

August 08/11: ANA detection by indirect immunofluorescence and EliA CTD Screen

Key messages:

- At equal specificity, the sensitivity of indirect immunofluorescence was lower than the sensitivity of EliA CTD Screen.
 - A positive result by EliA CTD Screen had a higher likelihood ratio than a positive result by indirect immunofluorescence.
 - On the other hand, as expected, the negative likelihood of IIF on HEp-2 is lower than that of EliA CTD Screen.
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Detection of antinuclear antibodies by indirect immunofluorescence and by solid phase assay
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Background: For the detection of antinuclear antibodies the indirect immunofluorescence (IIF) method is more and more replaced by solid phase assays. This study compares IIF on HEp-2000 cells (overexpressing SSA/Ro) with the EliA CTD Screen.

Summary: The study cohort consisted of 236 patients with different connective tissue diseases, 149 healthy blood donors, 139 patients with chronic fatigue syndrome, and 134 diseased controls.

The sensitivity of EliA CTD Screen for systemic lupus erythematosus, systemic sclerosis, primary Sjögren's syndrome, mixed connective tissue disease, and inflammatory myopathy was 74%, 72%, 89%, 100%, and 39%, respectively. The positivity in blood donors, in patients with chronic fatigue syndrome, and in diseased controls was <4%. However, among these controls a substantial portion was positive for antinuclear antibodies measured by IIF on HEp-2. 18 % of diseased controls tested positive at a cutoff titer of 1:160, while it was still 6% at a dilution of 1:640

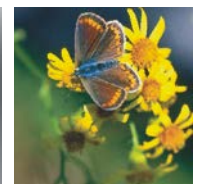
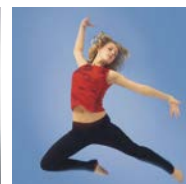
Negative likelihood ratios at a screening cutoff of 1:40 were sufficient (<0.1) for SLE, scleroderma and Sjögren's syndrome. Positive likelihood ratios were sufficient (>10) for SLE, scleroderma and MCTD, however, only at a cutoff of >1:640. In EliA CTD Screen, apart from scleroderma the other connective tissue diseases showed positive likelihood ratios exceeding a ratio of 10. Highest likelihood ratios were detected for SLE and Sjögren's syndrome patients; lowest for patients with inflammatory myopathies. At a cutoff titer which gives IIF the same specificity as EliA CTD Screen the sensitivity of IIF was considerably lower. While EliA CTD Screen detects a proportion of CTD patients who are missed by IIF the solid phase method also misses some patients detected by IIF, who show antibodies other than those included in the CTD Screen.

Generally, a positive test result by EliA CTD Screen had a higher likelihood ratio for systemic rheumatic disease than a positive test result by indirect immunofluorescence. A negative test result by indirect immunofluorescence, however, had a lower likelihood ratio than a negative test result by EliA CTD Screen, indicating that the negative predictive value was higher for indirect immunofluorescence than for EliA CTD Screen.

The examination of the individual antigens contained in the EliA CTD Screen assay confirmed the classical disease associations of specific antibodies.

Conclusions: EliA CTD Screen is superior in specificity, sensitivity at equal specificity, positive likelihood ratio and positive predictive value compared to indirect immunofluorescence. On the other hand, indirect immunofluorescence shows better negative likelihood ratio and negative predictive value than EliA CTD Screen.

Comment: There is a tendency in Europe and particularly in the US to go back to IIF on HEp-2 for the first step of ANA screening. The low specificity and low standardisation of this method is accepted with the argument that it is most important to find as many patients as possible. The result is an alarmingly high number of patients with false positive results. In this study it was shown that a positive result on HEp-2 with a titer of less than 1:640 is not a strong indication for connective tissue disease as the positive likelihood ratio is too low. Therefore, IIF is not very useful as indicative test in diagnosis but for *exclusion* of SLE, Sjögren's syndrome or scleroderma, as a patient is very unlikely to have one of these diseases when IIF is negative.





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Review

Detection of antinuclear antibodies by indirect immunofluorescence and by solid phase assay

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ABSTRACT

Testing for antinuclear antibodies is useful for the diagnosis of systemic rheumatic diseases. Solid phase assays are increasingly replacing indirect immunofluorescence for detection of antinuclear antibodies. In the most recent generation of solid phase assays, manufacturers attempt to improve the performance of the assays by adding extra antigens.

Solid phase assay (EliA CTD Screen, Phadia, in which antibodies to 17 antigens are detected) was compared to indirect immunofluorescence for the detection of antinuclear antibodies in diagnostic samples of 236 patients with autoimmune connective tissue diseases, in 149 healthy blood donors, 139 patients with chronic fatigue syndrome, and 134 diseased controls.

The sensitivity of EliA CTD Screen for systemic lupus erythematosus, systemic sclerosis, primary Sjögren's syndrome, mixed connective tissue disease, and inflammatory myopathy was 74%, 72%, 89%, 100%, and 39%, respectively. The reactivity in blood donors, in patients with chronic fatigue syndrome, and in diseased controls was <4%. At an immunofluorescence cutoff that corresponded to the specificity found with solid phase assays, the sensitivity of indirect immunofluorescence was lower than the sensitivity of solid phase assays. Likelihood ratios increased with increasing antibody concentrations. Generally, a positive test result by EliA CTD Screen had a higher likelihood ratio for systemic rheumatic disease than a positive test result by indirect immunofluorescence. A negative test result by indirect immunofluorescence, however, had a lower likelihood ratio than a negative test result by EliA CTD Screen, indicating that the negative predictive value was higher for indirect immunofluorescence than for EliA CTD screen.

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1. Introduction

Early diagnosis and treatment of systemic rheumatic diseases (e.g. systemic lupus erythematosus) improves remission rates and prognosis [1]. Antinuclear antibodies are useful for diagnosis of patients with systemic rheumatic diseases. According to the American College of Rheumatology ad hoc committee on immunologic testing, testing for antinuclear antibodies is (i) very useful for the diagnosis of systemic lupus erythematosus and systemic sclerosis, (ii) somewhat useful for the diagnosis of primary Sjögren's syndrome and polymyositis–dermatomyositis, (iii) very useful for monitoring or prognosis of juvenile chronic arthritis (to stratify the risk for uveitis), and (iv) a critical part of the diagnosis of drug-associated lupus, mixed connective tissue disease, and autoimmune hepatitis [1]. Antinuclear antibody testing is not useful for the diagnosis, monitoring, or prognosis of other diseases including rheumatoid arthritis and thyroid disease [2]. Antinuclear antibodies are also found in patients with non-rheumatic diseases such as infectious disease, malignant disease and thyroid disease, and even in individuals with no medical condition, particularly women >40 years old and elderly people [2,3]. Patients with no autoimmune disease and healthy individuals usually have low antinuclear antibody titers [3].

Antinuclear antibodies are directed against various nuclear antigens. Traditionally, indirect immunofluorescence on HEp-2 cells is used to screen for antinuclear antibodies. Systems that allow automatic classification of antinuclear antibodies on HEp-2 cells are currently being developed [4]. Some major patterns can be discerned: homogenous, speckled, centromeric, nucleolar, and speckled or diffuse cytoplasmic. Although not absolute, there exists a relationship between the pattern observed on indirect immunofluorescence and the presence of a specific antibody [3]. For example, a (high titer) homogeneous pattern is associated with antibodies to dsDNA, whereas a (high titer) speckled pattern is associated with antibodies to extractable nuclear antigens [5]. More specific second line tests are performed to identify the target antigen of the antibodies.

Quantitative automated solid phase methods are increasingly replacing indirect immunofluorescence methods for the detection of antinuclear antibodies. Substantial heterogeneity in the performance among various solid phase assays has been described [6]. By and large, solid phase assays are reported to have a lower sensitivity than indirect immunofluorescence [for recent review, see [7]]. Solid phase assays that employ HEp-2 extracts generally have a higher sensitivity and lower specificity for antinuclear antibodies than solid phase assays that do not employ HEp-2 [8]. Manufacturers of solid phase assays attempt to improve the performance of the assays by adding extra purified or recombinant antigens to the assays. For example, Phadia recently introduced a connective tissue disease screen assay that includes 17 different antigens (dsDNA, SSA/Ro (52 + 60), SSB/La, U1-RNP (RNP-70, A, C), Sm, centromere B, Jo-1, Scl-70, Rib-P, fibrillarin, RNA Pol III, PM-Scl, PCNA and Mi-2). In this report, the diagnostic performance of the Phadia newest generation of solid phase assays for the detection of antinuclear antibodies is compared to indirect immunofluorescence.

2. Materials and methods

2.1. Study population

The following patient groups were included: systemic lupus erythematosus (n = 80, male/female ratio 10:70, mean age 37 years, range 15–72 years), cutaneous lupus erythematosus (n = 10, male/female ratio 3:7, mean age 49 years, range 32–85 years), systemic sclerosis (n = 69, male/female ratio 25:44, mean age 53 years, range 18–79 years), mixed connective tissue disease (n = 13, male/female ratio 1:12, mean age 31 years, range 16–66 years), primary Sjögren's syndrome (n = 36, male/female ratio 5:31, mean age 50 years, range

21–75 years), polymyositis/dermatomyositis (n = 28, male/female ratio 11:17, mean age 54 years, range 26–77 years). All samples included in this study were obtained at the time of diagnosis.

The control groups included: healthy blood donors (n = 149, male/female ratio 75:74, mean age 44 years, range 19–65 years), patients with chronic fatigue syndrome (n = 139, male/female ratio 25:114, mean age 41 years, range 18–75 years), and diseased controls (n = 134, male/female ratio 34:100, mean age 46 years, range 17–81 years). The diseased controls were consecutive patients who consulted the rheumatology clinic and for whom the rheumatologist considered it necessary to request antinuclear antibodies. Afterwards, these patients were diagnosed not to have a systemic rheumatic disease (rheumatoid arthritis was also excluded).

All patients with primary Sjögren's syndrome had disease characteristics that conformed with the American–European consensus classification criteria [9]. Patients with systemic lupus erythematosus and scleroderma met the classification criteria of the American College of Rheumatology [10,11]. Patients with polymyositis/dermatomyositis met the criteria of Bohan and Peter [12], and patients with mixed connective tissue disease met the criteria of Alarcón-Segovia [13].

The serum samples that were used for this study were from the serum data bank. Samples were obtained from patients as part of routine screening for autoantibodies in the clinical laboratory. There was no informed consent for this study, but the study was approved by the local ethics committee.

2.2. Antinuclear antibodies by indirect immunofluorescence using HEp-2000®

SSA-transfected HEp-2000™ cells were from ImmunoConcepts (ImmunoConcepts, Sacramento, USA). The assay was performed according to the manufacturer's instructions, as described by Bossuyt et al. using a screening serum dilution of 1:40 [14]. The presence in serum of anti-SSA antibodies leads to development of a characteristic, so-called distinctive fluorescence pattern in the HEp-2000 cells. This characteristic pattern is a distinct bright speckled pattern with prominent staining of the nucleoli in 10–20% of the interphase nuclei. The chromosome region of metaphase mitotic cells is negative. The antibody isotype detected was IgG heavy and light chain.

2.3. Antibody detection by fluorescence enzyme immunoassay

Antibodies to nuclear target antigens were detected by fluorescence enzyme immunoassay from Phadia (Freiburg, Germany). In the Phadia EliA™ connective tissue disease (CTD) Screen, each well was coated with following antigens: dsDNA, SSA/Ro 52, SSA/Ro 60, SSB/La, U1-RNP (RNP-70, A, C), Sm, centromere B, Jo-1, Scl-70, Rib-P, fibrillarin, RNA Pol III, PM-Scl, PCNA, and Mi-2. All antigens are human recombinant, except dsDNA which is native purified. In addition to this screening assay, Phadia EliA™ assays were used to measure antibodies to individual antigens. Antibodies to individual antigens were determined in all patients and in controls that tested positive with the EliA CTD Screen assay. The assays were performed according to the instructions of the manufacturer (on a Unicap 250 instrument). The antibody isotype detected was IgG. Cutoff values for CTD screen was >1 (ratio), for dsDNA >15 IU/ml, SSA/Ro (52 + 60), SSB/La, U1RNP (RNP70, A, C), Sm, centromere B, Scl70, Jo-1: >10 U/ml, Rib-P: >10 µg/l (for research only), fibrillarin, RNA Pol III, PM-Scl, PCNA, Mi-2: >15 µg/l (for research only).

3. Results

A cohort of 236 patients with an autoimmune connective tissue disease was tested for antinuclear antibodies by indirect immunofluorescence using HEp-2000 cells (which over-express SSA). The

samples were obtained at the time of diagnosis. In addition, 149 healthy blood donors, 139 patients with chronic fatigue syndrome, and 134 diseased controls (i.e. patients who attended the rheumatology clinic, but who were diagnosed not to have a systemic rheumatic disease) were tested as controls. The results of the indirect immunofluorescence analysis are summarized in Tables 1 and 2.

The majority (>80%) of the patients with systemic lupus erythematosus had antinuclear antibody titers $\geq 1:160$. The most prevalent pattern (64%) in these patients was the homogeneous pattern. Patients with systemic sclerosis had high antinuclear antibody titers [peak titers 1:640–1:1280]. The two most prominent patterns found in systemic sclerosis patients were centromere (36%) and homogeneous (31%) (combined with nucleolar staining). Patients with mixed connective tissue disease typically had a speckled pattern in high titer ($\geq 1:320$). Patients with primary Sjögren's syndrome characteristically displayed either the distinctive fluorescence pattern of the SSA-transfected cells or a speckled pattern (at rather low titers [peak titer: 1:80]). Thirty five percent of the patients with inflammatory myopathy had no antinuclear antibodies. Those with antinuclear antibodies had rather low antibody titers (major patterns: cytoplasmic [18%] and speckled [25%]).

A substantial portion of the controls had antinuclear antibodies. At a cutoff of 1:40, 12–14% of blood donors and patients with chronic fatigue syndrome and 36% of diseased controls tested positive (Table 3). At a cutoff of 1:160, 4–6% of blood donors and patients with chronic fatigue syndrome and 18% of diseased controls tested positive. Six percent of the diseased controls tested positive at a cutoff of 1:640. The reactivity in patients also decreased with increasing cutoff values. At a cutoff of 1:40, 96%, 70%, and 64% of patients with systemic lupus erythematosus, systemic sclerosis, and inflammatory myopathy tested positive, respectively. At a cutoff of 1:160, 90%, 60%,

Table 3

Antinuclear antibodies by indirect immunofluorescence and by EliA CTD Screen.

	EliA CTD Screen	IIF or SSA pattern	IIF or SSA pattern	IIF or SSA pattern	IIF or SSA pattern	IIF or SSA pattern	IIF or SSA pattern
		1: ≥ 40	1: ≥ 80	1: ≥ 160	1: ≥ 320	1: ≥ 640	1: ≥ 1280
SLE	73.8	96.3	92.5	90.0	68.8	53.8	31.3
SCL	60.0	70.0	70.0	60.0	60.0	40.0	40.0
SSc	72.5	98.6	97.1	92.8	81.2	62.3	39.1
MCTD	100.0	100.0	100.0	100.0	100.0	92.3	69.2
SS	88.9	91.7	83.3	55.6	44.4	41.7	36.1
PM/DM	39.3	64.3	46.4	35.7	28.6	14.3	7.1
BD	2.7	12.1	8.7	6.0	2.7	1.3	0.7
CFS	2.9	14.4	7.9	3.6	2.9	2.9	2.9
DC	3.7	35.8	25.4	17.9	11.2	6.0	3.0

Antinuclear antibodies were determined by Phadia CTD screen and by indirect immunofluorescence (IIF) in patients with various connective tissue diseases (at the time of diagnosis) and in controls. For each pathology, the likelihood for reactivity is shown. Reactivity by indirect immunofluorescence as a function of antibody level (cutoff) is shown as well. Because the distinctive SSA pattern typically produces a strong fluorescence signal, it was considered equivalent to titer $\geq 1:1280$. For abbreviations, see legend to Table 1.

and 36% of patients with systemic lupus erythematosus, systemic sclerosis, and inflammatory myopathy tested positive, respectively.

For each antinuclear antibody test result (titer), the likelihood ratio (i.e. the likelihood for patients with a specific systemic rheumatic disease divided by the likelihood for controls) was calculated. Blood donors, patients with chronic fatigue syndrome and diseased controls were combined for this analysis. The results are shown in Table 4. The likelihood ratio increased with increasing antibody titer. A negative

Table 1

Distribution of antinuclear antibody titers (by indirect immunofluorescence) in various connective tissue diseases and in controls.

	Neg	SSA pattern	1/40	1/80	1/160	1/320	1/640	1/1280	1/>1280
SLE	3.75	8.75	3.75	2.5	21.25	15.0	22.5	11.25	11.25
SCL	30.0	40.0		10.0		20.0			
SSc	1.5		1.45	4.35	11.6	18.8	23.2	21.7	17.4
MCTD						7.7	23.1	23.1	46.1
SS	8.3	27.8	8.3	27.8	11.1	2.8	5.6	5.6	2.8
PM/DM	35.7		17.9	10.7	7.1	14.3	7.1		7.1
BD	87.9		3.4	2.7	3.4	1.3	0.7	0.7	
CFS	85.6	0.7	6.5	4.3	0.7			2.2	
DC	64.2	0.75	10.4	7.5	6.7	5.2	3.0	1.5	0.75

Antinuclear antibodies were determined by indirect immunofluorescence in patients with various connective tissue diseases (at the time of diagnosis) and in controls. For each pathology, the likelihood (in %) for a specific antinuclear antibody titer is shown. Likelihoods exceeding 20% are indicated in bold.

SSA pattern: characteristic distinctive fluorescence pattern of the SSA-transfected cells. The SSA pattern typically had a high intensity and, therefore, was not titrated.

SLE: systemic lupus erythematosus, SCL: subacute cutaneous lupus, SSc: systemic sclerosis, MCTD: mixed connective tissue disease, SS: primary Sjögren's syndrome, PM/DM: polymyositis/dermatomyositis, BD: blood donors, CFS: chronic fatigue syndrome, DC: diseased controls.

Table 2

Distribution of antinuclear antibody patterns (by indirect immunofluorescence) in various connective tissue diseases and in controls.

	Neg.	SSA pattern	Homogeneous	Speckled	Nucleolar	Centromere	Cytoplasmic
SLE	3.75	8.75	63.75	18.75	2.5		2.5
SCL	30.0	40.0	10.0		10.0		10.0
SSc	1.5		30.4	17.4	14.5	36.2	
MCTD				100.0			
SS	8.3	27.8	13.9	50.0			
PM/DM	35.7		14.3	25.0	7.1		17.9
BD	87.9		3.4	6.0	0.7	0.7	1.3
CFS	85.6	0.7	4.3	4.3	2.9	0.7	1.4
DC	64.2	0.7	17.2	6.7	7.5		3.7

Antinuclear antibodies were determined by indirect immunofluorescence in patients with various connective tissue diseases (at the time of diagnosis) and in controls. For each pathology, the likelihood (in %) for a specific antinuclear antibody pattern is shown. Likelihoods exceeding 20% are indicated in bold. When multiple patterns were present, the pattern with the highest titer was taken.

For abbreviations, see legend to Table 1.

Table 4
Test result specific likelihood ratios for antinuclear antibody detection by indirect immunofluorescence.

	Neg	SSA pattern	1/40	1/80	1/160	1/320	1/640	1/1280	1/>1280
SLE	0.05 (0.02-0.14)	19 (4-87)	0.6 (0.2-1.8)	0.5 (0.1-2.2)	6 (3-12)	7 (3-16)	19 (7-50)	8 (3-22)	48 (6-370)
SCL	0.38 (0.15-0.97)	84 (17-409)		2 (0.3-14)		9 (2-40)			
SSc	0.02 (0.00-0.13)		0.2 (0.0-1.6)	0.9 (0.3-3)	3 (1.4-7)	9 (4-20)	20 (7-52)	15 (6-38)	73 (10-556)
MCTD	0					4 (0.5-26)	20 (5-73)	16 (5-58)	195 (25-1504)
SS	0.10 (0.03-0.31)	59 (13-258)	1.3 (0.4-4)	6 (3-12)	3 (1-9)	1 (0.2-10)	5 (0.9-23)	4 (0.8-19)	12 (0.7-184)
PM/DM	0.45 (0.27-0.74)		3 (1-6)	2 (0.7-7)	2 (0.5-8)	7 (2-20)	6 (1-30)		30 (3-322)

Antinuclear antibodies were determined by indirect immunofluorescence in patients with various connective tissue diseases (at the time of diagnosis) and in controls. For each pathology, the likelihood ratio (and 95% confidence interval) for a specific antinuclear antibody test result is shown. Likelihood ratios are calculated by dividing the likelihood for patients by the likelihood for controls. Values >10 are indicated in bold. Blood donors, patients with chronic fatigue syndrome and diseased controls were pooled (and used as controls for this analysis).

For abbreviations, see legend to Table 1.

test result (for screening at 1:40) had a likelihood ratio ≤0.1 for systemic sclerosis, systemic lupus erythematosus, and primary Sjögren's syndrome. The distinctive SSA pattern had a likelihood ratio >10 for primary Sjögren's syndrome, systemic lupus erythematosus and subacute cutaneous lupus. The likelihood ratios that corresponded to antibody titers 1/40 and 1/80 varied between 0.2 and 6 [<1 for systemic sclerosis and systemic lupus erythematosus and >1 for primary Sjögren's syndrome and inflammatory myopathy]. For antibody titer 1/160, the likelihood ratios varied between 2 and 6. Antinuclear antibodies with titers $1/\geq 640$ had a likelihood ratio >10 for systemic sclerosis, systemic lupus erythematosus, and mixed connective tissue disease.

Next, we evaluated the presence of antinuclear antibodies in patients with a systemic rheumatic disease and in controls by solid phase assay (EliA CTD Screen). The results are shown in Fig. 1 and summarized in Table 3. The highest reactivity was found in patients with systemic lupus erythematosus and primary Sjögren's syndrome, whereas the lowest reactivity was found in patients with systemic sclerosis and inflamma-

tory myopathy (Fig. 1). The sensitivity of EliA CTD Screen for systemic lupus erythematosus, systemic sclerosis, primary Sjögren's syndrome, mixed connective tissue disease, and inflammatory myopathy was 74%, 72%, 89%, 100%, and 39%, respectively. The reactivity in blood donors, in patients with chronic fatigue syndrome, and in diseased controls was <4%. At an immunofluorescence cutoff that corresponded to the specificity found with solid phase assays, the sensitivity of indirect immunofluorescence was lower than the sensitivity of solid phase assays (see Table 3).

The distribution of the test results obtained by enzyme immunoassay is given in Table 5. Table 6 shows the likelihood ratios for test result intervals by solid phase assay for different systemic rheumatic diseases. For negative test results, the likelihood ratio was 0.1 for primary Sjögren's syndrome, 0.3 for systemic lupus erythematosus and systemic sclerosis, and 0.6 for inflammatory myopathy. Likelihood ratios clearly increased with increasing antibody concentration. The lowest likelihood ratio was observed for systemic sclerosis. For the other pathologies, likelihood ratios were infinite for EliA CTD Screen test results exceeding a ratio of 10.

Fig. 2 shows the distribution of the indirect immunofluorescence test results for each disease. For each antinuclear antibody test result, the part that was positive (filled bars) and negative (open bars) by EliA CTD Screen is shown. The figure shows that EliA CTD Screen detected antibodies in certain patients (with systemic lupus erythematosus, primary Sjögren's syndrome, and inflammatory myopathy) that tested negative or only weakly positive (titer 1:40 or 1:80) for antinuclear antibodies by indirect immunofluorescence. On the other hand, EliA CTD Screen failed to detect antibodies in some patients with high levels of

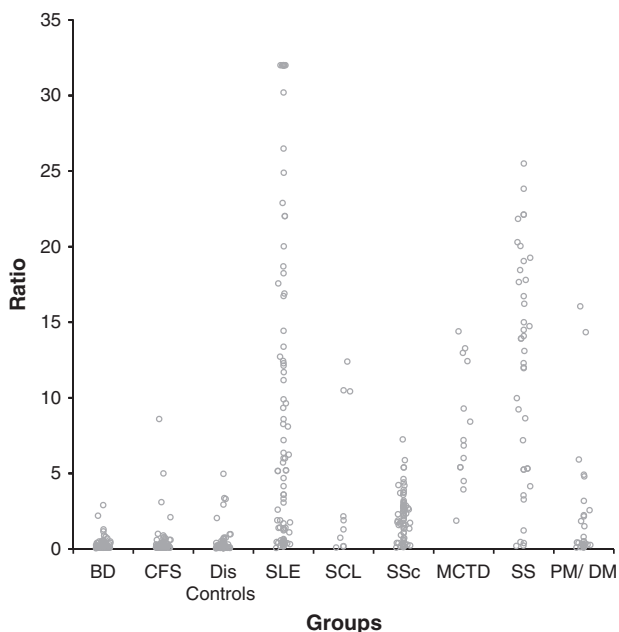


Fig. 1. EliA CTD Screen reactivity in patients with a systemic rheumatic disease and in controls. For abbreviations, see legend to Table 1.

Table 5
Distribution of EliA CTD Screen antibody level as a function of the pathology.

	EliA CTD Screen (ratio)			
	≤1	1-5	>5-10	>10
SLE	26	23	18	34
SCL	40	30	0	30
SSc	28	68	4	0
MCTD	0	23	46	31
SS	11	11	17	61
PM/DM	61	29	4	7
BD	97	3	0	0
CFS	96	3	1	0
DC	95	5	0	0

Antinuclear antibodies were determined by EliA CTD Screen in patients with various connective tissue diseases (at the time of diagnosis) and in controls. For each pathology, the likelihood (in %) for a specific antibody level (ratio) is shown. For abbreviations, see legend to Table 1.

Table 6

Test result interval specific likelihood ratios for antinuclear antibody detection by EliA CTD Screen.

	EliA CTD Screen (ratio)			
	≤1	>1–5	>5–10	>10
SLE	0.3 (0.2–0.4)	6 (3–12)	74 (10–554)	∞
SCL	0.4 (0.2–0.9)	8 (3–25)		∞
SSc	0.3 (0.2–0.4)	19 (11–32)	18 (2–174)	
MCTD		6 (2–20)	195 (25–1504)	∞
SS	0.1 (0.0–0.3)	3 (1–9)	70 (9–568)	∞
PM/DM	0.6 (0.5–0.9)	8 (4–17)	15 (1–235)	∞

Antinuclear antibodies were determined by EliA CTD Screen in patients with various connective tissue diseases (at the time of diagnosis) and in controls. For each pathology, the likelihood ratio (and 95% confidence interval) for a specific antinuclear antibody test result interval is shown. Likelihood ratios were calculated by dividing the likelihood for patients by the likelihood for controls. Blood donors, patients with chronic fatigue syndrome and diseased controls were pooled (and used as controls for this analysis). For abbreviations, see legend to Table 1.

antinuclear antibodies (especially in systemic lupus erythematosus and systemic sclerosis).

All samples from patients with a systemic rheumatic disease and from controls that tested positive by EliA CTD Screen were tested for antibodies to the individual antigens. Table 7 gives an overview of the results.

Antibodies to centromere, Scl-70, and RNA Pol III were specific for systemic sclerosis. Of the 69 patients with systemic sclerosis 24 (34.8%), 19 (27.5%), 5 (7.2%), and 2 (2.9%) had antibodies to centromere, Scl-70, RNA-Pol-III, or PM-Scl, respectively. In the majority of the systemic sclerosis patients, these antibodies were mutually exclusive. Only three patients had the combined presence of antibodies to centromere and to SSA and only one patient had the combined presence of antibodies to centromere and RNA Pol III. Two (2.9%) patients with systemic sclerosis had only anti-SSA antibodies.

Anti-PM-Scl antibodies were found in systemic sclerosis and in inflammatory myopathy. Antibodies to Jo-1, Mi-2, and PM-Scl were found in 29%, 4%, and 7.2% of patients with inflammatory myopathy, respectively. These antibodies were mutually exclusive.

Systemic sclerosis and inflammatory myopathy were two systemic rheumatic diseases in which most patients had reactivity to one or only few antigens (Table 7). This is in contrast to patients with systemic lupus erythematosus or primary Sjögren's syndrome who typically had the combined presence of several antibodies (see Table 8). Eighty-six percent of primary Sjögren's syndrome patients had the combined presence of at least two antibodies, the typical combination being anti-SSA and anti-SSB antibodies. Forty-nine percent of patients with systemic lupus erythematosus had the combined presence of at least two antibodies. Monospecific anti-dsDNA antibodies, anti-SSA antibodies and anti-U1RNP antibodies were found in 17.5%, 15%, and 2.5% of lupus patients, respectively. In our patient cohort, anti-Sm antibodies and anti-Rib-P antibodies were always combined with other antibodies in systemic lupus erythematosus. For example, 6 of the 7 patients with anti-Rib-P antibodies had antibodies to dsDNA.

Antibodies to Rib-P were found in 9% of patients with systemic lupus erythematosus and in 8% of patients with mixed connective tissue disease. Anti-Rib-P antibodies found in patients with mixed connective tissue disease had low reactivity (<12.1 µg/l). Anti-Sm antibodies were specific for systemic lupus erythematosus. Anti-dsDNA antibodies were found in 45% of patients with systemic lupus erythematosus and in 8% of patients with mixed connective tissue. The level of the anti-dsDNA antibodies in mixed connective tissue disease was low (<20 IU/ml).

Antibodies to U1-RNP and RNP-70 were associated with mixed connective tissue disease (in 100% and 92%, respectively) and with systemic lupus erythematosus (in 16% and 6%, respectively).

Antibodies to Ro60, Ro52, and La were associated with primary Sjögren's syndrome, systemic lupus erythematosus, and cutaneous lupus. These antibodies were also found in a minority (<10% was positive for SSA or SSB) of patients with systemic sclerosis. An association between anti-Ro52 and anti-Jo-1 has previously been reported [15].

4. Discussion

In this manuscript we documented screening for antinuclear antigens by indirect immunofluorescence and by EliA CTD Screen immunoassay, which detects antibodies to a mixture of 17 antigens. The evaluation was done on diagnostic samples of patients with a connective tissue disease. Controls included blood donors, patients with chronic fatigue syndrome, and diseased controls.

The highest antinuclear antibody levels by indirect immunofluorescence were typically found in patients with mixed connective tissue disease, systemic sclerosis and systemic lupus erythematosus. Patients with primary Sjögren's syndrome and cutaneous lupus typically had lower antibody titers, although a substantial portion of them displayed the distinctive pattern of the SSA-transfected cells (HEp2000 substrate). Our data confirmed the classical pattern associations [3], such as the centromere with systemic sclerosis, homogenous with systemic lupus erythematosus, speckled with mixed connective tissue disease or primary Sjögren's syndrome. At a low cutoff, indirect immunofluorescence had a high sensitivity, but a low specificity. At higher cutoffs, specificity increased, but sensitivity decreased.

EliA CTD Screen had a high specificity. Under three percent of blood donors and patients with chronic fatigue syndrome and under four percent of diseased controls tested positive. The indirect immunofluorescence cutoff that corresponded to the specificity of EliA CTD Screen was 1:320 for blood donors and chronic fatigue syndrome and 1:≥1280 for diseased controls. At a cutoff of 1:320, the sensitivity of indirect immunofluorescence was 69%, 81%, 44%, and 36% for systemic lupus erythematosus, systemic sclerosis, primary Sjögren's syndrome, and inflammatory myopathy, respectively. The sensitivity of EliA CTD Screen was 74%, 72%, 89%, and 39%, respectively.

A fraction of patients with systemic lupus erythematosus (23.8%), systemic sclerosis (21.7%), primary Sjögren's syndrome (5.6%), subacute cutaneous lupus (10%), and inflammatory myopathy (17.9%) tested positive by indirect immunofluorescence (titer 1:≥160) but negative by EliA CTD Screen. The fact that solid phase assays may miss clinically relevant antibodies has been illustrated by a case report of the Massachusetts General Hospital where a diagnosis of SLE was delayed because of a false negative antinuclear antibody result by solid phase method [16]. On the other hand, a fraction of patients with systemic lupus erythematosus (6.3%), systemic sclerosis (1.5%), primary Sjögren's syndrome (38.9%), subacute cutaneous lupus (10%), and inflammatory myopathy (21.4%) tested positive by EliA CTD Screen but negative or undecided/indefinite (titer 1:40 or 1:80) by indirect immunofluorescence. This observation confirmed previous reports that demonstrated that solid phase assays detect antibodies to extractable nuclear antigens that are missed by indirect immunofluorescence [17–19].

Overall, EliA CTD Screen was highly specific and detected antibodies that were missed by indirect immunofluorescence. However, the sensitivity of the solid phase assay could be further improved, for example, by adding extra relevant antigens [e.g. heterogeneous nuclear ribonucleoproteins [20]].

A negative indirect immunofluorescence test result (for screening at 1:40) had a likelihood ratio ≤0.1 for systemic sclerosis, systemic lupus erythematosus, and primary Sjögren's syndrome, indicating a significant difference in pre-test to post-test probability [21]. The

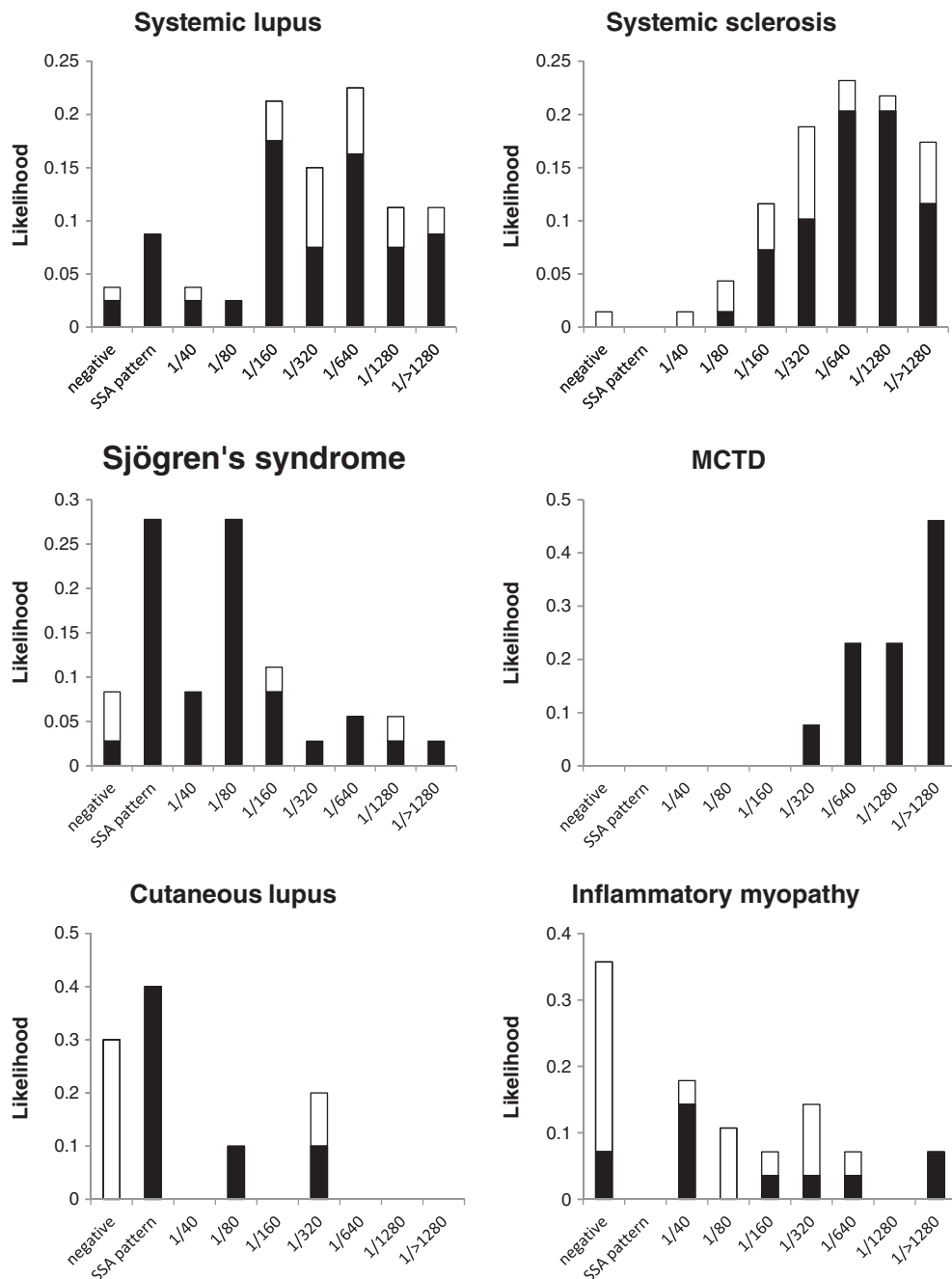


Fig. 2. Distribution of indirect immunofluorescence titer and EliA CTD Screen positivity/negativity in various connective tissue diseases. Antinuclear antibodies were determined by indirect immunofluorescence and by EliA CTD Screen in patients with various connective tissue diseases (at the time of diagnosis). For each pathology, the likelihood (in %) for a specific antinuclear antibody titer is shown. For each antinuclear antibody test result, the part that was positive (filled bars) and negative (open bars) by EliA CTD Screen is shown. SSA pattern: characteristic distinctive fluorescence pattern of the SSA-transfected cells.

likelihood ratio for an indirect immunofluorescence test result with titer 1:40 or 1:80 was between 0.2 and 6, indicating no or only small differences in pre-test to post-test probabilities. The typical SSA pattern and antibodies titers $1:\geq 640$ had likelihood ratios > 10 (up to 195), indicating a clinically significant change in pre-test to post-test probabilities.

With EliA CTD Screen, a negative test result had a likelihood ratio of 0.1 for primary Sjögren's syndrome and between 0.3 and 0.6 for the other diseases. Thus, for systemic lupus erythematosus and for systemic sclerosis, the likelihood ratios for a negative test result with EliA CTD Screen were higher than the likelihood ratios for a negative test result with indirect immunofluorescence (at screening titer 1:40). Accordingly, the post-test probability of a negative test

result was lower for indirect immunofluorescence (at screening 1:40) than for EliA CTD Screen. In other words, the negative predictive value was higher for indirect immunofluorescence than for EliA CTD Screen.

For a positive EliA CTD Screen test result, the likelihood ratio increased with increasing antibody concentrations. The likelihood ratios obtained with EliA CTD Screen were higher than those obtained with indirect immunofluorescence. Accordingly, the post-test probability for a connective tissue disease was higher for a positive CTD screen result than for a positive indirect immunofluorescence test result (with the exception of systemic sclerosis).

The highest antibody levels by EliA CTD Screen were characteristically found in patients with systemic lupus erythematosus and primary Sjögren's syndrome, whereas the lowest antibody levels were

Table 7
Distribution of reactivity to specific antigens by ELIA.

	dsDNA	Ro52	Ro60	La	U1-RNP	RNP-70	Sm	Centr	Scl-70	Jo-1	Rib-P	RNA Pol III	PM-Scl	Mi-2
SLE	45	38	48	19	16	6	6				9			
SCL		30	60	10										
SSc	1	3	7	1				35	28			7	3	
MCTD	8				100	92					8			
SS	3	81	86	58										
PM/DM		18			4			4		29			7	4
BD	1							1						
CFS	1	1	1					1						
DC	1	2			1									1

Antibodies to specific nuclear antigens were determined by ELIA in patients with various connective tissue diseases (at the time of diagnosis) and in controls. The likelihood (in %) for reactivity to a specific antigen is shown.

For abbreviations, see legend to Table 1.

Data for PCNA and for fibrillarlin are not shown, as no positive reactivity was found for these antigens.

found in patients with systemic sclerosis and inflammatory myopathy. Systemic lupus erythematosus and primary Sjögren's syndrome were diseases in which a substantial fraction of the patients had multiple antibodies, whereas systemic sclerosis and inflammatory myopathy were diseases in which the combined presence of antibodies was uncommon. Based on these observations we hypothesize that the high reactivity observed in systemic lupus erythematosus and primary Sjögren's syndrome by ELIA CTD Screen might be related to the fact that these patients had multiple antibodies, whereas the low reactivity in patients with systemic sclerosis and inflammatory myopathy by ELIA CTD Screen might be related to the fact that these patients typically had mono-specific antibodies. The Phadia ELIA CTD Screen has been reported to be insufficient for screening for anti-fibrillarlin and anti-RNA polymerase III antibodies [22].

In all patients with a connective tissue disease we quantified antibodies to the individual antigens contained within the ELIA CTD Screen assay. This analysis confirmed the classical disease associations of specific antibodies [3], e.g. anti-dsDNA, anti-Sm, anti-Rib-P [23,24] antibodies with systemic lupus erythematosus, anti-SSA and anti-SSB antibodies with primary Sjögren's syndrome, anti-Scl-70 [25], anti-centromere, and anti-RNA Pol III antibodies [26] with systemic sclerosis, and anti-Jo-1 and anti-Mi-2 antibodies with polymyositis [27].

In conclusion, ELIA CTD Screen had a high specificity. At equal specificity, the sensitivity of ELIA CTD Screen was higher than the sensitivity of indirect immunofluorescence. Generally, a positive

result by ELIA CTD Screen had a higher likelihood ratio than a positive result by indirect immunofluorescence, indicating that the positive predictive value was higher for ELIA Screen than for indirect immunofluorescence. A negative result by indirect immunofluorescence (at screening dilution 1:40) had a lower likelihood ratio than a negative result by ELIA CTD Screen, indicating that the negative predictive value was higher for indirect immunofluorescence than for ELIA CTD Screen.

Take-home messages

- At equal specificity, the sensitivity of indirect immunofluorescence was lower than the sensitivity of ELIA CTD Screen.
- With ELIA CTD Screen, likelihood ratios for systemic rheumatic diseases increase with increasing antibody concentrations.
- Generally, a positive result by ELIA CTD Screen had a higher likelihood ratio than a positive result by indirect immunofluorescence.
- A negative result by indirect immunofluorescence (at screening dilution 1:40) had a lower likelihood ratio than a negative result by ELIA CTD Screen.

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Table 8
Combined reactivity to antigens contained in ELIA CTD Screen.

%	≥1	≥2	≥3	≥4	≥5	≥6	≥7	≥8
SLE	72.5	48.8	32.5	17.5	7.5	6.3	1.3	
SCL	60.0	30.0	10.0					
SSc	73.9	10.1	1.4					
MCTD	100.0	92.3	15.4					
SS	88.9	86.1	52.8					
PM/DM	39.3	17.9	7.1					
BD	1.3							
CFS	2.9	0.7						
DC	4.5							

Antibodies to each of the specific antigens contained in ELIA CTD Screen (see Table 6) were determined in diagnostic samples of patients with a connective tissue disease and in controls. The combined presence of antibodies was evaluated and shown in the Table. The results presented in this Table (under ≥1) are slightly different from the results presented in Table 3 (ELIA CTD Screen). The reason is that in some cases the ELIA CTD Screen assay was more sensitive than the ELIAs for individual antibodies (this was the case for 1 SLE patient and 2 BDs), while in other cases the ELIA CTD Screen was less sensitive than the ELIAs for the individual antibodies (this was the case for 1 SSc patient and 1 DC).

For abbreviations, see legend to Table 1.

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