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Prevalence of anti-cyclic citrullinated peptide and anti-keratin antibodies in patients with primary Sjögren’s syndrome

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Keywords: primary Sjögren’s syndrome, anti-CCP antibodies, anti-keratin antibodies

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Running Title: Anti-CCP and anti-keratin antibodies in primary Sjögren’s syndrome.

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ABSTRACT

Objective. To investigate the prevalence of anti-cyclic citrullinated peptide (anti-CCP) and anti-keratin (AKA) antibodies in a cohort of patients with primary Sjögren’s syndrome (pSS).

Methods. One hundred forty-nine patients with a diagnosis of pSS according to the European/American consensus criteria (four criteria with a focus score ≥1 or presence of anti-SSA/SSB antibodies) were recruited from three French medical centers. The presence of anti-CCP antibodies was determined by ELISA and that of AKA antibodies by indirect immunofluorescence. Radiographs of hands and feet were evaluated at the time of the anti-CCP analysis.

Results. Six patients with radiological erosions were reconsidered to have rheumatoid arthritis (RA) and secondary SS. Of the 134 pSS patients, not fulfilling ACR classification criteria for RA, studied (mean disease duration of 11.1 ± 6.6 years), 80 patients tested positive for IgM rheumatoid factor (RF) (59%), 10 (7.5%) for anti-CCP antibodies, 7 (5.2%) for AKA antibodies, and 5 (3.7%) for both anti-CCP and AKA antibodies. There was no difference between anti-CCP positive and negative patients regarding clinical and biological features, including prevalence of RF. Additionally, we individualized a group of 9 anti-CCP positive patients fulfilling ACR classification criteria for RA but presenting like pSS patients with non erosive arthritis, whose response to DMARDs could be different from classical RA patients. Conclusion. In conclusion, the majority of pSS patients is negative for AKA and anti-CCP antibodies. However, positive test results for anti-CCP or AKA antibodies should not rule out the diagnosis of pSS. Anti-CCP-positive patients, who could be prone to develop RA, require a cautious clinical and radiographic follow-up to confirm the eventual evolution to RA in the future.
Anti-cyclic citrullinated peptide (anti-CCP) antibody has proved to be an efficient diagnosis marker for the diagnosis of rheumatoid arthritis (RA) (1, 2). Both anti-CCP and the so-called anti-keratin antibodies (AKA), which recognize the protein filaggrin in rat esophageal mucosa cells, specifically bind to substrates containing the modified amino acid citrulline (3). Numerous studies corroborate the high specificity (more than 95%) of the anti-CCP assay for the diagnosis of RA (1, 2). However, the control sera, used to assess the discriminative characteristics of this assay, were mostly derived from normal subjects, patients with infectious diseases, or cohorts of patients with a high variety of rheumatic diseases. Primary Sjögren’s syndrome (pSS) was almost absent in the latter cohorts. However, clinical manifestations of pSS and RA may be very close, and the prevalence of the rheumatoid factor (RF) is the same in pSS and RA. Additionally, polyclonal B activation and autoantibody synthesis is a hallmark of pSS. Since little is known about the presence of anti-CCP and AKA antibodies in pSS, we investigated the prevalence of such antibodies (anti-CCP and AKA) in a cohort of 149 patients with a diagnosis of pSS according to the American-European Consensus Group Criteria (including a focus score ≥1 on labial salivary gland biopsy or the presence of anti-SSA or anti-SSB antibodies) (4).

PATIENTS AND METHODS

Patients.

One hundred forty-nine patients were recruited from the Department of Rheumatology, Bicêtre Hospital, Le Kremlin Bicêtre, of Hautepierre Hospital, Strasbourg, and of Bichat Hospital, Paris, between October 2001 and June 2003. All patients fulfilled the diagnosis of pSS according to the American-European Consensus Group Criteria. Each patient was clinically assessed for the presence of synovitis and extraglandular involvement (including lung, kidney and skin involvement, vasculitis, neuropathy, and lymphoma). The ACR classification criteria for RA were assessed in each patient and the patients who fulfilled 4 or more of these criteria were reconsidered to have RA with secondary SS and were analysed separately.

Anti-keratin (AKA), anti-cyclic citrullinated peptide (anti-CCP) antibodies, rheumatoid factor (RF), and anti-SSA(Ro)/SSB(La) determination.

AKA IgGs were determined by two senior seroimmunologists using indirect immunofluorescence (IFI) as described by Young and colleagues (5). Serum samples were diluted 1:10. Positive sera were titrated, and the greatest serum dilution showing fluorescence was considered the titration end-point. A second-generation anti-CCP assay (CCP2) was performed with use of ELISA (Immunoscan RA, Eurodiagnostica Arnhem, The Netherlands), according to the manufacturer’s instructions. Patient serum samples were diluted 1:50 and were considered positive if the antibody titer was greater than 50 arbitrary units as determined by dilution of a positive standard serum. Sera with intermediate positivity
according to the manufacturer (with titers between 25 and 50 units) were considered negative for anti-CCP.

In the case of discordant results regarding anti-CCP and AKA, the same sera were reanalyzed by one of us (PNR) for a second independent determination using the same techniques (ELISA and IFI, respectively). IgM RF was determined by nephelometry and was considered positive if ≥ 20 IU/ml.

Anti-Ro(SSA)/La(SSB) were detected by the commercial Elisa assay (Varelisa Ro and La antibodies, Pharmacia-Upjohn, Freiburg, Germany), which used both baculovirus-expressed recombinant Ro52 and Ro60, coated in an unspecified ratio. All the positive results were confirmed by either counter-immunoelectrophoresis using purified antigens obtained from rabbit and rat thymus powder (Pel Freez, Arkansas, USA) and from human spleen extract (Laboratoire d’Immuno-Pathologie, Hôpital Saint-Louis, Paris, France), or by double radial immunodiffusion.

**Radiographic measurements.**
Radiographs of the hands and feet of all patients were taken at the time of anti-CCP analysis. In the case of erosions, the diagnosis of pSS was discarded, and the patient was reconsidered to have RA with secondary SS and excluded from the study. The radiographs of all patients were reviewed by two senior rheumatologists.

**Statistical analysis**
The chi-square test (with Yates’ correction when appropriate) and Student’s t test were used for testing the significance of differences in baseline parameters between anti-CCP-positive and anti-CCP-negative patients. All tests were two-sided, and P values less than 0.05 were considered significant.

**RESULTS**

**Patient characteristics**
Six patients with radiological erosions were reconsidered to have RA and secondary SS. Nine further patients, without erosions, who fulfilled the ACR classification criteria for rheumatoid arthritis (RA), were analysed separately. Thus, the study involved 134 pSS patients (12 men and 122 women) not fulfilling ACR criteria for RA. The mean age was 56.4 ± 13.6 years and mean disease duration was 11.1 ± 6.6 years (range 1 to 27 years). Anti-SSA activity was present in 79 patients (59%) and anti-SSB in 50 (37.3%) patients. The focus score was ≥ 1 in 105 patients (78.3%). Synovitis and extraglandular involvement was observed in 36 (26.9%) and 51 (38%) patients, respectively.

**Prevalence of anti-CCP and AKA antibodies, RF and cryoglobulinemia**
Ten samples (7.5%) tested positive for anti-CCP2 antibodies at > 50 units reactivity, including 8 (5.9%) >100 units. The mean value of the anti-CCP titer was 623.8 ± 584.8 in positive sera. Five samples, whose titer was measured between 25 and 50 units after two independent assays, were considered negative for anti-CCP. AKA antibodies were detected in 7 patients (5.2%). Five patients (3.7%) tested positive for both anti-CCP and AKA antibodies. Five patients had positive anti-CCP test results but negative AKA ones, whereas two patients had only positive AKA test results. A second determination, using the same techniques, confirmed the discordant results regarding AKA and anti-CCP results in these 7 patients. Of the 80 IgM RF positive sera (59.7%), 7 (8.7%) were positive for anti-CCP, 6 (7.5%) for AKA
and 4 (5%) for both anti-CCP and AKA antibodies. Cryoglobulinemia was detected in 7 patients, whose sera were all negative for anti-CCP and AKA antibodies.

**Characteristics of anti-CCP-positive patients**
Clinical and biological features of the 10 pSS patients with anti-CCP antibodies are summarized in Table 1. Three patients were treated with disease-modifying antirheumatic drugs (DMARDs) (methotrexate: n = 2, hydroxychloroquine: n = 1). Demographic factors, including age and disease duration, was not significantly different between anti-CCP-positive and -negative patients (Table 2). Neither the presence of synovitis, nor that of extraglandular involvement was associated with the presence of anti-CCP or AKA antibodies (Table 2).

Mean IgG serum level did not differ significantly between the anti-CCP-positive and -negative groups, nor did erythrocyte sedimentation rate at the first hour, presence of anti-SSA, anti-SSB, RF or focus score $\geq$ 1 (Table 2).

**DISCUSSION**
In this cohort of 134 patients with pSS, none of them fulfilling ACR classification criteria for RA, 7.5% and 5.2% of the sera were positive for anti-CCP and AKA antibodies, respectively. Comparatively, the prevalence of anti-CCP was 68.9% in RA patients, using the same second generation anti-CCP assay (Nicaise-Rolland P, et al, submitted manuscript). To our knowledge, this is the first study that analyzes the prevalence of anti-CCP and AKA antibodies in a cohort of patients with pSS according to the American-European consensus group criteria. The population study compares well with other cohorts of patients with pSS in the literature regarding clinical and immunological features (6).

Interestingly, the mean titer of anti-CCP antibodies was high and no positive serum had borderline values. Eight of the 10 positive sera (5.9% of patients) had a reactivity greater than 100 units, a threshold which definitely allows for decreasing the risk of false positive results with use of the second generation assay. The mean age of patients and mean disease duration was similar between patients with and without anti-CCP. Hypergammaglobulinemia could not account for the positive anti-CCP test results, since the mean IgG serum level was not significantly different according to anti-CCP status.

The current study confirms that anti-CCP and AKA antibodies may be detected in patients with no radiographic evidence of erosions after a long follow-up. This dissociation was already reported in patients with RA (7, 8).

Might anti-CCP-positive patients actually suffer from RA? First, the long period of disease duration without erosions (10 years) does not support the current diagnosis of RA associated with secondary SS. Secondly, anti-SSB prevalence was the same in patients with and without anti-CCP antibodies, whereas the frequency of anti-SSB is usually lower in secondary SS (9). Last, we chose to exclude the 15 patients who fulfilled the ACR classification criteria for RA, in order to be as stringent as possible in the definition of primary Sjögren’s syndrome. But, if it is quite certain that the 6 patients with erosions had RA with secondary SS, the diagnosis of the 9 others remains still debatable. Indeed, if they fulfilled ACR classification criteria for RA since they suffered from RF-positive symmetrical polyarthritis, they did not have erosions after a long period of follow-up (mean disease duration of 9 years) and were previously considered as primary Sjögren’s syndrome by their clinicians. Moreover, 6 of these 9 patients were treated with DMARDs, but without any response to therapy.
Last, the authors of the classification criteria for RA acknowledged that “pSS patients with arthritis could cause classification difficulties and required further study” (10). All of these 9 patients were anti-CCP positive. It can be interpreted as an additional marker of definite RA or as an argument for the possibly higher prevalence of anti-CCP and AKA antibodies in SS in daily clinical practice than observed in the present study. HLA genotyping could help to differentiate primary SS patients with polyarthritis and anti-CCP from RA patients, since HLA-DR3 is associated with pSS and the presence of anti-SSB antibody (11), whereas HLA-DR4 predisposes to RA and could be required for the presentation of citrullinated antigens (12). Further studies are required to evaluate the effect of DMARDs in this sub-population of patients fulfilling ACR criteria for RA but presenting like pSS with non erosive arthritis.

Additionally, the possibility that patients with anti-CCP antibodies could be prone to develop RA cannot be ruled out. Accordingly, it is known that anti-CCP can be present years before the first signs of RA (13). In three anti-CCP positive patients with polysynovitis, the prescription of DMARDs might have prevented the progression to RA. Additionally, the concomitant presence of IgM RF and anti-CCP, which was observed in 7 patients, is the best predictor of developing active RA (14). Therefore, a cautious clinical and radiographic follow-up is required to confirm the absence of evolution to RA of anti-CCP positive patients.

However, this study suggests that production of anti-CCP antibodies, which have also been recently reported in patients with juvenile idiopathic arthritis (15), could be less intimately related to RA pathogenesis, than was previously hypothesized. Polyclonal activation of B lymphocytes, which is a predominant feature of pSS, might account for the presence of anti-CCP and AKA antibodies. Likewise, an increased serum level for BLyS, a potent B lymphocyte costimulation molecule, is associated with an increased serum level of gammaglobulin, RF and anti-SSA/SSB in patients with SS (16).

In conclusion, the majority of pSS patients is negative for AKA and anti-CCP antibodies. However, clinicians should be aware that the presence of anti-CCP or AKA antibodies does not rule out the diagnosis of pSS: 7.5% of pSS patients, not fulfilling ACR criteria for RA, were anti-CCP positive. Moreover, we individualized another group of anti-CCP positive patients fulfilling ACR classification criteria for RA but presenting like pSS patients with non erosive arthritis, whose response to DMARDs could be different from classical RA patients. Anti-CCP-positive patients require a cautious clinical and radiographic follow-up to confirm the absence of evolution to RA in the future.

**Acknowledgements:** We thank Isabelle Marie (Clinical Research Unit, Bicêtre Hospital, AP-HP) for excellent technical assistance.
Table 1. Clinical and biological features of pSS patients with anti-CCP antibody.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Disease duration (years)</th>
<th>Synovitis (Y/N)</th>
<th>Extraglandular involvement (Y/N)</th>
<th>ESR (mm)</th>
<th>Serum IgG (g/L)</th>
<th>AKA (Y/N)</th>
<th>RF (Y/N)</th>
<th>Anti-SSA/anti-SSB (Y/N)</th>
<th>Focus score ≥ 1 (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38</td>
<td>10</td>
<td>N</td>
<td>N</td>
<td>3</td>
<td>12.3</td>
<td>N</td>
<td>N</td>
<td>Y/N</td>
<td>Y</td>
</tr>
<tr>
<td>2</td>
<td>33</td>
<td>2</td>
<td>Y</td>
<td>Y</td>
<td>76</td>
<td>18.1</td>
<td>Y</td>
<td>Y</td>
<td>N/N</td>
<td>Y</td>
</tr>
<tr>
<td>3</td>
<td>64</td>
<td>23</td>
<td>Y</td>
<td>Y</td>
<td>10</td>
<td>11.2</td>
<td>Y</td>
<td>Y</td>
<td>Y/Y</td>
<td>Y</td>
</tr>
<tr>
<td>4</td>
<td>54</td>
<td>7</td>
<td>N</td>
<td>N</td>
<td>40</td>
<td>19.4</td>
<td>N</td>
<td>N</td>
<td>Y/Y</td>
<td>N</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>8</td>
<td>N</td>
<td>N</td>
<td>7</td>
<td>9.7</td>
<td>N</td>
<td>N</td>
<td>Y/Y</td>
<td>Y</td>
</tr>
<tr>
<td>6</td>
<td>53</td>
<td>8</td>
<td>N</td>
<td>N</td>
<td>45</td>
<td>15.7</td>
<td>Y</td>
<td>Y</td>
<td>N/N</td>
<td>Y</td>
</tr>
<tr>
<td>7</td>
<td>55</td>
<td>14</td>
<td>N</td>
<td>N</td>
<td>41</td>
<td>39</td>
<td>Y</td>
<td>Y</td>
<td>Y/N</td>
<td>N</td>
</tr>
<tr>
<td>8</td>
<td>65</td>
<td>8</td>
<td>N</td>
<td>N</td>
<td>30</td>
<td>11.4</td>
<td>N</td>
<td>Y</td>
<td>N/N</td>
<td>Y</td>
</tr>
<tr>
<td>9</td>
<td>43</td>
<td>14</td>
<td>N</td>
<td>N</td>
<td>32</td>
<td>NA</td>
<td>Y</td>
<td>Y</td>
<td>Y/N</td>
<td>Y</td>
</tr>
<tr>
<td>10</td>
<td>55</td>
<td>7</td>
<td>N</td>
<td>N</td>
<td>27</td>
<td>NA</td>
<td>N</td>
<td>Y</td>
<td>N/N</td>
<td>Y</td>
</tr>
</tbody>
</table>

NA: not assessed. Y/N: present/absent.
Table 2. Characteristics of the study population.

<table>
<thead>
<tr>
<th></th>
<th>Anti-CCP-positive patients (n = 10)</th>
<th>Anti-CCP-negative patients (n = 124)</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years, mean ± SD)</td>
<td>49.5 ± 11.5</td>
<td>56.8 ± 13.7</td>
<td>0.11</td>
</tr>
<tr>
<td>Disease duration (years, ± SD)</td>
<td>10.1 ± 5.7</td>
<td>11 ± 6.7</td>
<td>0.65</td>
</tr>
<tr>
<td>Synovitis¹</td>
<td>2 (20)</td>
<td>34 (27.4)</td>
<td>0.9²</td>
</tr>
<tr>
<td>Extraglandular involvement¹</td>
<td>2 (20)</td>
<td>49 (39.5)</td>
<td>0.5²</td>
</tr>
<tr>
<td>ESR (mm at 1¹st hour, mean ± SD)</td>
<td>31.1 ± 22.1</td>
<td>32.1 ± 27.7</td>
<td>0.91</td>
</tr>
<tr>
<td>Serum IgG level (g/l, mean ± SD)</td>
<td>17.1 ± 9.5</td>
<td>16.6 ± 8.2</td>
<td>0.87</td>
</tr>
<tr>
<td>AKA¹</td>
<td>5 (50)</td>
<td>2 (1.6)</td>
<td>&lt;0.001²</td>
</tr>
<tr>
<td>Rheumatoid factor¹</td>
<td>7 (70)</td>
<td>73 (58.9)</td>
<td>0.9²</td>
</tr>
<tr>
<td>Anti-SSA¹</td>
<td>6 (60)</td>
<td>73 (58.9)</td>
<td>&gt;0.9²</td>
</tr>
<tr>
<td>Anti-SSB¹</td>
<td>3 (30)</td>
<td>47 (37.9)</td>
<td>&gt;0.9²</td>
</tr>
<tr>
<td>Focus score ≥1¹</td>
<td>8 (80)</td>
<td>97 (78.2)</td>
<td>&gt;0.9²</td>
</tr>
</tbody>
</table>

¹ Results are expressed as number of patients, (%).
² Value with Yates'correction.
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