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Screening for connective tissue disease-associated antibodies by automated immunoassay

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Abstract

Background: Antinuclear antibodies (ANAs) are useful for the diagnosis of ANA-associated systemic rheumatic disease (AASRD). The objective of this study was the evaluation of an immunoassay that detects antibodies to a mixture of 17 antigens as an alternative to indirect immunofluorescence (IIF).
Methods: Nine thousand eight hundred and fifty-six consecutive patients tested for ANAs were tested by IIF and EliA connective tissue disease screen (Thermo-Fisher). Medical records were reviewed for 2475 patients, including all patients that tested positive/equivocal by either test and a selection of 500 patients that tested negative.
Results: Concordance between IIF and EliA was 83.1%. AASRD was found in 12.8% of IIF-positive patients, 30.2% of EliA-positive patients and 0.4%, 46.6%, 5.8% and 3.0% of patients that tested, respectively, double negative, double positive, single positive for EliA and single positive for IIF. The association with AASRD increased with increasing antibody level. IIF and EliA were positive in, respectively, 90.4% and 69.9% of systemic lupus erythematosus (n = 83), 100% and 84.1% of systemic sclerosis (n = 63), 86.7% and 93.3% of Sjögren's syndrome (n = 45), 88.2% and 52.9% of polymyositis/dermatomyositis (n = 17), and in all cases of mixed connective tissue disease (n = 8). The specificity was projected to be 94%–96% for EliA and 86% for IIF. When all AASRDs were taken together, the areas under the curve of receiver operator curves were similar between IIF and EliA.
Conclusions: The positive predictive value for AASRD was higher for EliA than for IIF, but, depending on the disease, EliA might fail to detect antibodies that are detected by IIF. Combining immunoassay with IIF adds value.

Keywords: antinuclear antibodies; autoantibody(ies); autoimmune diseases; enzyme immunoassay; indirect immunofluorescence.

Introduction

Antinuclear antibodies (ANAs) are useful for the diagnosis of ANA-associated systemic rheumatic diseases (AASRD), including systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), mixed connective tissue disease (MCTD), polymyositis/dermatomyositis (PM/DM) and systemic sclerosis (SSc) [1, 2]. At disease onset, patients with SRD can present with nonspecific symptoms such as fatigue, joint pain or muscle weakness. At this stage, differential diagnoses are manifold.

Indirect immunofluorescence (IIF) is considered the gold standard for ANA screening [3]. At a low cutoff, IIF has a high sensitivity but low specificity [4]. Because SRD have a low prevalence, most positive low-titer IIF results in the context of aspecific symptoms will be clinically false positive. These false-positive results may trigger unnecessary additional analyses, stressing the need for more specific tests.

Recently, fully automated systems for autoantibody screening have been developed, such as BioPlex 2200 ANA screen (BioRad), which is an automated multiplexed system that allows the simultaneous detection of 13 antibodies [5], and EliA™ connective tissue disease (CTD) screen (Thermo-Fisher, Freiburg, Germany), which is a solid phase fluorescence enzyme immunoassay (FEIA) that detects antibodies to a mixture of 17 autoantigens. Such systems are attractive alternatives to IIF, not only because of automation but also because of the improved specificity compared to IIF [4].

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Automated FEIA like EliA™ CTD screen is increasingly introduced in clinical laboratories, and the question arises whether such assay can replace IIF for AASRD screening. Only few studies have evaluated CTD screen. Op de Beeck et al. [4] reported the specificity of EliA CTD screen to be 97% in healthy controls and 96% in diseased controls, compared to, respectively, 94% and 82% for IIF (cutoff 1:160). The sensitivity of CTD screen was lower than the sensitivity of IIF for SLE and SSc, but not for SS or PM/DM [4]. Robier et al. [6] compared CTD screen to IIF in 1708 consecutive samples. They demonstrated that the sensitivity of EliA CTD screen for anti-dsDNA, anti-SSA, anti-SSB, anti-U1RNP and anti-Jo-1 antibodies was higher than the sensitivity of IIF. On the other hand, the sensitivity for anti-CENP-B antibodies was highest by IIF. Obviously, IIF also had a higher sensitivity for antibodies to antigens not included in EliA CTD screen assay, such as histone, nucleosome and Pl-12. The authors reported a higher sensitivity of CTD screen for SS and a higher sensitivity of IIF for SLE and limited SSc. They concluded that sequential or parallel screening with IIF and CTD screen is reasonable when the clinical suspicion for CTD is high [6]. The strength of the study of Robier et al. [6] is that it reflects a real-life routine laboratory situation, the weakness that only few AASRD patients (n = 61) were included.

In order to better comprehend the performance of automated immunoassay in comparison to IIF in a routine clinical setting, we systematically performed IIF and automated immunoassay on all samples submitted to the clinical laboratory for ANA testing over a 2-year period.

Materials and methods

Study population

Between May 8, 2013, and April 30, 2015, IIF and EliA CTD screen were simultaneously performed on all serum samples submitted to the clinical laboratory for ANA testing (n = 18,432 samples). Samples from patients that had already been tested for ANAs before May 8th were excluded (n = 7474). If multiple samples from the same patient were present in the cohort, then all but the first sample were excluded. This resulted in the inclusion of 9856 samples from unique patients. The flow of the sample selection is illustrated in Supplementary Data, Figure 1.

The IIF and CTD screen results were correlated to the clinical diagnosis conform the diagnostic criteria. For diagnosis of PM/DM, the criteria of Bohan et al. [7] were used. For SLE and SS, the classification criteria of the American College of Rheumatology (ACR) were applied [8, 9]. For MCTD the criteria of Alarcón-Segovia and Cardiel [10] were used, and for SSc the ACR/EULAR classification criteria were used [11]. The study was approved by the Ethics Committee of the University Hospitals Leuven (s57553).

The study included 83 patients with a new and 133 patients with a known AASRD diagnosis: SLE (n = 83, male/female ratio 11:72, median age 45 years, range 8–78 years), SSc (n = 45, male/female ratio 3:42, median age 55 years, range 16–85 years), SS (n = 63, male/female ratio 16:47, median age 60 years, range 22–87 years), PM/DM (n = 17, male/female ratio 7:10, median age 43 years, range 22–78 years), and MCTD (n = 8, male/female ratio 1:7, median age 31 years, range 12–55 years).

ANA detection by IIF

ANA was performed using SSA-transfected HEp-2000® cells (Immunoconcepts, Sacramento, CA, USA) (screening dilution 1:40) [12]. Samples with a titer ≥1:80 were considered positive.

ANA detection by FEIA

In the EliA™ CTD screen, each well is coated with following antigens: dsDNA, SSA/Ro 52, SSA/Ro 60, SSB/La, U1-RNP (RNP-70, A, C), Sm, Jo-1, Scl-70, CENP, fibrillarin, RNA Pol III, PM-Scl, Mi-2, Rib-P and PCNA. In case of an equivocal (ratio 0.7–1.0) or positive (ratio >1.0) result, a standard confirmatory autoantibody panel was tested: anti-SSA-60, anti-SSB, anti-U1 RNP, anti-RNP-70, anti-SmD, anti-Scl-70s and anti-Jo-1 antibodies. In the presence of a centromere IIF pattern, anti-CENP antibodies were tested by EliA. In case the CTD screening result was positive and no antibodies were detected with the standard confirmatory panel, antibodies to RNA polymerase III, PM-Scl, fibrillarin, Mi-2, SSA-52, PCNA, Rib-P and dsDNA antibodies were tested. The assays were performed on an ImmunoCAP 250 instrument (Phadia, Freiburg, Germany) (cutoffs for all antibody results with exception of U1RNP: <7 U/mL = negative, 7–10 U/mL = equivocal and >10 U/mL = positive; cutoff for U1RNP antibody results: <5 U/mL = negative, 5–10 U/mL = equivocal and >10 U/mL = positive).

During the study period, native Sm was replaced by recombinant SmD on June 6, 2013 and Scl70 by Scl70 sensitive on January 16, 2015.

Categorization of patients

SRD patients were categorized as ANA-associated SRD (AASRD) including SLE, SS, MCTD, PM/DM and SSc or non-ANA-associated SRD (non-AASRD), i.e. different types of vasculitis, polymyalgia rheumatica and sarcoidosis (categorized according to clinical diagnosis). Other clinical categories were cutaneous lupus, rheumatic diseases (RD; e.g. rheumatoid arthritis, psoriatic arthritis), other inflammatory diseases (e.g. colitis ulcerosa, Crohn's disease, auto-immune hepatitis, auto-immune thyroiditis, psoriasis and immune thrombocytopenic purpura) and absence of inflammatory diseases. Patients with insufficient data for proper categorization were excluded. The patients were further categorized considering whether ANAs were tested on a diagnostic sample ('new…') or on a follow-up sample ('known with…') and whether all the necessary classification criteria were fulfilled or not. If not all classification criteria were fulfilled, the diagnosis was labeled as 'doubtful'.

Statistics

Fisher Exact and receiver operator curve (ROC) analysis was performed using Analyse-it Software® for Microsoft Excel. Comparisons of the area under the curve (AUC) was done by the method of De Long (Analyse-it). For ROC curve analysis, samples positive for the SSA-transfected cells (IIF) were assigned a titer of 1:1280. For multiple comparisons, 'Steel-Dwass-Critchlow-Fligner' all pairs comparisons test was performed (Analyse-it.)

Results

Concordance between IIF and CTD screen

ANA was tested by IIF and by CTD screen in 9856 consecutive patients. The results are summarized in Table 1. Positivity by IIF was found in 1665 (16.9%) patients and positivity by immunoassay in 623 (6.3%) patients. In 8191 patients (83.1%), there was concordance between IIF and immunoassay (382 [3.9%] concordant positive and 7813 [79.3%] concordant negative).

Association of (combined) antibody positivity with AASRD

Medical records were reviewed of (i) all patients with discordant results (n = 1661), (ii) all patients with concordant positive results (n = 382) and (iii) a random selection of 517 consecutive patients with concordant negative results (n = 361 with IIF titer <1:40 and n = 156 with IIF titer 1:40). Of these 2560 patients, 85 (3.3%) were excluded (17 of which were double negative) due to insufficient data for proper clinical categorization. In 83 patients, a new diagnosis of AASRD was established. In 133 patients, an AASRD had previously been diagnosed in another medical center (primary or secondary care) and the patients were referred to a tertiary hospital for further guidance. Most of these patients had received immunosuppressive therapy before referral. In 62 patients, the clinician strongly considered the presence of an AASRD and initiated immunosuppressive therapy, but the patient did not fulfill the diagnostic criteria ('doubtful' AASRD). Twenty two patients had cutaneous lupus.

Figure 1 shows the results obtained by CTD screen in the various disease groups. AASRD (new and known) patients had significantly higher antibody levels than patients with a non-AASRD (n = 110) or patients with a RD, an inflammatory disease or absence of an inflammatory disease (n = 2065) ($p < 0.0001$ for all comparisons). Figure 2 shows the results of IIF as well as CTD screen for the different disease groups and illustrates that the majority of patients with AASRD had high antibody levels by IIF and CTD screen.

Table 2 shows the distribution of patients with AASRD as a function of IIF and CTD screen results. AASRD was found in 12.8% of IIF-positive patients, in 30.2% of CTD screen-positive patients, in 46.6% of IIF-positive/CTD screen-positive patients and in 13.3% of IIF-positive/CTD screen-equivocal patients (Table 2). AASRD was found in 3.0% of IIF-positive/CTD screen-negative patients and in 5.8% of IIF-negative/CTD screen-positive patients ($p = 0.046$ for comparison with the IIF-positive/CTD screen-negative group). Yet, the number of AASRD patients in the IIF-positive/CTD-negative group was higher than in the IIF-negative/CTD-positive group. Results including doubtful (i.e. not fulfilling diagnostic criteria) diagnoses are shown in Supplementary Data Table 1 but are not discussed further. Overall, including uncertain diagnosis did not change the conclusions. A more detailed analysis of the results according to the different disease groups is shown in Supplementary Data, Table 2. The results indicate that the positive predictive value (PPV) of double positivity (IIF and CTD screen) was higher than the PPV of single positivity (either IIF or CTD screen) and that the PPV was higher for CTD screen than for IIF. However, the overall PPV was low.

Table 1: Classification (number and proportion) of patient samples according to IIF and EliA CTD screen results.

	Elia CTD screen			Total
	Negative	Equivocal	Positive	
IIF negative	7813 (79.3%)	137 (1.4%)	241 (2.4%)	8191 (83.1%)
IIF positive	1217 (12.3%)	66 (0.7%)	382 (3.9%)	1665 (16.9%)
Total	9030 (91.6%)	203 (2.1%)	623 (6.3%)	9856 (100.0%)

Proportions are given as a percentage of the total number of included samples (n = 9856).

Effect of antibody level on association with AASRD

Next, we evaluated how the association with AASRD depended on the antibody level. AASRD was found in 7.4% (4/54), 32.7% (17/52), 44.7% (17/38), 75.0% (27/36), 79.6% (43/54) and 47.6% (49/103) of patients with a positive CTD screen (ratio >1) in combination with an IIF titer of, respectively, 1:80, 1:160, 1:320, 1:640, 1:1280 or the

Figure 1: Distribution of EliA CTD-Screen titers in the different clinical subgroups.
The groups included ANA-associated systemic rheumatic disease (AASRD) (n = 83 new AASRD, 133 known with AASRD, 62 doubtful AASRD), cutaneous lupus (n = 22), not ANA-associated systemic rheumatic disease (non-AASRD) (n = 30 new, 40 known with, 40 doubtful), rheumatic disease (RD) (n = 54 new, 110 known with, 19 doubtful) and inflammatory disease (n = 241) and no inflammatory disease (n = 1641).

typical SSA pattern. AASRD was found in 12.0% (3/25), 44.8% (13/29), 59.1% (13/22), 82.1% (23/28), 82.4% (42/51) and 46.5% (46/99) of patients with a positive CTD screen ratio ≥2.5 in combination with an IIF titer of, respectively, 1:80, 1:160, 1:320, 1:640, 1:1280 and the typical SSA pattern. AASRD was found in 0.8% (4/517), 2.9% (12/412), 5.0% (7/141), 10.9% (6/55), 15.0% (6/40) and 0.0% (0/4) of patients with a negative CTD screen results (ratio <0.7) in combination with an IIF titer of, respectively 1:80, 1:160, 1:320, 1:640, 1:1280 or the typical SSA pattern. These data clearly indicate that the higher the antibody level, the higher the chance for AASRD.

Effect of pretest probability on PPV

Subsequently, we separated clinical units that are specialized in SRD (rheumatology, general internal medicine, clinical immunology, dermatology, cardiology, clinic for coagulation disorders and pediatric hematology) (high prevalence units) from units that are less specialized in SRD (all other units) (low prevalence units). The outcome is shown in Table 3 and illustrates a significant higher PPV for AASRD when ANA was requested by clinicians specialized in SRD. The results also show that the PPV for AASRD was higher for CTD screen-positive/IIF-negative than for IIF-positive/CTD screen-negative. Results including doubtful (i.e. not fulfilling diagnostic criteria) diagnoses are shown in Supplementary Data, Table 3. Overall, including uncertain diagnosis did not change the conclusions.

Approximation of performance characteristics

Of the 83 newly diagnosed AASRD patients, 77 (92.8%), 70 (84.3%) and 64 (77.1%) were, respectively, IIF-positive, CTD screen-positive and IIF-positive/CTD screen-positive. Of the 133 known AASRD patients, 123 (93.2%), 100 (75.8%) and 93

Figure 2: Distribution of indirect immunofluorescence and Elia CTD-screen results for the different disease subgroups.
AASRD, ANA-associated systemic rheumatic disease; non-AASRD, not ANA-associated systemic rheumatic disease; RD, rheumatic disease. The non-AASRD and RD subgroup include new and known patients. Doubtful diagnoses were excluded.

Table 2: Number and proportion (%) of patients with AASRD according to IIF and EliA CTD screen results.

	CTD screen negative	CTD screen equivocal	CTD screen positive
IIF negative	2 (0)/495	1 (0)/126	13 (6)/226
	0.4%	0.8%	5.8% (2.7%)
IIF positive	35 (11)/1169	8 (2)/60	157 (64)/337
	3.0% (0.9%)	13.3% (3.3%)	46.6% (19.0%)

Table includes new and known AASRD. Values in parenthesis indicate the number or proportion of newly diagnosed patients. p-Values were calculated using the Fisher exact analysis. Proportion of new and known AASRD's is statistically significantly different between IIF negative CTD-positive and IIF positive CTD-negative subgroups (p = 0.0456). AASRD, ANA-associated systemic rheumatic disease; IIF, indirect immunofluorescence; CTD, connective tissue disease screening.

(70.5%) were, respectively, IIF-positive, CTD screen-positive and IIF-positive/CTD screen-positive. A detailed overview of the results is given in Supplementary Data, Table 2.

CTD screen was positive or equivocal in 12 (70.6%) out of 17 PM/DM patients, in 43 (95.6%) out of 45 SS patients, in 62 (74.7%) out of 83 SLE patients and in 54 (85.7%) out of 63 SSc patients whereas IIF was positive in 15 (88.2%) out of 17 PM/DM patients, 39 (86.7%) out of 45 SS patients, 75 (90.4%) out of 83 SLE patients and 63 (100%) out of 63 SSc patients. Both IIF and CTD screen were positive in all MCTD patients (n = 8). The distribution of the different newly diagnosed AASRDs among the serological subgroups defined by IIF and CTD screen results are given in Supplementary Data, Table 4. Six new AASRD diagnoses were made in the IIF-negative/CTD screen-positive subgroup (four Sjögren and two PM/DM diagnoses), whereas 11 new diagnoses were made in the IIF-positive/CTD screen-negative subgroup (three SLE, two PM/DM and six SSc diagnoses). Thus, CTD screen detected reactivity in SS patients that was missed by IIF, whereas IIF detected reactivity in SLE and SSc patients that was missed by CTD screen. In patients known with AASRD, seven (three SLE

Table 3: Number and proportion (%) of patients with AASRD according to IIF and EliA CTD screen results and as a function of the unit requesting the ANA.

	IIF neg CTD neg		IIF neg CTD equiv		IIF neg CTD pos	
	HPU	LPU	HPU	LPU	HPU	LPU
Total number of patients	221	274	52	74	98	128
AASRD abs.	2 (0)	0 (0)	0 (0)	1 (0)	11 (5)	2 (1)
AASRD, %	0.9%	0.0%	0.0%	1.4%	11.2% (5.1%)	1.6% (0.8%)
	IIF pos CTD neg		IIF pos CTD equiv		IIF pos CTD pos	
Total number of patients	568	601	30	30	217	120
AASRD abs.	28 (10)	7 (1)	6 (2)	2 (0)	121 (47)	36 (17)
AASRD, %	4.9% (1.8%)	1.2% (0.2%)	20.0% (6.7%)	6.7%	55.8% (21.7%)	30.0% (14.2%)

This table includes new and known AASRD. Values in parenthesis indicate the number or proportion of newly diagnosed patients. p-Values were calculated using the Fisher exact analysis. Proportion of new and known AASRD's is statistically significantly different between HPU and LPU in IIF negative CTD-positive and IIF-positive CTD-negative subgroups (p = 0.0027 and 0.0002, respectively). Proportion of new and known AASRD's is statistically significantly different between HPU of IIF negative CTD-positive and IIF-positive CTD-negative subgroups (p = 0.0321) but not for LPU (p = 0.6618). Neg, negative; pos, positive; AASRD, ANA-associated systemic rheumatic disease; abs., absolute; HPU, high prevalence unit (unit with high AASRD prevalence); LPU, low prevalence unit (unit with low AASRD prevalence).

and one SS) were IIF-negative/CTD screen-positive and 24 (16 SLE, 2 SS, 3 SSC and 3 PM/DM) were IIF-positive/CTD screen-negative.

Of the 2197 patients who did not have AASRD, 1366 (62.2%), 393 (17.9%) and 180 (8.2%) were, respectively, IIF positive, CTD screen positive and positive for both IIF and CTD screen, indicating that IIF had a lower specificity than CTD screen. A positive/equivocal CTD screen result was found in 393/177 (in total 570) patients without AASRD. Based on these data we could project the specificity of CTD screen for the total population to be around 94% (if equivocal results are considered positive) or around 96% (if equivocal results are considered negative) and the specificity of IIF to be around 86. The specificity was calculated as 1 – (false positives/controls) = 1 – (393/[9856 (total population) – 245 (estimated AASRD patients, see below)] = 0.96 or 1 – (570/[9856 – 245]) = 0.94). Assuming that 0.4% of seronegative patients had AASRD (Table 2), the prevalence of AASRD in our total population (n = 9856) was estimated to be 2.5% (245/9856). When cutaneous lupus was included as well, then the prevalence was estimated to be 2.7%. When doubtful diagnoses were also considered AASRD, then the prevalence was estimated to be 3.9% (or 4.1% when cutaneous lupus was included as well).

Association of specific antibodies with AASRD

All positive/equivocal CTD screen samples (n = 826) were further tested by EliA for common antibodies including anti-SSA-60, SSB, U1 RNP, RNP-70, SmD, Scl-70, Jo-1, and CENP antibodies (if IIF revealed centromere antibodies). In 341 samples (41.3%), a common antibody was detected. In this group, AASRD/doubtful AASRD was present in 48.7%/7.9%. In 485 samples, none of the above-mentioned antibodies were detected. In 462 of these samples, we tested for the less common antibodies, i.e. antibodies to RNA polymerase III, PM-Scl, fibrillarin, Mi-2, SSA-52, PCNA, Rib-P and dsDNA. In 65 samples (7.9%), a less common specific antibody (with the exception of anti-dsDNA and anti-SSA-52) was found. In these patients, AASRD/doubtful AASRD was present in 21.5%/7.7%. In 50 patients (6.1% of total CTD screen equivocal or positive results) only anti-SSA-52 antibodies were detected. An AASRD/doubtful AASRD was present in 10%/0% of these patients. In 203 patients (24.6% of total CTD screen equivocal or positive results), only anti-dsDNA antibodies were detected. An AASRD/doubtful AASRD was present in 4.2%/1.7% of these patients. In 144 samples (17.4% of total CTD screen equivocal or positive results), no specific antibody could be detected. These concerned mainly low antibody levels (data not shown). In this group of patients, four (2.8%) had AASRD. In 23 samples, there was insufficient sample volume for further testing.

Table 4 shows the association of various specific antibodies with AASRD. Some antibodies such as anti-RNP-70 were highly associated with AASRD, whereas other antibodies such as anti-Ro52 were weakly associated with AASRD. In general, the higher the antibody level, the higher the chance AASRD was present (Figure 3).

Table 4: Number of patients with positive or equivocal results for specific antibodies.

Antibody	Positive results	AASRD	PPV, %	Equivocal results	AASRD	EPV, %
EliA-Ro60	158/171	76/89	48.1/52	11	2	18.2
EliA-La (SSB)	56/60	37/41	66.1/68.3	6	2	33.3
EliA-U1 RNP	28/34	20/26	71.4/76.5	23/25	6/8	24.0/35
ELiA-RNP 70	14/20	13/19	92.8/95	3	2	66.7
EliA-SmD	31/33	15/17	48.4/51.5	8	1	12.5
EliA-Scl 70 Old	20/21	16/17	80/81	1	–	0.0
EliA-Scl 70 Sens.	2	1	50.0	–	–	NPR
EliA-Jo1	6/9	3/6	50/66.7	1	–	0.0
EliA-CENP	67/72	37/42	55.2/58.3	3	–	0.0
EliA-Fibrillarine	6	–	0.0	3	–	0.0
EliA-Mi-2	14	4	28.6	3	–	0.0
EliA-PCNA	1	–	0.0	1	–	0.0
EliA-PM-Scl	3	2	66.7	4	–	0.0
EliA-Rib-P	4	–	0.0	1	–	0.0
EliA-RNA Pol III	1/2	–/1	0.0/50	3	–	0.0
EliA-Ro52	56/57	7/8	12.5/14	10	–	0.0

The table also indicates the number of patients with AASRD. The first number excludes patients with doubtful diagnosis, whereas the second number includes patients with doubtful diagnosis. AASRD, ANA-associated systemic rheumatic disease; PPV, positive predictive value; EPV, equivocal predictive value; NPR, no positive results in the cohort; Sens., sensitive.

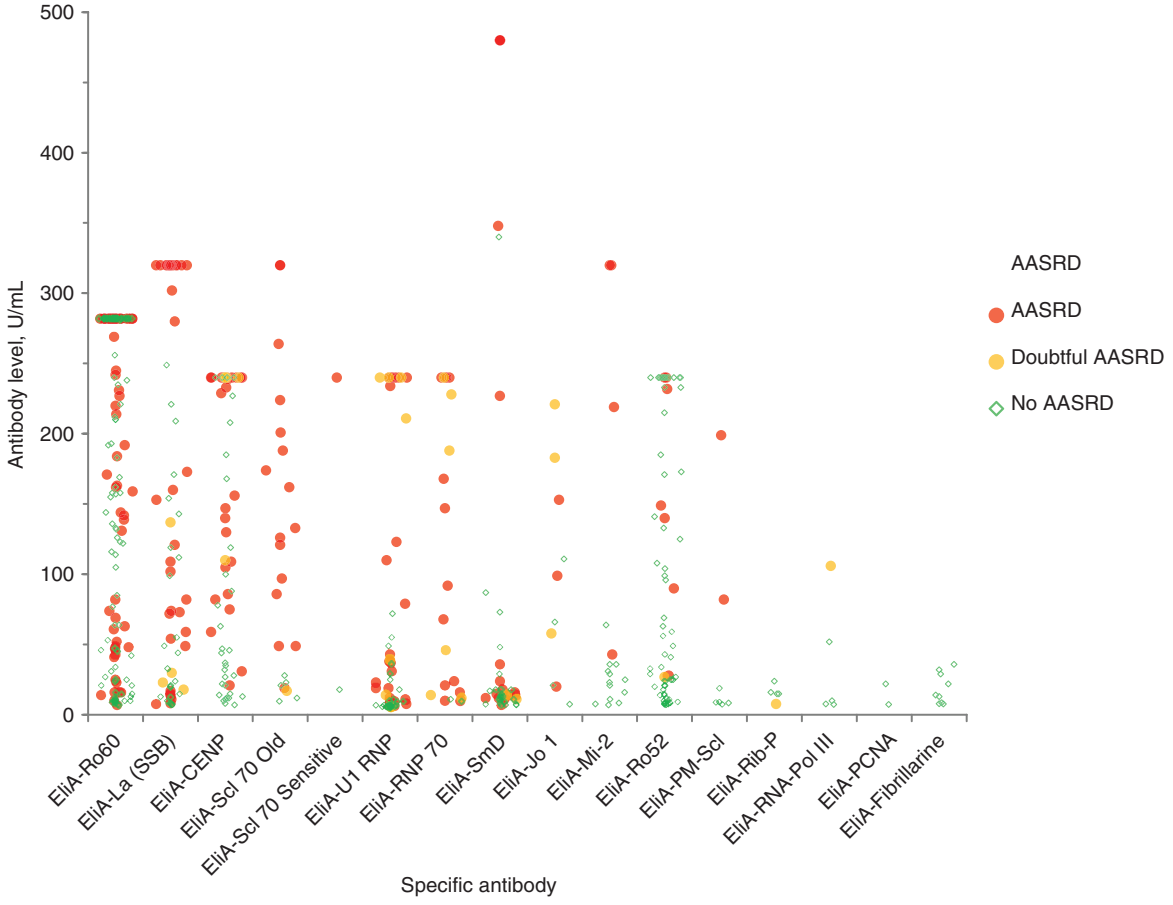

Figure 3: Antibody level of specific antibodies.
Antibody levels of antibodies to various 'extractable nuclear antigens'. Only positive results are shown, with indication of presence/absence of AASRD.

ROC curve analysis

ROC curves were made based on patients for whom clinical information was available (n = 216 AASRD patients and 2197 patients with no AASRD [with exclusion of patients not fulfilling the classification criteria]). The results are shown in Figure 4. When all AASRDs were taken together, then the AUC values (95% CI) of IIF and CTD screen were similar: 0.841 (0.811–0.872) for IIF and 0.856 (0.826–0.886) for CTD screen (p = 0.43). The AUC for SS (n = 45) was significantly (p = 0.003) higher for CTD screen (0.924 [0.876–0.971]) than for IIF (0.803 [0.799–0.892]), whereas the AUC for SSc (n = 63) was higher for IIF (0.889 [0.867–0.912]) than for CTD screen (0.546 [0.799–0.892]), but this did not reach statistical significance (p = 0.0728). For SLE (n = 82), the AUC of IIF (0.771 [0.718–0.824]) was similar to the AUC of CTD screen (0.784 [0.726–0.841]) (p = 0.70).

As the population for which the medical records were consulted was biased towards inclusion of patients with a positive test result, we extrapolated the data to the whole population. For extrapolation we made two assumptions. First we assumed that AASRD was present in 0.9%, which corresponds to the prevalence of AASRD in HPU (Table 3). Second, we assumed that AASRD was present in 0% of the double-negative patients, which corresponds to the prevalence of AASRD in LPU (Table 3). The results are shown in Figure 4. The AUC for IIF and CTD screen was, for the first assumption, respectively, 0.852 (0.819–0.884) and 0.844 (0.811–0.876) (p = 0.57) and for the second assumption, respectively, 0.934 (0.913–0.956) and 0.920 (0.895–0.945).

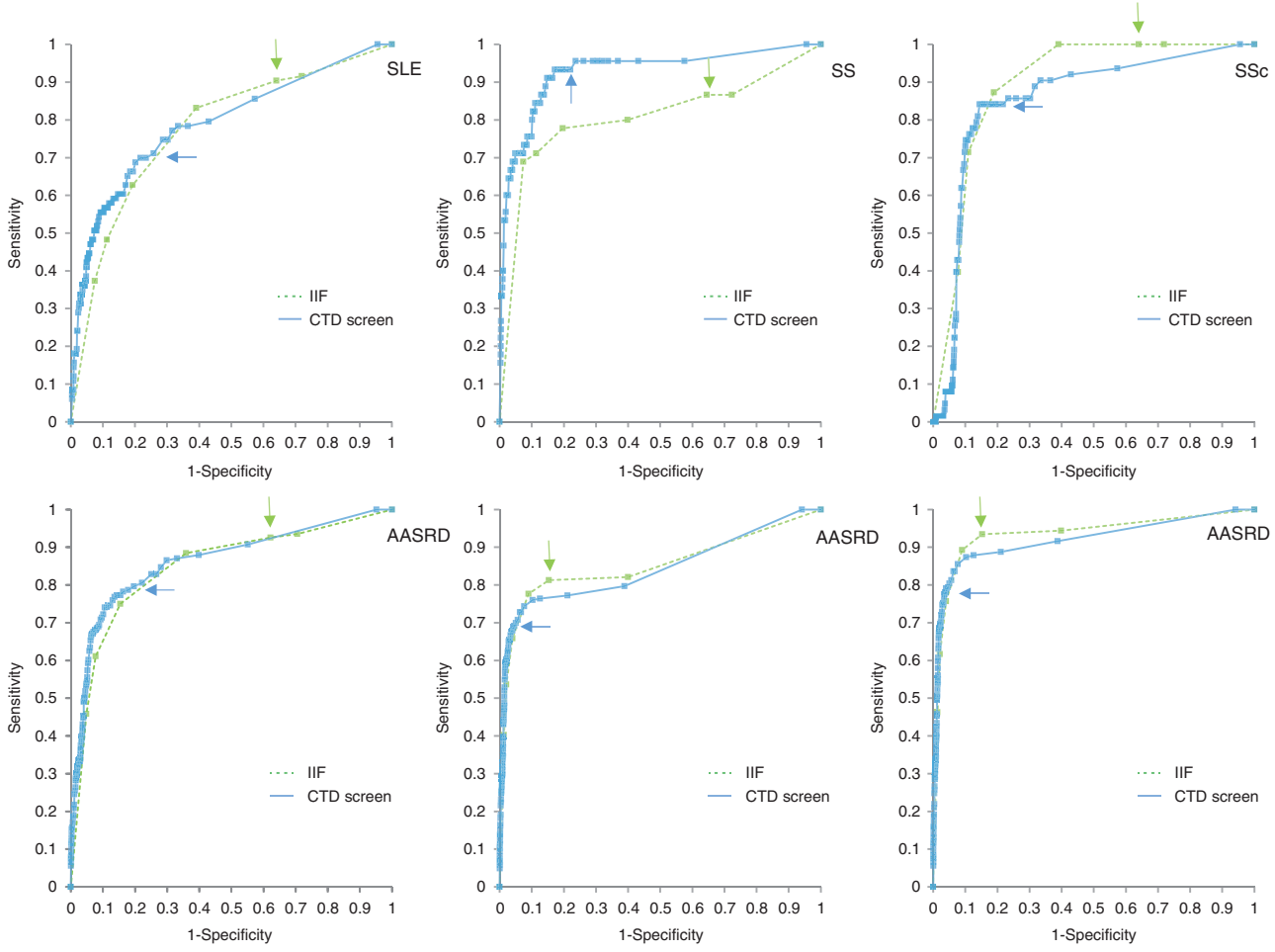

Figure 4: Receiver operating characteristics curves for EliA CTD screen and IIF to discriminate patients with AASRD from patients who did not have AASRD.
Upper panels and lower left-hand panel. Analysis performed on all samples for which clinical data was available (n = 216 AASRD and n = 2197 controls). In the upper panels, AASRD was broken down in SLE, SS and SSc. Lower-, middle- and right-hand panels panels. ROC curve analysis extrapolated for the whole study population (n = 216 AASRD and n = 9856 controls). The prevalence of AASRD in the IIF/EliA double-negative population was assumed to be 0.9% (lower middle panel) or 0% (lower right hand panel). Doubtful diagnoses were excluded. Arrows indicate cutoff point of 1:80 for IIF (green) and cutoff point of CTD screen ratio 1 (blue).

Discussion

In the present study, 9856 consecutive unique patients for whom ANA testing was requested for the first time at the university hospitals Leuven were analyzed by IIF and by EliA CTD screen. This study is unique because of the large number of patients included.

The concordance between IIF and CTD results was 83.1%, which is similar to the concordance between the two methods reported by Robier et al. [6] (78.8%). The PPV of IIF (12.8%) and of CTD screen (30.2%) for AASRD was low. It was significantly higher for double positivity (46.6%). The PPV of double positivity was higher (55.8%) when ANA was requested from a clinical unit with experience with AASRD (e.g. rheumatology) than when it was requested from clinical units with less experience with AASRD (30%). PPV also increased with increasing antibody levels. Overall, low antibody levels (for IIF and CTD screen) were less predictive for AASRD than high antibody levels, even for double-positive samples. Thus, the PPV was higher for double positivity, for high antibody levels and a high pretest probability. Of note, patients with cutaneous lupus had high antibody levels by CTD screen, due to anti-Ro60 antibodies.

The overall low PPV is related to the low prevalence of AASRD (2.5%–2.7%) and the specificity of the assays: 94%–96% for CTD screen and ~86% for IIF. Under such conditions (false positivity rate exceeding the prevalence), there are more false-positive than true-positive results.

CTD screen had a higher specificity but a lower sensitivity than IIF (cutoff 1:80), which is concordant with a previous study [4]. However, ROC curve analysis revealed that the AUC was similar for both assays. Similar findings have been reported for comparison of IIF with BioPlex [13]. The differences in sensitivity and specificity are therefore (partly) related to setting of the cutoff. Breaking down the analysis to the separate AASRD diseases revealed that for SS the AUC was significantly higher for CTD screen than for IIF, whereas for SSc, the AUC was higher for IIF than for CTD screen, albeit not significantly. The lower sensitivity of CTD screen for SSc might be partly related to a suboptimal sensitivity of this assay for anti-fibrillarin and anti-RNA polymerase III antibodies, as previously reported [14]. These findings confirm previous conclusions that the performance of immunoassay and CTD screen is disease-dependent and that combining both assays may add value [15].

The PPV of an equivocal CTD screen result was intermediate between the PPV of a negative and a positive result, which provides evidence to consider an equivocal result as separate from either a negative or a positive test result.

CTD screen detected relevant antibodies that were missed by IIF, thereby confirming previous similar observations [16, 17]. *Vice versa*, IIF detected relevant antibodies that were missed by CTD screen.

It has been suggested that in a low prevalence setting (e.g. general practitioners), a more specific immunoassay should be preferred over sensitive but non-specific IIF [18]. In the low prevalence setting in our study, CTD screen picked up 2/48 AASRD patients that were missed by IIF and IIF picked up 7/48 AASRD patients that were missed by CTD screen. However, in the same low prevalence setting, out of 1179 patients with no AASRD, IIF reported 594 false-positive results compared to 126 for CTD screen. In this situation, the increased sensitivity of IIF should be balanced against the increased number of false positives, potentially triggering unnecessary referral of patients. It might be justifiable to screen with EliA CTD screening in a setting with a low pretest probability. In case of high clinical suspicion of AASRDs, we recommend transferring the patient to an AASRD specialist, regardless of CTD screen or IIF results.

A positive CTD screen should be followed by identification of the specific antibody. In 659 patient samples, the presence of at least one specific antibody was detected. The PPV of a specific antibody for AASRD depended on the antibody and could reach >90%, e.g. for anti-RNP-70. Anti-Ro52 antibodies had a low PPV value for AASRD (<15%), confirming a previous report [19] that anti-Ro52 can be found in diseases other than AASRD. It should be noted, however, that the presence of autoantibodies might predate the presence of disease. Also of note is the low PPV of EliA anti-SmD (48%) and anti-CENP (55%), two antibodies that are classically associated with a high specificity. In that respect, we observed a drop in PPV when Sm was replaced by SmD on the EliA system (unpublished data). Finally, the PPV of mono-specific dsDNA by EliA was low. False-positive anti-dsDNA might negatively affect the specificity of the overall EliA CTD screen.

The EliA system depends on coating of wells with a mixture of 17 antigens. The affinity of the antigens for binding to the wells depends on various parameters (such as hydrophobicity, pI, and glycosylation) and thus might differ between antigens. Such complex assay set up might impact on the sensitivity and specificity. Although the specificity of EliA is clearly higher than the specificity of IIF, it is not 100%. Consequently, in a low prevalence setting, the PPV of EliA was low, with (weak) positivity observed in many clinical conditions. EliA results lack information on staining patterns. Some of these patterns such as centromeric or nucleolar are associated with certain diseases.

In conclusion, our study revealed that the performance of immunoassay and IIF depends on the specific disease and that combining immunoassay with IIF adds value, if the results of both tests are correctly judged in the context of the clinical manifestations of the patient.

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