Diagnostic associations in a large and consecutively identified population positive for anti-SSA and/or anti-SSB: the range of associated diseases differs according to the detailed serotype

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Objective: To determine the diagnostic distribution in a consecutive anti-SSA and/or anti-SSB positive population.

Methods: A total of 15 937 serum samples from 10 550 consecutive patients were analysed for antinuclear antibodies (ANAs) using either immunoassay and line immunodiffusion with recombinant SSA-Ro52, natural SSA-Ro60, and recombinant SSB. Disease associations were systemic lupus erythematosus (SLE) (45.3%), primary Sjögren’s syndrome (pSS) (14.4%), scleroderma (8.8%), RA (7.7%), cutaneous lupus (7.7%), and dermatomyositis (2.2%). The ratio of diagnoses differed according to the anti-SSA/anti-SSB serotype. Scleroderma and dermatomyositis were enriched among mono-Ro52 reactive serum samples (34.2% and 10.5% respectively). Single reactivity towards Ro60 or anti-Ro60 with anti-Ro52 predisposed for SLE (80.0% and 52.2% respectively). Triple reactivity towards Ro52, Ro60, and SSB was primarily linked with SLE (55.8%) followed by pSS (20.9%). Anti-SSA on immunodiffusion increased the chance for SLE (62.8%), whereas isolated anti-SSB reactivity on immunodiffusion was less indicative for SLE (14.3%) and predisposed more for cutaneous lupus (23.8%) and pSS (33.3%).

Conclusion: The diagnostic range associated with anti-SSA or anti-SSB reactivity differs significantly according to the detailed serotype defined by line immunodiffusion and immunofluorescence.

Anti-SSA autoantibodies are the most often identified antinuclear antibodies (ANAs). They were initially recognised in systemic lupus erythematosus (SLE) and Sjögren’s syndrome (SS). It soon became evident that anti-SSA antibodies were also present in serum samples from patients with other autoimmune diseases.

Anti-SSA antibodies may react against at least two different protein determinants: a 52 kD protein (Ro52) and a 60 kD protein (Ro60). The Ro60 molecule and the SSB protein have been shown to be components of the same ribonucleoprotein complex. It remains uncertain, however, whether the Ro52 protein is also a component of this complex. Evidence has been presented that Ro52 and Ro60 are structurally unrelated and, if they are associated on the molecular level, that this association is most likely transitory.

The anti-SSA response may be directed towards either of these components. Anti-SSB antibodies often occur together with anti-SSA antibodies. Therefore, we consider anti-Ro52, anti-Ro60, and anti-SSB reactivity altogether.

Various serological assays exist for the detection of anti-SSA and anti-SSB reactivity. Enzyme linked immunosorbent assays (ELISAs), double immunodiffusion, and counter-immunoelectrophoresis techniques are the commonly used tests in clinical laboratories. Recently, recombinant Ro52 and recombinant or native Ro60 have been introduced in different assays to detect autoantibodies towards one or both components. The diagnostic value of these subsets of antibodies is, however, not clear. We describe here the diagnostic association of anti-SSA and anti-SSB antibodies identified in a large consecutive population, from whom serum samples were referred to the laboratory for ANA testing.

Patients and Methods
A total of 15 937 serum samples from 10 550 consecutive patients were referred to the rheumatology laboratory (Ghent University Hospital) over a three year period (1996–9) for ANA detection and identification. These samples were referred by in house rheumatologists (25% of the samples), internal medicine specialists (15%), gastroenterologists (7%), dermatologists (5%), neurologists (5%), nephrologists (3%), and external hospitals or laboratories (23%).

Serum samples positive for ANA were further analysed in parallel by double immunodiffusion with thymus/spleen nuclear extract (mammalian extracted nuclear antigen, Immunoconcepts, Sacramento, CA, USA) and by line immunosassay coated with nuclear antigens, including recombinant Ro52 and SSB, and natural Ro60 (INNO LIA ANA K1090, Innogenetics, Gent, Belgium). For each patient showing anti-SSA (Ro52 and/or Ro60) and/or anti-SSB reactivity, clinical information was asked from the doctor who had ordered the test. Thus, diagnostic information could be obtained in 181 patients. Patients who were classified as having SLE, rheumatoid arthritis (RA), scleroderma (Scl), primary Sj (pSS), or dermatomyositis (DM) met the classification criteria for the respective disorders.

Abbreviations: ANA, antinuclear antibodies; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CLE, cutaneous lupus erythematosus; DM, dermatomyositis; ELISA, enzyme linked immunosorbtent assay; pSS, primary Sjögren’s syndrome; RA, rheumatoid arthritis; Ro52, 52 kD protein; Ro60, 60 kD protein; Scl, scleroderma; SLE, systemic lupus erythematosus; SS, Sjögren’s syndrome.
Patients classified with cutaneous lupus erythematosus (CLE) had CLE established by biopsy but did not meet the criteria for SLE.

**Indirect immunofluorescence on HEp-2 cells**
Serum diluted 1:40 in phosphate buffered saline (PBS) was overlaid onto fixed HEp-2 cells (Medica inc, Carlsbad, CA, USA) for 30 minutes at room temperature. Slides were washed twice for five minutes each with PBS, overlaid with fluoresceinated total immunoglobulin, and incubated for an additional 30 minutes. After washing twice, a coverslip was placed over the slide, and the slides were read using a fluorescence microscope at 40x power.

**Double immunodiffusion**
Precipitating antibodies against extractable nuclear antigens were detected by double immunodiffusion on Ouchterlony plates with thymus/spleen nuclear extract (mammalian extracted nuclear antigen, Immunoconcepts, Sacramento, CA, USA). Antibody specificity was determined by comparison with a reference serum.

**Line immunoassay**
A line immunoassay coated with nuclear antigens, including full size *Escherichia coli* derived recombinant Ro52, recombinant SSB, and natural Ro60 (INNO-LIA ANA K1090), was used. The test was performed according to the manufacturer’s instructions. Briefly, the nylon strips were incubated with serum at a 1:200 dilution. A goat antihuman IgG labelled with alkaline phosphatase was allowed to bind to the antigen-antibody complex. The enzyme substrate and chromogen 5-bromo-4-chloro-3-indolyl phosphatase (BCIP) produces a dark brown colour in proportion to the amount of specific autoantibody in the test sample. Sulphuric acid stops the colour development (fig 1).

**Statistics**
Percentages and their corresponding 95% confidence intervals (95% CIs) (one binomial) and Fisher’s exact test were performed by StatXact.

**RESULTS**
Testing for ANA consecutively performed on 15 937 serum samples from 10 550 patients referred to our laboratory over a three year period, was positive in 4691 samples from 2669 patients. Anti-SSA and/or anti-SSB reactivity was found in 11.8% of ANA positive serum samples. We identified 181 consecutive patients with anti-SSA and/or anti-SSB antibodies.

### Table 1: Distribution of diagnoses in the anti-SSA and/or anti-SSB positive population according to the fine reactivity defined by line immunoassay

<table>
<thead>
<tr>
<th>n</th>
<th>CLE % (95% CI)</th>
<th>SLE % (95% CI)</th>
<th>SCl % (95% CI)</th>
<th>RA % (95% CI)</th>
<th>PM/DM % (95% CI)</th>
<th>pSS % (95% CI)</th>
<th>Other % (95% CI)</th>
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<tr>
<td>181</td>
<td>45.3 (47.1 to 62.1)</td>
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<td>10</td>
<td>80.0 (44.4 to 97.5)</td>
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<td>40.0 (12.2 to 73.8)</td>
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<td>9</td>
<td>33.3 (2.8 to 60.0)</td>
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CLE, cutaneous lupus without systemic involvement; ID, immunodiffusion; PM/DM, polymyositis/dermatomyositis; pSS, primary Sjögren’s syndrome; RA, rheumatoid arthritis; SCl, scleroderma; SLE, systemic lupus erythematosus.

Figure 1 Different combinations of reactivities towards Ro52, Ro60, and SSB defined by line immunoassay. Lane a: anti-Ro52 antibodies; lane b: anti-Ro60 antibodies; lane c: anti-SSB antibodies; lane d: anti-Ro52 and anti-Ro60 antibodies; lane e: anti-Ro52 and anti-SSB antibodies; lane f: anti-Ro52 and anti-SSB antibodies; lane g: anti-Ro52 and anti-Ro60 and anti-SSB antibodies.

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Table 2 gives the distribution of diagnoses according to the fine reactivity defined by immunodiffusion. Table 3 gives the distribution of diagnoses according to the detailed anti-SSA/anti-SSB reactivity defined by line immunoassay with recombinant Ro52, recombinant SSB, and natural Ro60. Single anti-Ro52 reactivity increased the probability for systemic sclerosis (34.2%, 95% CI 19.6–51.4 versus 8.8% in the total anti-SSA and/or anti-SSB positive population) whereas the chances for SLE (15.8%, 95% CI 6.0 to 31.3), PM/DM (10.5%, 95% CI 2.9 to 24.8) and pSS (7.9%, 95% CI 1.7 to 21.4) became comparable. Single reactivity towards Ro60 or anti-Ro60 combined with anti-Ro52 increased the probability for SLE (respectively 80.0%, 95% CI 44.4 to 97.5 and 52.2%, 95% CI 30.6 to 73.2). Triple reactivity towards Ro52, Ro60 and SSB predisposed primarily to SLE (55.8%, 95% CI 44.7 to 66.5), followed by pSS (20.9%, 95% CI 12.9 to 31.1). On the other hand, triple reactivity was significantly less indicative for Scl (1.2%, 95% CI 0.03 to 6.3), CLE (5.8%, 95% CI 1.9 to 13.1), and RA (5.8%, 95% CI 1.9 to 13.1).

Table 2 gives the distribution of diagnoses according to the immunodiffusion result. A positive result for anti-SSA on immunodiffusion, independent of the result for anti-SSB reactivity, strongly predisposed to SLE (66.7%, 95% CI 44.7 to 83.5 and 61.4%, 95% CI 49.0 to 72.8) and to a significantly smaller extent to pSS (18.5%, 95% CI 6.3 to 38.1 and 15.7%, 95% CI 8.1 to 26.4). Isolated anti-SSB immunodiffusion reactivity instead decreased the chances for SLE (14.3%, 95% CI 3.0 to 34.63) whereas in the sub-group, CLE (23.8%, 95% CI 14.0 to 36.2) and pSS (33%, 95% CI 14.6 to 57.0) became more probable. Sixty three of the 181 patients (34.8%) were solely identified by line immunoassay and not by immunodiffusion. Negative results for immunodiffusion in the line immunoassay positive group were less indicative for pSS (4.8%, 95% CI 1.0 to 13.3) whereas SLE (28.6%, 95% CI 17.9 to 41.3) and Scl (23.8%, 95% CI 14.0 to 36.2) were found in almost equal percentages.

Combining immunodiffusion and line immunoassay had only additional diagnostic value in the case of combined reactivity towards Ro52, Ro60, and SSB (table 3). A positive test result for anti-SSA on immunodiffusion in this group gave a probability for SLE of 69.2% (95% CI 48.2 to 85.7) and 64.1% (95% CI 47.2 to 78.8) and a significantly lower chance for pSS (19.2%, 95% CI 6.6 to 39.4 and 15.4%, 95% CI 5.9 to 30.5). On the contrary, the finding of a precipitin line on immunodiffusion for SSB but not for SSA in this profile decreased the probability for SLE (13.3%, 95% CI 1.7 to 40.5) whereas in that case there was a tendency towards an increased chance for pSS (46.7%, 95% CI 21.3 to 73.4; table 3).

DISCUSSION

The description of a large, consecutively identified cohort of anti-SSA and/or anti-SSB positive serum samples in the routine setting of a rheumatology laboratory offers the opportunity to look at a realistic representation of the diagnostic range associated with this type of autoreactivity. Most of the work on the value of autoantibodies has been carried out by...
testing samples from selected patients with well defined clinical disease. By contrast, by looking at sensitivity and specificity of autoantibody markers such as anti-SSA and anti-SSB, the current study rather provides clues for estimating the probability for a certain diagnosis given the anti-SSA/anti-SSB status, taking into account that the a priori probabilities can differ according to the type of clinical practice and the specialty of the doctor ordering the test. Serum samples in our laboratory had a mixed origin, with about one third of the ANA positive samples coming from the rheumatology department. A positive ANA result itself has only weak predictive value for diagnosing SLE or other connective tissue diseases, even in a group whose serum samples are specifically referred for ANA testing.

Identification of more specific antinuclear reactivities significantly increases the predictive diagnostic value up to a level that is of real diagnostic value in specialist practice. Anti-SSA and/or anti-SSB reactivity were identified in 11.8% of the ANA positive patients. The most prevalent disease associated with anti-SSA/SSB autoreactivity was SLE. Especially, the combined triple reactivity (anti-Ro52, anti-Ro60, and anti-SSB) and anti-Ro60 with or without anti-Ro52 reactivity makes this diagnosis highly probable. Our data confirm that anti-Ro60 reactivity without anti-Ro52 and anti-Ro52 reactivity is very indicative for SLE.

By contrast, none of the 26 patients with pSS had only antibodies to Ro60, whereas anti-Ro52 reactivity was present in 25 of the 26 patients. Previous evidence has been presented that the major anti-SSA response consists of anti-Ro52 antibodies in pSS and anti-Ro60 antibodies in SLE. Patients with Scl or DM rarely present with combined anti-Ro52, anti-Ro60, or anti-SSB antibodies. Our present study, representing a DM rarely present with combined anti-Ro52, anti-Ro60, or anti-SSB antibodies.

To our knowledge, no other studies examined the diagnostic range associated with the detailed anti-SSA/anti-SSB serology in a large consecutive ANA-positive cohort. This analysis underscores the interest in identifying the detailed reactivity of anti-SSA/SSB autoantibodies, as this alters the ratios of associated diagnoses, and thus the diagnostic probabilities. Evidence has been provided that patients with undifferentiated connective tissue disease and antibodies to SSA can progress in a relatively short period to well defined connective tissue diseases. The possibility exists that some of our patients classified as “other” will evolve to defined connective tissue diseases over time.

It seemed that immunodiffusion had somewhat higher diagnostic value than line immunoadsays (table 2). However, 63 serum samples positive on line immunoadsay were not identified by immunodiffusion versus two serum samples that were solely retrieved by immunodiffusion. Most of these 63 patients had a defined connective tissue disease. The higher sensitivity of the line immunoadsay could mainly be attributed to the earlier described better performance of this assay in detecting anti-Ro52 and anti-SSB antibodies. Based on this higher detection level and on the fact that the diagnostic range associated with anti-SSA/anti-SSB reactivity differs significantly according to the fine serotype, we suggest screening for anti-SSA/anti-SSB reactivity by line immunoadsay. When confronted with triple reactivity to Ro52, Ro60, and SSB on line immunoadsay, we found that additionally performed immunodiffusion discriminates between SLE and pSS. An additional advantage of the line immunoassay technique in clinical practice is that with one test result information can be obtained on the range of simultaneous occurrence of autoantibodies in connective tissue disease. Besides anti-Ro52 and anti-Ro60, this assay also detects antireactivity towards the different antigen determinants of the RNP-antigen (RNP-A, C, and 70) and the Sm antigen (SmB and SmD). A major challenge for autoantibodies in general and for anti-SSA/anti-SSB in particular is now to find out whether reactivities to subtypes of antigens orientate towards a specific diagnosis or a specific feature common to different clinical entities, as well as to understand which mechanisms induce these different reaction patterns in autoimmune patients.

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