Clinical usefulness of anti-RNA polymerase III antibody measurement by enzyme-linked immunosorbent assay

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Objective. To evaluate the clinical usefulness of measuring anti-RNA polymerase (RNAP) III antibody in Japanese patients with SSc.

Methods. This multicentre study involved 354 patients with SSc, 245 with non-SSc CTDs and 102 healthy controls. ELISAs were used to detect anti-RNAP III antibody and ACA. The presence of anti-RNAP III antibody in selected serum samples was confirmed by immunoprecipitation (IP) assay.

Results. By ELISA, anti-RNAP III antibody was detected in 38 (10.7%) patients with SSc, 3 (1.2%) with non-SSc CTD and no healthy controls. The clinical specificity for SSc was excellent (98.8%), although a small number of false positives occurred. The sensitivity of the anti-topo I and ACA ELISAs for SSc was 59.9%, which increased to 68.2% without a reduction in specificity when the anti-RNAP III measurement was added. Clinical features associated with positivity for the anti-RNAP III antibody include dcSSc, a high total skin score and a trend towards high prevalence of renal crisis, consistent with previous studies that used an IP assay. Furthermore, on clinical severity scales, SSc patients with anti-RNAP III antibody scored highest for skin and renal involvement among patients subgrouped by the presence of individual SSc-related antibodies.

Conclusions. The measurement of anti-RNAP III antibody by ELISA is useful in routine clinical practice, because it helps diagnose SSc and identify a disease subset with severe skin and renal involvement.

Key words: Autoantibody, RNA polymerase, Systemic sclerosis, ELISA, Renal crisis, SSc severity scale.

Introduction

Serum ANAs are detected in nearly all patients with SSc, a CTD characterized by fibrosis of the skin and internal organs as well as microvascular injury [1]. Two major SSc-related ANAs are anti-topo I antibody and ACA. The presence of the anti-topo I antibody is associated with dcSSc and interstitial lung disease (ILD), whereas ACA is detected primarily in patients with lcSSc [1, 2]. A higher mortality rate is reported in patients with anti-topo I than with ACA [2, 3]. These two SSc-related ANAs are useful for the diagnosis and subtyping of SSc, and are used widely in clinical practice.

Materials and methods

Patients and controls

This multicentre prospective study was conducted at 15 medical centres across Japan from September 2005 to December 2006.
Three hundred and fifty-four consecutive Japanese patients with SSc who fulfilled the ACR preliminary classification criteria for SSc [12] were evaluated in this study. They consisted of 48 men and 306 women, with a mean (s.d.) age of 57.8 (13.4) years. According to the disease classification by Medsger [13], 175 patients had dcSSc and 179 had lcSSc. Disease controls included 245 Japanese patients who were randomly selected from patients with non-SSc CTDs who visited the participating medical centres during the same period. This control group included 27 men and 218 women, with a mean age of 50.1 (15.3) years, of whom 122 had SLE, 46 had DM, 29 had RA, 22 had primary SS, 12 had MCTD, 9 had PM, 3 had Behçet’s disease, 1 had EF and 1 had Takayasu’s arteritis. None of these patients satisfied the ACR classification criteria for SSc. One hundred and two healthy volunteers were also enrolled as a control group. Serum samples obtained from the subjects were stored at −20°C until use. The study was approved by the individual Institutional Review Boards, and written informed consent was obtained from each participant.

Clinical features of SSc patients

The clinical and laboratory features were retrospectively collected by the review of medical charts. These included sex, disease subset, maximum modified Rodnan total skin score (mTSS) and the presence or absence of the following clinical findings: RP, digital pitting scars, digital tip ulceraions, oesophageal hypomotility, ILD, isolated pulmonary arterial hypertension (PAH), conduction defects on ECG, myocardial dysfunction and renal crisis. The criteria for individual organ involvement were described previously [2].

SSc severity scale

We adapted an SSc severity scale from the one proposed by Medsger et al. [14]. Since these scales have been developed based on the University of Pittsburgh Scleroderma Databank, we modified them to conform to Japanese SSc patients, who have less extensive skin and tendon involvement (supplementary table available as supplementary data at Rheumatology Online). Briefly, we selected 10 systems for evaluation: general health, peripheral vascular, skin, joint/tendon, upper gastrointestinal (GI) tract, lower GI, lung (ILD and PAH), heart and kidney. Each was graded with a score of 0 (normal) to 4 (end stage), as in Medsger’s original report. Specific changes we made in grading systems included skin (reallocation of scales based on mTSS), joint/tendon (use of range of motion of wrists, elbows and knees in scales) and ILD (re-allocation of scales based on percentage of vital capacity).

Detection of anti-RNAP III antibody by ELISA

Serum anti-RNAP III antibody was measured using an ELISA kit developed and manufactured by MBL (Nagoya, Japan). For this kit, a recombinant fragment encoding amino acid residues 891–1080 of human RPC155 was produced using a bacterial expression system and used as an antigen. This kit contained pooled anti-RNAP III-positive and -negative reference sera. The anti-RNAP III antibody index was calculated from the following formula:

\[
\frac{100 \times (\text{sample OD}_{450} - \text{negative control OD}_{450})}{(\text{positive control OD}_{450} - \text{negative control OD}_{450})}
\]

The cut-off value was set at 28, according to the manufacturer’s recommendation.

Detection of anti-RNAP III antibody by IP assay

We did the IP assay using 35S-labelled K562 cell extracts as an antigen source to confirm the presence of anti-RNAP III antibody, as described earlier [4]. The identification of anti-RNAP III antibody was based on the profiles of immunoprecipitated RNAP III large subunits (138 and 155 kDa) in comparison with the reference serum.

Detection of anti-topo I antibody and ACA

Anti-topo I antibody was measured using a commercial ELISA kit with purified rabbit topo I as the antigen (SRL, Tokyo, Japan). ACA was also measured using ELISA with a recombinant fragment encoding the carboxyl terminal portion of human CENP-B as the antigen (MESACUP-2 CENP-B; MBL). The cut-off levels were set at 10 U/ml and an index of 16 for anti-topo I and ACA, respectively, according to the manufacturers’ recommendations. In some experiments, the presence of ACA was assessed by IIF on HeLa cell chromosomal spread slides (MBL).

Statistical analysis

All continuous variables are shown as the mean (s.d.). Frequencies between two groups were tested for statistical significance using the chi-square test or Fisher’s two-tailed exact test, when applicable. Differences in continuous variables were examined by the non-parametric Mann–Whitney U-test. Correlation coefficients were determined using a single regression model.

Results

Detection of anti-RNAP III antibody by ELISA

We screened 354 patients with SSc, 245 patients with non-SSc CTDs and 102 healthy controls for the anti-RNAP III antibody using ELISA (Fig. 1). Anti-RNAP III antibody was detected in 38 patients with SSc (10.7%), of whom 32 (18.3%) had dcSSc and 6 (3.3%) had lcSSc. However, three patients with non-SSc CTDs (1.2%) also showed a positive result. None of the healthy controls was positive for the anti-RNAP III antibody. Thus, the clinical sensitivity and specificity of the anti-RNAP III antibody detected by ELISA for SSc were 10.7 and 98.8%, respectively, with a positive predictive value of 92.7% and a negative predictive value of 43.4%.

![Fig. 1. Anti-RNAP III antibody levels measured by ELISA in 354 sera from SSc patients (175 dcSSc and 179 lcSSc), 245 sera from patients with non-SSc CTDs and 102 sera from healthy controls. Sera with a false positive result confirmed by IP assay are indicated as open circles. A broken line denotes the cut-off level for positivity (index = 28).](https://academic.oup.com/rheumatology/article-abstract/48/12/1570/1786922/fig1?download=true)
Results of ELISA compared with IP assay

To validate the results obtained by ELISA, the IP assay was performed with 41 sera that gave a positive result with ELISA, including 38 patients with SSC and 3 with non-SSc CTDs. Of these sera, 35 (85.4%) were confirmed to be positive by the IP assay. A false-positive result was obtained in six sera, consisting of three with SSC (one dcSSc and two lcSSc) and all three with non-SSc CTDs. Four of the false-positive samples showed a low antibody index of <50 in the ELISA (Fig. 1). We also evaluated nine serum samples that gave an antibody index just below the cut-off by ELISA (index >15), including four from SSC patients, three from non-SSc CTD patients and two from healthy controls, and found that all were negative by the IP assay.

Effect of anti-RNAP III antibody measurement

Table 1 summarizes the sensitivity, specificity and positive and negative predictive values of three SSc-related ANAs (anti-RNAP III, anti-topo I and ACA) measured by ELISA for the diagnosis of SSC in 597 patients with CTDs, including 352 with SSC. The anti-RNAP III antibody showed the lowest sensitivity, but its specificity was the highest among the three SSc-related ANAs. Anti-topo I or ACA, or both, which are routinely measured in most laboratories, were detected in 59.9% of the SSC patients, and this proportion was increased to 68.2% when the anti-RNAP III antibody was also measured. This increase in clinical sensitivity was statistically significant (P = 0.03), and there was no reduction in specificity.

Coexistence of anti-RNAP III antibody with other SSc-related ANAs

We further examined potential correlations between the antibody levels of anti-RNAP III and those of anti-topo I or ACA in 352 patients with SSC (Fig. 2). The antibody levels did not correlate at all (correlation coefficient <0.1), and the coexistence of two or more SSc-related ANAs was infrequent: 1 with anti-RNAP III, anti-topo I and ACA; 1 with anti-RNAP III and anti-topo I; 7 with anti-RNAP III and ACA; and 11 with anti-topo I and ACA. Anti-RNAP III and anti-topo I, two major ANAs associated with dcSSc, were mutually exclusive, but the coexistence of ACA in anti-RNAP III-positive sera was relatively common (21%). In all eight sera positive for both anti-RNAP III and ACA by ELISA, the presence of ACA was confirmed by staining the centromeric regions of individual chromosomes by IIF on chromosomal spreads.

Clinical features associated with the anti-RNAP III antibody

Clinical features were compared among SSC patients subgrouped by the presence of anti-RNAP III, anti-topo I and ACA measured by the ELISA (Table 2). Patients with two or three coexisting SSC-related ANAs were excluded in this analysis. Females were significantly less frequent in patients with anti-RNAP III than in those with ACA (P = 0.008). The dcSSc was more frequent in anti-RNAP III-positive and anti-topo I-positive patients compared with ACA-positive patients (P < 0.0001 for both comparisons), but the maximum mTSS was higher in the anti-RNAP III-positive than anti-topo I-positive patients (P = 0.02). Prevalence of ILD in patients with anti-RNAP III was lower than those with anti-topo I, but higher than those with ACA (P = 0.004 and <0.0001, respectively). Renal crisis tended to be more frequent in patients with anti-RNAP III antibody compared with other two groups, but this difference did not reach statistical significance (P = 0.08). This is probably due to the low frequency of renal crisis in our patients (2.5%). In seven patients with anti-RNAP III and ACA, but not with anti-topo I, all but one had dcSSc with maximum mTSS comparable with that in anti-RNAP III alone. None of them developed renal crisis, but the number of patients was very few.

SSc severity scale values in patients with anti-RNAP III in comparison with those with other SSc-related ANAs

Scores from the SSc severity scale were compared among SSC patients subgrouped by positivity of anti-RNAP III, anti-topo I or ACA alone (Table 3). The anti-RNAP III-positive patients had higher skin severity scores than anti-topo I-positive or ACA-positive patients (P = 0.009 and <0.0001, respectively), and higher joint/tendon severity score than in ACA-positive patients (P < 0.0001). In patients with anti-RNAP III, ILD was less severe.
Table 2. Clinical features in SSc patients positive for anti-RNAP III, anti-topo I or ACA detected by ELISA

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Anti-RNAP III alone, n = 29</th>
<th>Anti-topo I alone, n = 96</th>
<th>ACA alone, n = 95</th>
<th>Overall P-value</th>
<th>Anti-RNAP III and ACA, n = 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex: female, %</td>
<td>76</td>
<td>82</td>
<td>95</td>
<td>0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td>dSSc, %</td>
<td>86</td>
<td>77</td>
<td>12</td>
<td>&lt;0.0001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>86</td>
</tr>
<tr>
<td>Maximum mTSS, (s.d.)</td>
<td>21.5 (10.7)</td>
<td>15.7 (10.4)</td>
<td>7.2 (6.8)</td>
<td>&lt;0.0001&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20.3 (8.3)</td>
</tr>
<tr>
<td>RP, %</td>
<td>100</td>
<td>95</td>
<td>96</td>
<td>0.8</td>
<td>100</td>
</tr>
<tr>
<td>Digital pitting scars, %</td>
<td>45</td>
<td>62</td>
<td>31</td>
<td>0.0001&lt;sup&gt;d&lt;/sup&gt;</td>
<td>43</td>
</tr>
<tr>
<td>Digital tip ulcerations, %</td>
<td>21</td>
<td>41</td>
<td>15</td>
<td>0.008&lt;sup&gt;e&lt;/sup&gt;</td>
<td>14</td>
</tr>
<tr>
<td>Oesophageal hypomotility, %</td>
<td>66</td>
<td>71</td>
<td>65</td>
<td>0.7</td>
<td>71</td>
</tr>
<tr>
<td>ILD, %</td>
<td>66</td>
<td>89</td>
<td>18</td>
<td>&lt;0.0001&lt;sup&gt;c&lt;/sup&gt;</td>
<td>57</td>
</tr>
<tr>
<td>Isolated PAH, %</td>
<td>10</td>
<td>14</td>
<td>6</td>
<td>0.3</td>
<td>14</td>
</tr>
<tr>
<td>Conduction defects, %</td>
<td>14</td>
<td>12</td>
<td>5</td>
<td>0.4</td>
<td>14</td>
</tr>
<tr>
<td>Myocardial dysfunction, %</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>0.9</td>
<td>14</td>
</tr>
<tr>
<td>Renal crisis, %</td>
<td>10</td>
<td>4</td>
<td>0</td>
<td>0.08</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>P = 0.008 between the anti-RNAP III and ACA groups, and P = 0.01 between the anti-topo I and ACA groups. <sup>b</sup>P = 0.001 between the anti-RNAP III and ACA groups, and between the anti-topo I and ACA groups. <sup>c</sup>P = 0.02 between the anti-RNAP III and anti-topo I groups, P = 0.001 between the anti-RNAP III and ACA groups and between the anti-topo I and ACA groups. <sup>d</sup>P = 0.001 between the anti-RNAP III and anti-topo I groups, P = 0.001 between the anti-RNAP III and ACA groups and between the anti-topo I and ACA groups.

Table 3. Scores for the SSc severity scales in SSc patients who were positive for anti-RNAP III, anti-topo I or ACA alone

<table>
<thead>
<tr>
<th>SSc severity scale</th>
<th>Anti-RNAP III, n = 29</th>
<th>Anti-topo I, n = 96</th>
<th>ACA, n = 95</th>
<th>Overall P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>General health</td>
<td>0.8 (1.0)</td>
<td>0.6 (0.8)</td>
<td>0.3 (0.7)</td>
<td>0.05</td>
</tr>
<tr>
<td>Peripheral vascular</td>
<td>1.3 (0.6)</td>
<td>1.5 (1.0)</td>
<td>1.2 (0.7)</td>
<td>0.1</td>
</tr>
<tr>
<td>Skin</td>
<td>2.5 (1.2)</td>
<td>1.9 (1.0)</td>
<td>1.2 (0.7)</td>
<td>&lt;0.0001&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>Joint/tendon</td>
<td>0.7 (0.6)</td>
<td>0.8 (1.0)</td>
<td>0.2 (0.5)</td>
<td>&lt;0.0001&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Upper GI tract</td>
<td>1.4 (0.9)</td>
<td>1.5 (1.0)</td>
<td>1.2 (1.0)</td>
<td>0.3</td>
</tr>
<tr>
<td>Lower GI tract</td>
<td>0.2 (0.4)</td>
<td>0.3 (0.7)</td>
<td>0.2 (0.5)</td>
<td>0.7</td>
</tr>
<tr>
<td>Lung (ILD)</td>
<td>1.0 (0.9)</td>
<td>1.4 (1.0)</td>
<td>0.3 (0.7)</td>
<td>&lt;0.0001&lt;sup&gt;j&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lung (PAH)</td>
<td>0.2 (0.5)</td>
<td>0.2 (0.8)</td>
<td>0.1 (0.5)</td>
<td>0.8</td>
</tr>
<tr>
<td>Heart</td>
<td>0.1 (0.3)</td>
<td>0.2 (0.4)</td>
<td>0.1 (0.4)</td>
<td>1.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.3 (1.2)</td>
<td>0.1 (0.6)</td>
<td>0.01 (0.1)</td>
<td>0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results are shown as mean (s.d.) *SSc severity scales used were modified from those proposed by Medsger et al. [14] to conform to Japanese SSc patients (supplementary table available as supplementary data at Rheumatology Online). <sup>a</sup>P = 0.009 between the anti-RNAP III and anti-topo I groups, P < 0.0001 between the anti-RNAP III and ACA groups and between the anti-topo I and ACA groups. <sup>b</sup>P = 0.001 between the anti-RNAP III and ACA groups, and between the anti-topo I and ACA groups. <sup>c</sup>P = 0.001 between the anti-RNAP III and ACA groups, and between the anti-topo I and ACA groups. <sup>h</sup>P < 0.04 between the anti-RNAP III and anti-topo I groups, P < 0.0001 between the anti-RNAP III and ACA groups and between the anti-topo I and ACA groups. <sup>i</sup>P = 0.04 between the anti-RNAP III and anti-topo I groups, P = 0.04 between the anti-RNAP III and ACA groups, and between the anti-topo I and ACA groups.

Discussion

We measured anti-RNAP III antibody using a commercially available ELISA and found it is useful in rheumatology practice based on the following findings: (i) the clinical sensitivity of anti-RNAP III for SSc was 10.7%, but the specificity was excellent at 98.8%; (ii) adding the measurement of anti-RNAP III antibody to the conventional measurements of anti-topo I and ACA increased the clinical sensitivity for SSc from 59.9 to 68.2% without a reduction in specificity; (iii) anti-RNAP III in patients with lcSSc was extremely uncommon (2.2%); (iv) patients positive for anti-RNAP III antibody represented high prevalence of dSSc and high maximum mTSS, and a trend towards increased frequency of renal crisis, clinical characteristics known to be correlated with anti-RNAP III antibody detected by the IP assay [1–8]; and (v) anti-RNAP III antibody was associated with higher severity scores on the measures of skin, joint/tendon and kidney involvement. Therefore, in clinical settings, the anti-RNAP III antibody should be measured in conjunction with anti-topo I and ACA in patients who are suspected to have SSc or in whom SSc has already been diagnosed.

In this study, difference in prevalence of renal crisis among patients positive for anti-RNAP III, anti-topo I and ACA alone did not reach statistical significance. This could be explained by the low prevalence of renal crisis in Japanese patients with SSc, even in those with the anti-RNAP III antibody: 9% in this study compared with 12–33% in studies conducted in North America and the UK [5, 6, 16]. In addition, potential patient selection bias is one of the limitations of this study, since we used the ACR preliminary classification criteria for the selection of SSc patients, resulting in exclusion of some patients with lcSSc or patients with SSc sine scleroderma who do not satisfy these criteria. Collecting data on the anti-RNAP III antibody measurement by ELISA in SSc patients would be necessary to further establish utility of this ELISA system in routine rheumatology practice.

Two previous reports examined the clinical usefulness of ELISA kits for the detection of anti-RNAP III antibody: one examined the MBL kit used in this study [15], and the other used a kit manufactured by INOVA Diagnostics (San Diego, CA, USA) [16]. The INOVA kit uses a recombinant fragment that encodes the same amino acid sequence of RPC155, but it is produced by a baculovirus expression system. All three studies, including ours, showed a high clinical specificity (>96%) of the anti-RNAP III antibody for SSc diagnosis, validating the use of an ELISA system in which the antigen includes an immunodominant epitope on RPC155 [10]. However, the clinical sensitivity for SSc varied among the studies: 18.3% in the Italian study [15], 19.4% in the Canadian study [16] and 10.7% in this study. This variability appears to be due to differences in the ethnic backgrounds of the patients enrolled rather than differences between kidney sensitivity. In fact, the frequency of anti-RNAP III antibody in SSc patients is reported to be different among ethnic groups: it is higher in North American Caucasian patients than in Japanese or French patients [8, 11, 17, 18].

Disease severity scales were developed by Medsger et al. [14] to assess the disease status, including the disease activity and damage, in patients with SSc. We modified these scales to conform to Japanese patients with SSc, and used them to assess the potential relation between disease severity status and anti-RNAP III antibody. The presence of anti-RNAP III antibody was associated with severe manifestations of poor skin, joint/tendon and kidney involvement. Interestingly, SSc patients with anti-topo I, another antibody associated with dSSc, had a distinct set of severe organ system involvements: skin and tendon/joint involvement and ILD. The use of severity scales should be helpful for evaluating the clinical significance of other SSc-related ANAs.

Earlier studies indicated that anti-RNAP III, anti-topo I and ACA are mutually exclusive in SSc patients [2, 4–6, 8, 18]. In this study, the coexistence of anti-RNAP III and anti-topo I in SSc was infrequent, but ACA was detected in 21% of the anti-RNAP
III-positive patients. ACA was also detected in 6 of 43 patients (14%) with anti-RNAP III in a Canadian cohort [16]. By IIF, it is likely that weak nuclear staining of the anti-RNAP III antibody is obscured within a bright discrete speckled staining produced by ACA. Thus, it is clinically relevant to evaluate the presence of anti-RNAP III antibody in patients with ACA, especially in those in early disease, since such patients might have an increased risk for developing diffuse cutaneous involvement and renal crisis if positive for the anti-RNAP III antibody.

We carried out the gold standard IP assay in selected sera that gave a positive or borderline result in the ELISA, and found that the ELISA yielded a false-positive result in a small number of SSc and non-SSc sera. Although we were unable to obtain analytical specificity because the IP assay was performed only for selected sera, the analytical specificity of the same ELISA kit was reported to be 99.1% in the Italian cohort [15]. Thus, we should recognize that a false positive result can occur with the ELISA, but its frequency is very low. False-positive ELISA results can be caused by antibodies reactive with bacterial components contaminating the antigen preparation [11]. In this regard, Chang et al. [19] developed an ELISA system using affinity-purified RNAP antigen, which yielded results that were correlated with IP results. However, that system requires the preparation of a large quantity of the antigen from cultured cells by affinity purification using the patient’s serum, which is not practical. In the future, the specificity should be improved by using a highly purified antigen preparation or by modifying the serum dilution solution to absorb antibodies to bacterial components more efficiently. Since the majority of the false positive sera showed a low anti-RNAP III antibody index (<50), one potential way to eliminate false positive results at present is to set the cut-off index to 50 instead of 28. With our data, this modification improved the clinical specificity of the ELISA from 98.8 to 99.6%. Because one serum with an index under 50 in the ELISA was positive by the IP assay (clinical sensitivity down from 10.7 to 10.5%), we propose setting the index between 28 and 50 as a ‘grey’ zone, for which an IP assay should be done to confirm the presence or absence of anti-RNAP III antibody in cases lacking typical SSc features.

In conclusion, anti-RNAP III antibody detected by a commercially available ELISA is useful for the diagnosis and disease subtyping of SSc, and therefore its use may be adopted in routine clinical practice.

**Rheumatology key messages**

- A commercial ELISA kit for anti-RNAP III antibody can substitute the IP assay.
- Anti-RNAP III measured by ELISA is useful for diagnosing SSc and predicting renal crisis and severe skin thickening.

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**Disclosure statement:** M.K. has owned a patent on an assay system for detecting anti-RNAP III antibody, and has received licensing fees from MBL and INOVA Diagnostics. All other authors have declared no conflicts of interest.

**Supplementary data**

Supplementary data are available at Rheumatology Online.

**References**