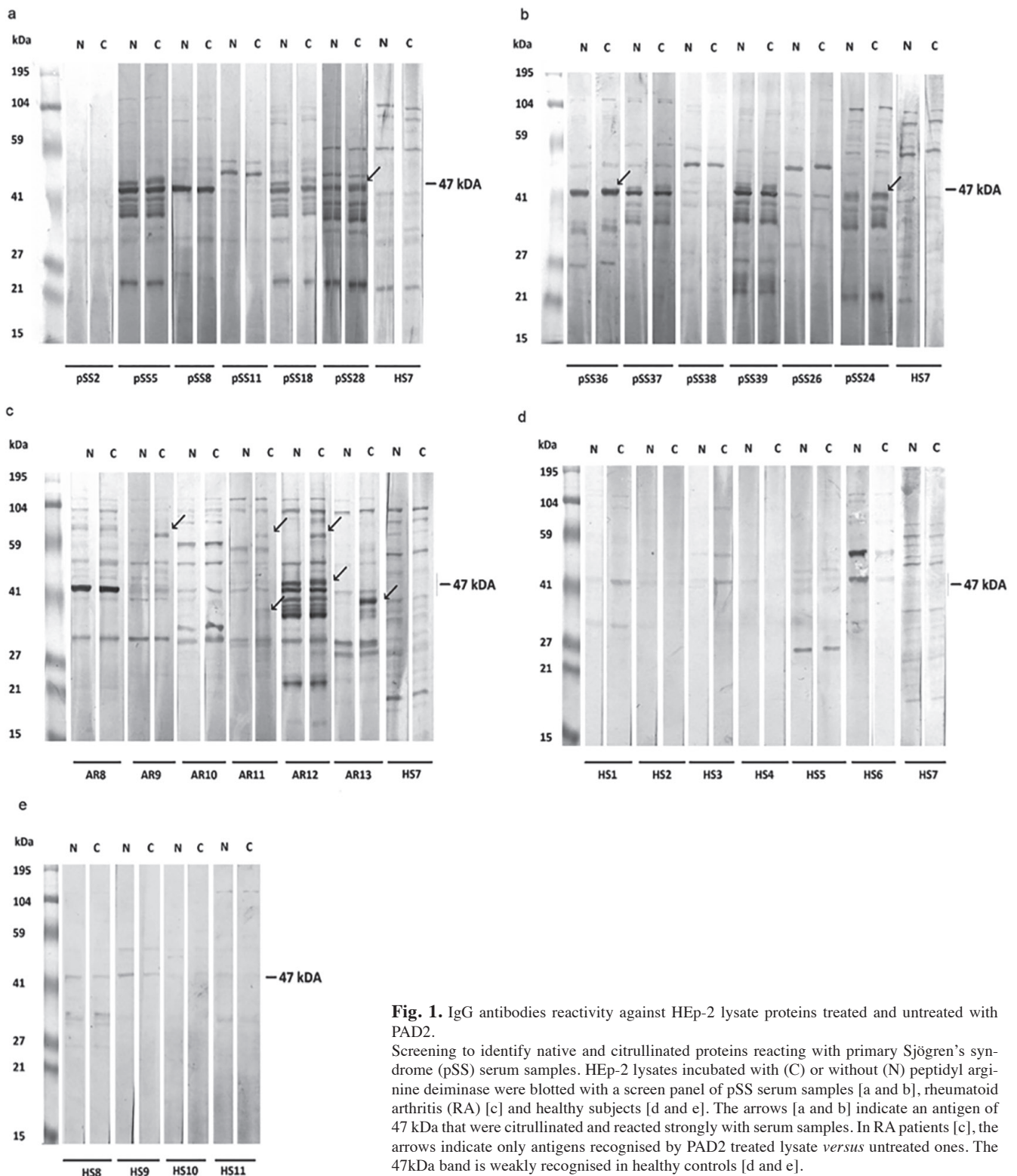


Fig. 1. IgG antibodies reactivity against HEP-2 lysate proteins treated and untreated with PAD2. Screening to identify native and citrullinated proteins reacting with primary Sjögren's syndrome (pSS) serum samples. HEP-2 lysates incubated with (C) or without (N) peptidyl arginine deiminase were blotted with a screen panel of pSS serum samples [a and b], rheumatoid arthritis (RA) [c] and healthy subjects [d and e]. The arrows [a and b] indicate an antigen of 47 kDa that were citrullinated and reacted strongly with serum samples. In RA patients [c], the arrows indicate only antigens recognised by PAD2 treated lysate *versus* untreated ones. The 47kDa band is weakly recognised in healthy controls [d and e].

restricted UniProt PDB Human Proteome in Discoverer 1.4 search.

Enzyme-linked immunosorbent assay (ELISA) for α -enolase

ELISA plates (Costar, Corning, NY, USA) were coated with 0.1 μ g/mL of

non-citrullinated α -enolase (ABNO-VA, Montluçon, France) or citrullinated α -enolase (see above) in carbonate-bicarbonate buffer (0.1 M, pH 9.5) overnight at 4°C. Plates were washed with wash solution (PBS/0.2% Tween) three times, blocked with 1.5% albumin in

wash solution for an hour, and probed with 1:100 diluted human serum for 2 hours at 37°C. All samples including those of controls were analysed on the same plate. Plates were washed three times for 5 minutes and incubated with alkaline phosphatase-conjugated anti-

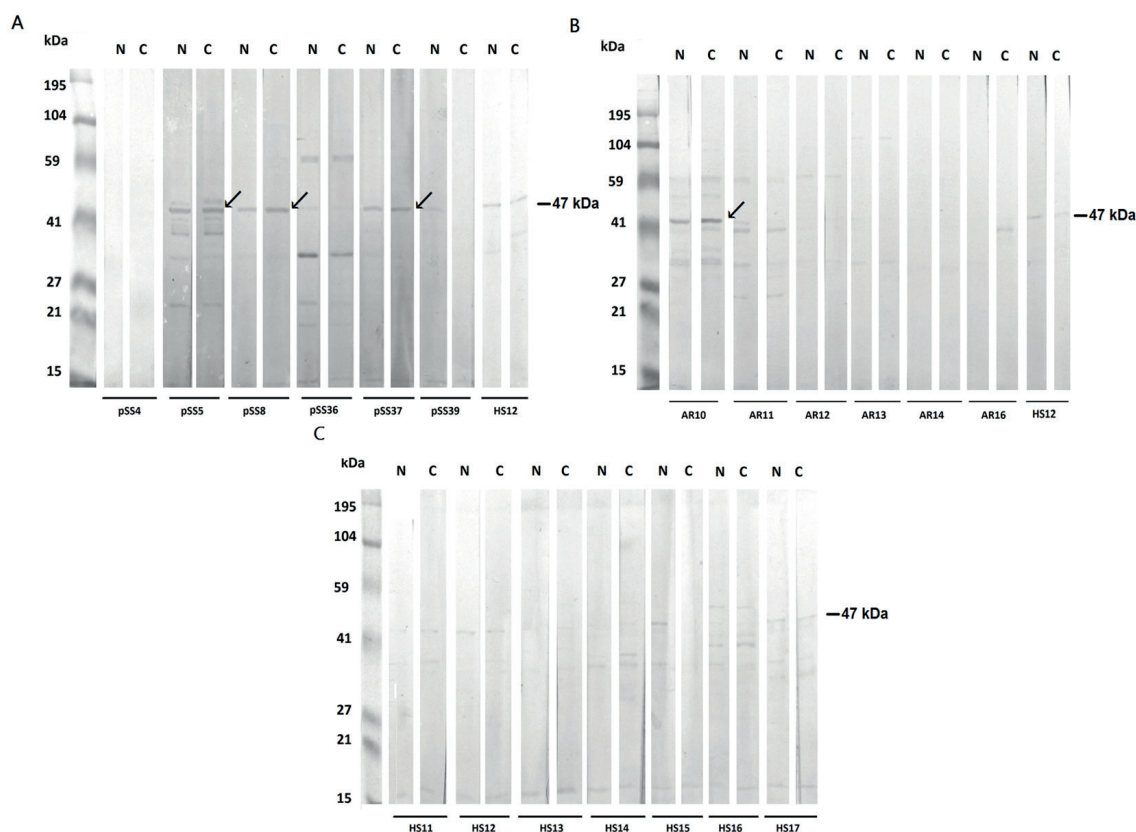


Fig. 2. IgA antibodies reactivity against HEP-2 lysate proteins treated (C) and untreated (N) with PAD2.

The arrows indicate an antigen of 47 kDa which is preferentially recognised in the PAD2 treated lysate in primary Sjögren's syndrome (pSS) [A] and rheumatoid arthritis (RA) patients [b]. Reactivity against the 47 kDa band was minimal in healthy subjects [HS].

human IgA and IgG (SIGMA, St Louis, MO, USA), respectively at a 1:10,000 dilution in wash solution. After the last wash, plates were incubated with 4-nitrophenyl phosphate disodium salt hexahydrate in diethanolamine buffer for an hour at 37°C. Plates were read at 405nm subtracting the blank lecture. A plate was performed under identical experimental conditions exclusively with PAD2 (same concentration) as correction for α -enolase reading treated with PAD2. The cut-off level was set as 99th percentile healthy controls.

Statistical analysis

We used Kruskal-Wallis test, Mann-Whitney U-test, Chi-square test, Fisher's exact test and Wilcoxon pair matched test to compare groups according to the variable distribution. We also used the Spearman correlation coefficient. Statistical analysis was performed using GraphPad software v. 5.0 software (GraphPad software, Inc., La Jolla, CA, USA). *p*-values of less than 0.05 were considered significant.

Results

Patients

The clinical and serologic features of the pSS group are shown in Table I. Among the pSS group most of them were females (98%), aged 56 ± 11 years old, and had a disease duration of 12.6 ± 11 years. In the RA group, also the females predominated (95%), had a similar disease duration 13.6 ± 12 years ($p=0.73$) but were younger (45.2 ± 13.5 , $p=0.0009$) than the pSS group. The controls age was 40 ± 13.8 years and 98% of them were females. Regarding the anti-cyclic citrullinated peptide (anti-CCP) overall reactivity, the pSS group had anti-CCP positivity in 4% and the RA group in 75%.

Reactivity of pSS sera against cellular antigens

The reactivity of IgG and IgA antibodies was analysed in 12 random samples from patients with pSS, 6 with RA and 10 healthy controls against proteins from HEP-2 lysates incubated in the presence or absence of PAD2.

For IgG, 8 samples reacted strongly with a band with an apparent mass of 47 kDa, 3 of them had higher reactivity against the citrullinated form (Fig. 1a and 1b). The pSS sera, particularly patients numbers pSS8 and pSS36 have limited reactivity against cellular antigens, although reactivity against the 47 kDa polypeptide in PAD-treated and untreated HEP-2 lysates was predominant. Among the sera of RA patients, only two samples reacted strongly against the 47 kDa band in treated and untreated lysates with PAD2, and also a greater number of recognised bands were observed in the lysate treated with PAD2 (Fig. 1c). The reactivity against the 47 kDa band in normal subjects was weak (Fig. 1d and 1e).

For IgA, 6 samples from pSS patients were analysed and fewer bands were observed. In three of them the reactivity prevailed against the band of 47 kDa and preferably in the lysate treated with PAD2 (Fig. 2a) In RA patients the reactivity against this same band was minimal as in healthy subject (Fig. 2b and c).

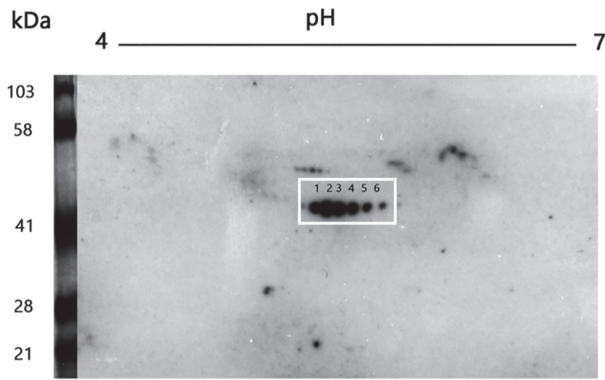


Fig. 3. Purification of the 47 kDa antigen by two dimensional electrophoresis (2DE). Proteins around 47 kDa were cleaved and eluted from the polyacrylamide gel after separated by 2DE and blotted with primary Sjögren's syndrome (pSS) serum. Six spots around 47 kDa were recognised by pSS serum. The six spots were excised from Coomassie blue stained 2DE, digested with trypsin, and analysed by tandem mass spectrometry.

Identification of citrullinated α -enolase

Secondly, 2DE of HEP-2 lysates were blotted onto nitrocellulose and probed with pSS sera. In six of the 12 random pSS samples tested recognised 6 spots with an apparent molecular mass of 47 kDa (Fig. 3). These spots were excised from the Coomassie stained 2DE, digested with trypsin and analysed by tandem mass spectrometry. Altogether thirteen peptides were sequenced all of which mapped α -enolase. Four spots were identified by mass spectrometry as α -enolase isoforms. The other two spots could not be identified due to low protein content.

Reactivity against citrullinated and native α -enolase

Finally, we evaluated the presence of non-citrullinated and citrullinated α -enolase anti-IgG and anti-IgA antibodies by ELISA among patients and controls.

The prevalence of IgG anti non-citrullinated α -enolase antibodies in pSS and RA patients was 92% and 95% respectively; moreover the prevalence of IgG anti-citrullinated α -enolase antibodies was of 98% for pSS and 90% for RA patients.

We observed that pSS serum samples had higher levels of IgG antibodies against citrullinated α -enolase compared to RA patients ($p=0.003$) and healthy subjects ($p<0.0001$) (Fig. 4a).

The reactivity of IgG antibodies against citrullinated α -enolase was higher than with non-citrullinated α -enolase in pSS patients ($p=0.0013$) (Fig. 4b). In contrast, in RA patients no differences were found in non-citrullinated or citrullinated α -enolase IgG antibodies (Fig. 4c). Thirty four (68%) of pSS patients had IgA anti-citrullinated α -enolase antibodies, and 11 patients (55%) of the RA group. On the other hand, 82% of pSS and 70% of RA patients had IgA native α -enolase antibodies. There were no differences in IgA non-citrullinated or citrullinated anti- α -enolase antibodies when compared patients with RA and pSS.

Clinical association

We did not find any differences regarding clinical and serological data among

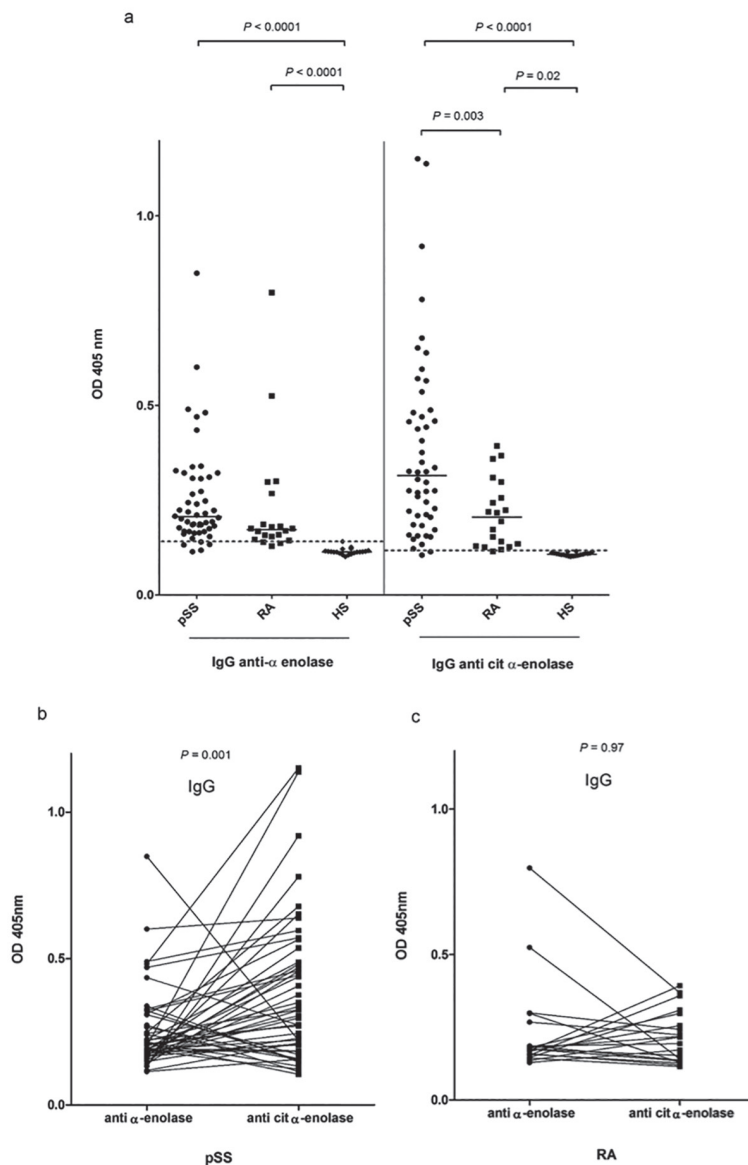


Fig. 4. Detection of IgG antibodies against non-citrullinated and citrullinated α -enolase.

(a) ELISA of *in-vitro* non-citrullinated and citrullinated α -enolase with serum samples from primary Sjögren's syndrome (pSS) and rheumatoid arthritis (RA) patients and healthy subjects (HS). First using Kruskal-Wallis test we obtained a statistical differences among the three groups ($p<0.0001$). Also patients with pSS have higher levels IgG antibodies anti-citrullinated α -enolase than RA patients by Mann-Whitney test. (b) The reactivity of IgG antibodies against citrullinated α -enolase was higher compared with non-citrullinated α -enolase in pSS patients by Wilcoxon pair matched test. (c) There was no difference in reactivity in RA by Wilcoxon pair matched test.

Table II. Anti-citrullinated enolase IgA antibodies.

	Positive Anti-citrullinated α -enolase IgA (n=34)	Negative Anti-citrullinated α -enolase IgA (n=16)	<i>p</i>
Smoking, n (%)	1 (2.9)	3 (18.7)	0.05
Non-erosive arthritis, n (%)	18 (52)	9 (56)	0.82
Raynaud's phenomenon, n (%)	6 (17.6)	3 (18.7)	0.92
Lung involvement, n (%)	10 (29.4)	5 (31.2)	0.89
Neurological involvement, n (%)	8 (23.5)	6 (37.5)	0.33
Skin vasculitis, n (%)	3 (8.8)	2 (12.5)	0.62
Lymphadenopathy, n (%)	13 (38.2)	4 (25)	0.52
Parotid enlargement, n (%)	20 (58.8)	8 (50)	0.55
Rheumatoid factor, n (%)	14 (41)	4 (25)	0.35
Anti-Ro/SSA antibody, n (%)	29 (85)	8 (50)	0.008
Anti-La/SSB antibody, n (%)	11 (32)	2 (12.5)	0.17

pSS patients with these IgG antibodies (data not shown). Interestingly, pSS patients with IgA anti-citrullinated α -enolase antibodies had a higher prevalence of anti-Ro/SSA antibodies (85% vs. 50%, $p=0.008$) and smoked less frequently (2.9% vs. 18.7%, $p=0.05$). No differences were found when we analysed clinical manifestations including non-erosive arthritis nor with other laboratory data (Table II).

Discussion

Despite the high specificity of ACPA in RA, these antibodies can be detected in other autoimmune diseases including pSS, where its prevalence ranges from 3-22% (15-22). However, until now there is no agreement of the clinical significance, as some studies had showed an association with non-erosive synovitis while others did not (18-19, 21-22). In addition, some authors consider that ACPA in pSS may be a predictor of future progress to RA (22). A previous study that evaluated the ACPA reactivity in patients with RA, SLE and pSS showed that RA patients presented reactivity against one to six citrullinated peptides whereas the SLE and the pSS group mostly had one reactivity. The specificity of antibodies in pSS patients was PAD 14.8%, vimentin 12.9%, enolase 7.4%, fibrin 3.7%, col-II 5.5% and flaggrin 1.85% (5). Herein we analysed the reactivity of pSS serum samples against non-citrullinated and citrullinated cellular proteins. We found high reactivity against citrullinated and native lysates from HEP-2 cells and, in some sera, even

greater against citrullinated lysates. We characterised α -enolase as a dominant antigen in citrullinated lysates of HEP-2 cells by screening a panel of pSS sera.

Human α -enolase is a ubiquitous glycolytic enzyme, highly expressed in the kidney and on the surface of several cell types including endothelial cells. α -enolase antibody has been reported in 6% to 66% of RA patients (26), 21% to 27% in SLE (27, 28), and 30% in scleroderma (28). In RA the presence of this antibody may identify a subset of patients with a higher frequency of joint erosions and rheumatoid factor (14), and has been noted as a candidate of molecular mimicry with *Porphyromonas gingivalis* (*P. gingivalis*), as anti-*P. gingivalis* antibody titres correlated with anti α -enolase antibody titres (29). On the other hand in SLE, α -enolase has been recognised as a component of NETosis, supporting the idea that externalisation of this protein during a flare is a mechanism that leads to formation of circulating antibodies (30). Indeed anti- α -enolase IgG2 antibodies have been identified in both, kidney and serum samples of patients with lupus nephritis, and highest levels were associated with proteinuria (30). Interestingly, we found that pSS patients have higher levels of anti-citrullinated α -enolase antibodies of the IgG isotype even greater than that found in RA patients. Unfortunately, we were not able to evaluate the IgG subclasses. The reason why patients with pSS have a preferential reactivity to citrullinated α -enolase and not against other citrul-

linated proteins is, as of today, a conundrum. It could be, otherwise, a characteristic serological finding of pSS.

Regarding the association of anti-non-citrullinated and anti-citrullinated α -enolase antibodies (IgG and IgA isotype) with clinical and serological features of pSS, we did not find any differences (including non-erosive arthritis), with the exception of an association of IgA anti-citrullinated α -enolase and anti-Ro/SSA antibodies. In contrast, the presence of anti-Ro/SSA and anti-La/SSB antibodies has not been associated with ACPA positivity in pSS (18, 22).

We also found that patients with positive IgA anti-citrullinated α -enolase smoked less frequently. This finding is contrary to the notion that smoking can trigger potentially pathogenic anti-citrullinated protein antibodies in genetically susceptible individuals. Indeed, in patients with RA, a history of ever smoking has been associated with seropositivity for ACPA IgA isotype (31). Recently Nezos *et al.* reported increased anti-CEP-1 antibody titres in 60% ACPA+ pSS patients vs. 41.7% ACPA+ RA patients, whereas no reactivity was detected in ACPA- pSS patients. Furthermore, the subgroup of patients with anti-CEP-1 antibodies was characterised by high urinary pH levels (23). These results coincide with ours, since α -enolase is a major antigenic target recognised by pSS patients. However, in our group only two patients had ACPA+, which indicates possibly that α -enolase contains at least other four peptides (identified by mass spectrometry) different from CEP-1 that, when citrullinated might become targets in ACPA negative pSS patients.

Furthermore, α -enolase as well as cofilin-1, annexin A2 and RGI2 have been found to be over-expressed in salivary gland tissue of patients with pSS but mainly in patients with pSS/MALT lymphoma. Even though current knowledge does not enable us to establish a relationship between these citrullinated peptides and the pathogenesis of SS, the link between them is amply suggestive that this indeed could be the case (32).

Certainly, we must acknowledge that the number of patients and healthy in-

dividuals is low, so an extrapolation of our data to the clinical setting cannot yet be established.

Summing up, we characterised α -enolase as a dominant antigen in citrullinated lysates of HEP-2 cells in pSS in comparison with RA and healthy subjects. The presence of IgA and IgG anti-citrullinated α -enolase was not associated with any clinical manifestation whatsoever in pSS patients, however, an association of IgA anti-citrullinated α -enolase with anti-Ro/SSA antibodies was documented. Our work sheds light on the role of citrullinated peptides in pSS, however further research is still needed.

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