Measurement of antinuclear antibodies and their fine specificities: time for a change in strategy?

H.G. Otten[1], W.J. Brummelhuis[2], R. Fritsch-Stork[2,3], H.L. Leavis[2], B.W. Wisse[1], J.M. van Laar[2], R.H.W.M. Derksen[2]

[1]Laboratory of Translational Immunology, [2]Department of Rheumatology and Clinical Immunology, University Medical Center Utrecht, The Netherlands; [3]Ludwig Boltzmann Institute of Osteology and AUVA Trauma Centre Meidling, Hanusch Hospital, and Sigmund Freud University, Vienna, Austria.

Abstract

Objective

The current strategy for antinuclear antibody (ANA) analysis involves screening for presence with a subsequent detailed analysis of their specificity. The aim of this study is to compare the clinical and financial efficacy of this strategy between different commercial tests in a large cohort of unselected patients.

Methods

In all consecutive 1030 patients associations were defined between results from different ANA test systems and the pre-test probability for connective tissue disease (CTDs). Test systems were used for screening (ANA-IIF vs. CTD screen) and definition of their fine specificity (profile 3 line blot vs. CTD single analytes).

Results

Positive ANA-IIF and/or CTD screen results were found in 304 sera. Further analysis for ANA-specificity by profile 3 line blot and CTD single analytes showed 86 discrepant results of which more than a third are clinically relevant, with the CTD single analyte assay performing better than the line blot in supporting or confirming the presence of a CTD. Autoantigens present in one test but absent in the other were of minor practical use. The ANA screening and identification strategies currently employed are not cost-effective as 83% of tests were performed in order to find specific autoantibodies in patients without the fitting clinical signs or symptoms. This causes many unexpected positive results and subsequent confusion with regard to interpretation.

Conclusion

We advocate that some autoantigens should be excluded from the line blot and CTD assays and propose the use of a cost-effective and selective ANA specificity testing purely based on clinical guidance.

Key words

autoantibodies, antinuclear antibodies, line blot, ELiA

Henny G. Otten, PhD
Walter J. Brummelhuis, MD
Ruth Fritsch-Stork, MD, PhD
Helen L. Leavis, MD, PhD
Bram W. Wisse, PhD
Jacob M. van Laar, MD, PhD, Prof.
Ronald H.W.M. Derksen, MD, PhD, Prof.

Please address correspondence to:
Dr Henny G. Otten,
Laboratory of Translational Immunology, UMC Utrecht,
Heidelberglaan 100,
3584 CX Utrecht, The Netherlands.
Email: h.g.otten@umcutrecht.nl

Received on August 26, 2016; accepted in revised form on November 7, 2016.

Competing interests: J.M. van Laar has received consultancy fees and honoraria from BMS, Eli Lilly, Janssen, MSD, Pfizer, Roche, and research grants from Genetech, and MSD; the other co-authors have declared no competing interests.

Introduction

As the presence of antinuclear antibodies (ANA) detected in blood by indirect immunofluorescence (IIF) is a characteristic of many autoimmune disorders (AID), this assay is frequently used by clinicians as a screening assay for autoimmunity (1). Both the classification criteria for systemic lupus erythematosus (SLE) and the diagnostic criteria for autoimmune hepatitis (AIH) include a positive ANA test (2, 3). The nuclear (and in part cytoplasmic) antigens recognised by ANA are very diverse, but certain specificities strongly associate with certain rheumatologic connective tissue diseases (CTD), providing them with clinically useful diagnostic capacity. Examples are anti-dsDNA and anti-Sm antibodies for SLE, anti-Ro/SSA and anti-La/SSB for Sjögren's syndrome, anti-RNP for mixed connective tissue disease (MCTD), anti-centromere antibodies for limited cutaneous systemic sclerosis and anti-Scl-70 (topoisomerase 1) for diffuse systemic sclerosis and anti-RNA polymerase III for a particular subset of systemic sclerosis (2, 4-8). Fine specificities of ANA are detected by solid phase assays (9). Over the years preferred (or advocated) detection techniques have constantly changed, both with respect to the nature of the antigens used (from purified native antigens to recombinant proteins) and efforts aimed at automation to reduce hands-on time. Using state of the art recombinant DNA technology and proteomics, highly purified antigens and novel targets are coupled to matrices, thus continuously improving sensitivity and reproducibility on multi-analytic platforms (10-14). In general, sensitivity and specificity of the assays as provided by manufacturers are determined from patient cohorts with well-defined different rheumatologic AIDs and from healthy and diseased controls. However, in daily clinical practice ANA testing in the laboratory is performed on sera from patients with widely varying pre-test probabilities for AID in general and rheumatologic AID. The majority of ANA screening requests are performed for patients with a low pretest probability for CTD (14). Especially in these patients a positive result in an assay for ANA specificity may cause confusion and influence clinical decision making (15, 16) Therefore, it is important to know the performance and characteristics of tests that are currently in use in immunological laboratories for daily patient care.

In this study we used all 1030 sera that were consecutively sent to the laboratory of our university hospital for ANA testing in a predefined time period. Blinded for laboratory test results an expert panel categorised all 1030 patients according to pre-test probability for CTD (high, intermediate, low, absent) based on information from the patient records. We compared the results of ANA testing by IIF with that of CTD screen (a solid phase test for IgG antibodies against a mixture of different purified nuclear antigens), and the results of tests for antigenic specificity of ANA detected by a commercial line blot versus that of the CTD single analytes. The purpose of this study was to define the clinical relevance of discrepant ANA test results between these assays without bias with regard to patient selection for any category included in ANA ordering, and to evaluate the efficacy of ANA screening and identification strategy commonly employed.

Patients and methods

Samples and patients

A total of 1030 serum samples from individual patients were consecutively tested for the presence of autoantibodies as requested by clinicians from the department Rheumatology and Clinical Immunology or from other departments between April 2013 and May 2014, and October 2013 and May 2014, respectively. Informed consent for diagnostic assays was obtained from all participants, as approved by the Medical Ethics Committee of the University Medical Center Utrecht. All laboratory methods and experimental protocols were carried out in accordance with the guidelines approved by the local Medical Ethics Committee. Within 2 weeks after the sample for ANA testing reached the laboratory, one of us (R.D.) read the medical chart of the patient to identify the most prominent signs/symptoms present at the time of the ANA request, unaware of any immu-

nological laboratory test result. Clinical charts were reviewed independently by R.F.S and H.L.L. and discrepancies in classification were resolved. The samples/patients were classified according to one of five categories (Table I). The five categories were:

Patient fulfills established criteria for the classification or diagnosis of SLE (2), systemic sclerosis (SSc; 17), rheumatoid arthritis (RA; 18), juvenile idiopathic arthritis (JIA; 19), dermatomyositis/polymyositis (20), primary Sjögren's syndrome (pSS; 4), mixed connective tissue disease (MCTD; 5), or undifferentiated connective tissue disease (UCTD; 21).

Patient has at least one sign/symptom that is relatively frequent in some connective tissue disease (CTD). Examples are: cutaneous lupus (chronic discoid lupus erythematosus (CDLE), subacute cutaneous lupus (SCLE), lupus tumidus), cutaneous abnormalities compatible with systemic sclerosis (morphea, linear scleroderma, sclerodactyli), cutaneous abnormalities compatible with dermatomyositis (heliotropic rash, Gottron's sign, erythroderma, mechanic's hands), leucocytoclastic vasculitis, Raynaud's phenomenon, malaise, fatigue, fever of unknown origin, arthralgia/myalgia, arthritis, proximal muscle weakness, sicca complaints, serositis, renal failure, proteinuria, microscopic haematuria, interstitial keratitis, haemocytopenia, generalised lymphadenopathy, interstitial lung disease and primary antiphospholipid syndrome (PAPS; 22). In this category the clinician has the intention to find an indication that a CTD is present.

Patient has a sign/symptom that is a rare presenting symptom of CTD. Examples are a psychiatric disorders (like schizophrenia and psychosis), abnormal cerebral CT- or MRI scan with cerebral infarction or suspicion of vasculitis, cognitive impairment, epilepsy, tinnitus, chorea, peripheral neuropathy, sudden deafness or tinnitus, aphthous mucosal ulcers, swelling of salivary or lacrimal glands, uveitis, chorioretinitis, scleritis, ocular myositis, perniones, urticarial rash, livedo reticularis, swan-neck deformities.

Patient has unspecific complaints or symptoms, and ANA are tested to exclude AID. Examples are: erythema nodosum, pyoderma gangrenosum, encephalitis, blepharitis, amblyopia, papilloedema, headaches, abdominal pain, inflamed ears, and thrombosis. Also ANA used as screening test for AID before heart transplantation and in humoral immunodeficiency.

Patient has abnormal liver function tests and the ANA request refers to a search for autoimmune hepatitis.

Autoantibody analysis

All autoantibody analyses were performed according to the respective manufacturers' instructions. The ANA-IIF was performed with a serum dilution of 1:100 using the Hep-20-10 cell line (EuroImmun, Lubeck, DE). Hep-20-10 cells are Hep-2 cells transfected with SS-A (60kDa) cDNA causing hyper-expression of the encoded protein, and demonstrate more mitotic cells allowing easy identification of mitotic structures (such as centromeres). Weak positive/dubious ANA-IIF results were considered negative in this study. Screening for ANA was also performed on a Unicap 250 machine by a fluorescence enzyme immunoassay from Thermo Fisher scientific (Freiburg, DE) designated as the CTD screen assay. In this solid-phase assay each well is coated with a mixture of autoantibodies. The supplier provided cut-off levels for the CTD screen measuring specific IgG concentrations by ratio with serum diluted 1:10. Cut-off ratios were used of <0.7 (negative), 0.7–1 (equivocal) and >1 U/ml (positive). Equivocal results were considered negative in this study, thus an equivocal CTD screen result with a positive ANA-IIF is considered discrepant as well as an equivocal (or dubious) ANA-IIF result with a positive ANA-IIF. After analysis by ANA-IIF and CTD screen, all sera positive in either or both assays were further analysed by the Profile 3 line blot (EuroImmun), and CTD single analytes (Thermo Fisher scientific) to identify quantities of individual antibody specificities. Both assays contain Sm proteins, SS-A/Ro 52 kDa, SS-A/Ro 60 kDa, Centromere B, Scl-70, Jo-1, Ribosomal P protein, PM-Scl, PCNA, and dsDNA. In addition, the CTD single analytes contains U1-RNP (RNP70, A, C), RNP-70, fibrillarin, Mi-2 proteins, and RNA polymerase III, whereas the Profile 3 line blot contains U1-nRNP/Sm, AMA-M2, histones and nucleosomes. Sera which were negative both by ANA-IIF and CTD screen were not further analysed for autoantibody specificities. In this study we compared antibody reactivity against the same type of proteins (expressed either native or recombinant), and proteins present in one assay only.

Interpretation of autoantibodies

Specificities found after ANA testing and relevant for patient categories 1, 2 and 5 were scored as "compatible" and displayed in yellow and green (i.e. compatible with the diagnosis of CTD or predictive for disease). These specificities were defined as follows: SLE: SS-A, SS-B, snRNP/Sm complex, U1-snRNP, RNP-70, nucleosomes, histones and PCNA. Skin abnormalities compatible with lupus: Sm, dsDNA, SS-A, SS-B, nRNP/Sm complex, U1-snRNP, RNP-70, nucleosomes, histones and PCNA. Skin abnormalities compatible with scleroderma: Scl-70 and Centromere B. Sicca complaints: SS-A, SS-B. APS, Raynaud and livedo reticularis: dsDNA. Abnormal liver tests: CENP-B, AMA-M2. Dermatomyositis/polymyositis: PM-Scl and Mi-2. Interstitial lung disease: Jo-1. Specificities found for patient categories 3 and 4 were considered "not relevant" (not compatible with the clinical signs and symptoms and not relevant for the current disease). A separate analysis was made for specificities that are supportive in the diagnosis (*i.e.* part of the disease classification criteria used by the American College of Rheumatology), which are displayed in red. Differences in sensitivity and specificity between both the ANA-IIF and CTD screen assay and the profile3 line blots and CTD single analyte essays were evaluated with McNemar's Chi-squared test for marginal homogeneity.

Results

Patients characteristics and screening for ANA

In our cohort, 162 patients were diagnosed with CTD (category 1 in Table

I), 424 patients were categorised with a disease in which ANA testing can aid decision making (category 2), 232 patients had symptoms which rarely present as part of a connective tissue disease (category 3), 144 patients had diseases or symptoms which are not related to rheumatologic AID (category 4), and 68 patients had abnormal liver function tests (category 5). ANA-IIF positive results were found in all 5 patient categories, although in different frequencies (Table II). In patient category 1 positive results by ANA-IIF were found in 87 of 162 cases (54%), occurring in all CTDs with a prevalence of 96% in SLE and 86% in pSS. In category 2, 112 patients (26%) were ANA-IIF positive mainly consisting of patients with skin abnormalities compatible with lupus (22 out of 50 cases), arthritis (19 out of 49 cases), cytopenia (all 5 cases), Raynaud (9 out of 39 cases), and sicca (16 out of 72 cases). Besides positive results for SS-A and/or SS-B in 34% of patients with skin abnormalities compatible with lupus, no other discernable fine specificities of autoantibody patterns were present in this patient category. Positive ANA-IIF results in patient categories 3-5 ranged from 1318% but no specific autoantibodies were found to be compatible with reported signs or symptoms.

Comparison between ANA-IIF and CTD screen

When results of autoantibodies from all 1030 sera were analysed as either supportive / compatible with CTD or not relevant, the ANA-IIF and CTD screen assays showed similar sensitivities (37%; 95% CI=32-42% and 35%; 95% CI=30-41% respectively) to detect antinuclear antibodies in diseases. In contrast, the specificity of ANA-IIF (83%; CI=79-86%) was lower than that of the CTD screen assay (91%; CI=89-94%). The ANA-IIF and CTD screen have the same association with all diseases listed in Table II, with the exception of RA, JIA and UCTD in which the ANA-IIF is supportive or associated (orange or red) whereas the CTD screen is not associated (blue). When this is taken into account, results from 939 sera can be used to compare the sensitivity and specificity between the ANA-IIF and CTD screen. For these sera no significant difference in sensitivity was found (p=0.08), whereas a significantly higher specificity of the CTD screen was observed (p<0.0001). The sensitivity of the ANA IIF for the remaining 91 cases of either RA, JIA or UCTD was with 32% significantly lower (p=0.001) than for the other diseases (39%).

Results of ANA-IIF and CTD screen were concordantly positive in 129 (13%) patients, and negative in 726 (70%) patients. Discordant results were found in the other 175 (17%) sera. Each autoantibody subsequently found was classified as supportive (*i.e.* part of the ACR disease classification criteria), or compatible (*i.e.* compatible with the diagnosis of an autoimmune disease or predictive for disease), or not relevant (not compatible with the clinical signs and symptoms and not relevant for the current disease). Sera scoring ANA-IIF positive but CTD screen negative (n=131) were further analysed by line blot and CTD single analytes, which identified 20 autoantibody specificities. None of these autoantibodies were supportive for diagnosis (Table I category 1) and

Table I. Categorisation of patients tested for ANA.

Category 1	nr
RA	52
JIA	38
SLE	23
pSS	21
SSC	13
Dermato/polymyositis	11
MCTD	3
UCTD	1
Total	162
Category 2	nr
Arthralgia/myalgia	93
Sicca	72
Skin abnormalities compatible with lupus	50
Arthritis	49
Raynaud	39
Malaise, fatigue	21
Serositis	13
Skin abnormalities compat. with scleroderma	13
Proteinuria	10
Fever	10
APS	8
Renal failure	8
Haematuria	7
Leucocytoclastic vasculitis	7
Cytopenia	5
Lymphadenopathy	7
Other (less than 5 per disease)	12
Total	424
Category 3	nr
Psychiatric disease	76
Uveitis	61
Peripheral neuropathy	19
Cerebral infarction(s) incl. suspicion vasculitis	12
Scleritis	12
Tinnitus	12
Sudden deafness	10
Aphthous ulcers	7
Urticaria	7
Perniosis	5
Other (less than 5 per disease)	11
Total	232
Category 4	nr
Heart transplantation	15
Humoral immunodeficiency	15
Erythema nodosum	6
Papilloedema	5
Other (less than 5 per disease)	103
Total	144
Category 5	nr
Abnormal liver tests	68

All patients were categorised as described in Patients and Methods according to their diagnosis, clinical symptoms or reason for analysis. Category 1 are patients with an established diagnosis. Category 2 are patients with at least one sign or symptom frequently present in CTD in which diagnostics is ordered to support a clinical diagnosis or acquire a prognostic marker. Category 3 are patients with signs or symptoms rarely presenting prior to CTD. Category 4 are patients with symptoms not related to CTD, or they are screened for ANA by protocol. Category 5 are patients with abnormal liver function tests.

Supplementary Table I. Antigens used in identification of autoantibodies.

CD single analytes	Profile 3
U1-RNP (RNP70, A, C)*	-
RNP-70*	-
-	U1-nRNP/Sn
Sm proteins*	Sm proteins
SS-A/Ro 52 kDa*	SS-A/Ro 52
SS-A/Ro 60 kDa*	SS-A/Ro 60
SS-B/La*	SS-B/La*
Centromere B*	Centromere
Scl-70*	Scl-70
Jo-1*	Jo-1
Fibrillarin*	-
RNA polymerase III*	-
Ribosomal P protein*	Ribosomal P
PM-Scl*	PM-Scl*
PCNA*	PCNA
Mi-2 proteins*	-
native purified DNA	dsDNA
-	AMA-M2
-	Histones
-	Nucleosome

Autoantigens present in the CTD single analytes (EliA) and the profile 3 (line blot). All autoantigens present in the 2 different techniques are listed in a side-by-side comparison. Autoantigens absent in kits used are indicated with a "– " and those marked with and asterisk are recombinant proteins.

Test	Specificity	SLE cat.1 (n=23)	pSS cat.1 (n=21)	SSc cat.1 (n=13)	MCTD cat.1 (n=3)	PM/DM cat.1 (n=11)	RA cat.1 (n=52)	JIA cat.1 (n=38)	UCTD cat.1 (n=1)	cat.2 (n=424)	cat.3 (n=232)	cat.4 (n=144)	cat.5 (n=68)
IIF	ANA	22	18	9	3	6	14	14	1	112	30	19	12
CTDscreen	ANA mixture	21	19	9	3	4	4	4		86	9	12	2
CTD SA /Line blot	dsDNA	18/8					2/1			14/7	3/1	1/1	1/0
Line blot	Histones	11	1				2	1		9	1	2	
Line blot	Nucleosomes	4								3	1	1	
CTD SA / Line blot	PCNA									1/2			
CTD SA / Line blot	Sm-D	2/1	1/1			1/1	1/0			3/1		1/0	
CTD SA	U1-RNP (RNP70, A, C)	2			2	1				7	2	1	
Line blot	U1-nRNP/Sm	3			2	1		1		9			
CTD SA	RNP-70	1			2	1				6			
CTD SA / Line blot	Ribosomal P	3/4								2/2		1/1	
CTD SA	Fibrillarin			1									
CTD SA / Line blot	SS-A/Ro 52 kDa	8/8	18/18	1/1	0/2	0/1	1/1	2/1		24/27	1/1	2/4	2/2
CTD SA / Line blot	SS-A/Ro 60 kDa	7/7	17/16			1/1		2/2		30/27	2/2	4/3	1/1
CTD SA / Line blot	SS-B/La	4/1	13/10					1/1		8/4	1/1	0/1	1/1
CTD SA / Line blot	Centromere B	2/1	1/1	3/3	1/1		1/0	2/2		10/7			1/0
CTD SA / Line blot	Scl-70			2/4						1/1			
CTD SA	RNA polymerase III							1					
CTD SA / Line blot	Jo-1							0/2		1/1			
CTD SA	Mi-2 proteins					1				2		1	
CTD SA / Line blot	PM-Scl		0/2	2/2		1/1			1/0				
Line blot	AMA-M2		2				2			6	1	2	

Part of classification criteria
associated with disease
association depends on disease
not associated with disease

Abbreviations: CTD SA = CTD single analytes

Table II. Interpretation of autoantibodies. Specificities found after ANA testing and relevant for patients categories 1, 2 and 5 were scored as "compatible" displayed in yellow and green (*i.e.* compatible with the diagnosis of an autoimmune disease or predictive for disease) as defined in Patients and Methods. All results are displayed according the method used. One number per cell means testing by one test whereas two numbers separated by a "/" indicates the result per assay. A separate analysis was made for specificities which are supportive in the diagnosis (*i.e.* part of the ACR disease classification criteria) which are displayed in red. Twenty-nine percent (n=304) tested positive for ANA-IIF and/or CTD screen, with mean age of 41.7 (±21.2, range 1-92) years and 28% (n=84) male. ANA-IIF and CTD screen negative patients (n=726) had a mean age of 40.7 (±20.8, range 2-88) years and 44% (n=312) were male. Female patients represent 74%, 67%, 54%, 48% and 41%, respectively in cases of category 1 to 5.

only 2 were compatible with disease. The other 44 discordant results, ANA-IIF negative and CTD screen positive, were mainly caused by failure of the ANA-IIF to detect SS-A 52kDa (n=11), SS-A 60kDa (n=9), and low quantities of dsDNA antibodies (n=9). Antibodies found in 2 sera were supportive for diagnosis and a further 7 test results were compatible with clinical symptoms. These data indicate that further analysis of discordant results between ANA and CTD screen, especially ANA-IIF negative and CTD screen positive, can be of value. Taken together, analysis of the 175 discordant results between ANA-IIF and CTD screen yielded autoantibodies supportive for diagnosis in 4 patients, in 14 patients the antibodies were compatible with clinical symptoms, in 31 patients the autoantibodies found were not relevant to clinical symptoms, whereas no autoantibody specificities were found in the remaining 132 patients.

Occurrence of autoantibodies in different diseases

Two-hundred and twenty-two sera were further analysed by both line blot and CTD single analytes. In these sera 257 autoantibody specificities were found by line blot and 267 by CTD single analytes. Their occurrence in the different disease categories is displayed in Table II. Autoantibody specificities determined by line blot were in 60 (23%), 89 (35%), and 108 (42%) cases supportive, compatible, or not relevant for a given diagnosis, whereas for the CTD single analytes these numbers were 73 (27%), 79 (30%), and 115 (43%) respectively. When results of autoantibodies were analysed supportive, compatible with CTD or not, both assays showed a significant difference in sensitivity and specificity, although the differences themselves were too small to be relevant: 27% sensitivity for the line blot *versus* 31% for CTD single analyte, and 97% specificity for the line blot *versus* 96% for CTD single analyte. In total 186 antibody results were found fitting with the ACR disease classification criteria (Table II displayed in red). The prevalence of autoantibodies included in the respective classification criteria ranged from 0% (RNA-polymerase III in SSc) to 86% (SS-A (52kD) in pSS) with a large variation in number of patients (3-23) fulfilling these criteria in our cohort. Furthermore, 102 positive antibody results were compatible with signs or symptoms, which are relatively frequent present in some CTD (Table II displayed in orange). All autoantibodies investigated were found in a wide range of disease specificities, and were often found in patients with a disease not known to be compatible with the autoantibodies investigated. For instance, Sm-D autoantibodies were found in 11 patients of which 9 did not have SLE, whereas for SSA (60kD) autoantibodies 70% (21/30) of the positive patients had a compatible disease in category 2.

Of note is that in 28 out of 173 (16%) positive CTD screen results a subsequent analysis by CTD single analytes did not detect autoantibodies. In these cases, CTD screen results were positive with low ratio's between 1.1 and 3.3 (the manufacturer's cut-off is 1.0). Vice versa, 5 sera negative by CTD screen did contain auto-antibodies detected by CTD single analytes. One of these contained a high titre of antibodies against Sm-D (112 U/mL) and against Rib-P antibodies (397 U/mL), and 4 sera contain low titres against dsDNA, CENP-B, or PM-Scl. This shows that results of CTD screen are not always in accordance with subsequent antibody analysis by CTD single analytes.

Clinical relevance of discrepant autoantibody results

Analysis of autoantigens present in both screening assays revealed that 202 autoantibody specificities were detected by line blot and 236 by CTD single analytes (Fig. 1). Discrepant results were found in 82 cases of which 58 were found by CTD single analytes only and 24 by line blot only (Fig. 2a). Further analysis to determine the clinical significance of these indicated differences, especially with regard to detection of dsDNA, SS-A, SS-B, and Sm-D autoantibodies. Fifteen out of 58 autoantibodies found by CTD single analytes only supported diagnosis and 10 were compatible with diagnosis (Fig. 2b). Only 2 out of 24 autoantibodies found by line blot only were supportive for diagnosis and 5 were compatible (Fig. 2c). Positive results obtained by CTD single analytes or line blot only were not relevant in 34 and 20 patients, respectively.

Clinical relevance of autoantibody results unique for one test system

Fifty-five positive results were obtained on autoantigens occurring on line blot only (U1-nRNP/Sm, AMA-M2, histones, and nucleosomes, Fig. 1). In these cases the nRNP/Sm complex was recognised in 16 sera. In 2 patients these antibodies were supportive of a diagnosis of MCTD, in 4 patients compatible with diagnosis, whereas in 10 patients these autoantibodies were not relevant to disease. Histones and nucleosomes were recognised in 25 and 8 cases respectively, which was compatible with the diagnosis of 14 and 4 patients. Analysis of their additive value in diagnostics showed that 10 out of 11 SLE patients with anti-histone antibodies and all 4 SLE patients with anti-nucleosome antibodies also had dsDNA antibodies, and although a low number of patients were included, this suggests a limited value of inclusion of histones and nucleosomes in the line blot and a lower prevalence of these antibodies in SLE in comparison with anti-dsDNA antibodies (Table II). Antibodies against mitochondrial antigens of the M2 subtype were found in 6 patients, none of whom had abnormal liver test results. Thirty-one positive results were obtained by single analytes available in CTD only (U1-RNP (RNP70, A, C), RNP-70, fibrillarin, Mi-2 proteins, and RNA polymerase III). In these cases, antibodies against U1-snRNP were found in 15 patients. In 2 patients these antibodies were supportive for the diagnosis of MCTD, in 3 patients they were compatible with diagnosis, whereas in 10 patients these autoantibodies were not relevant to disease. Antibodies against RNP-70 were found in 10 sera and 2 of these results were supportive for the diagnosis of MCTD, in 2 patients they were compatible with diagnosis, and the

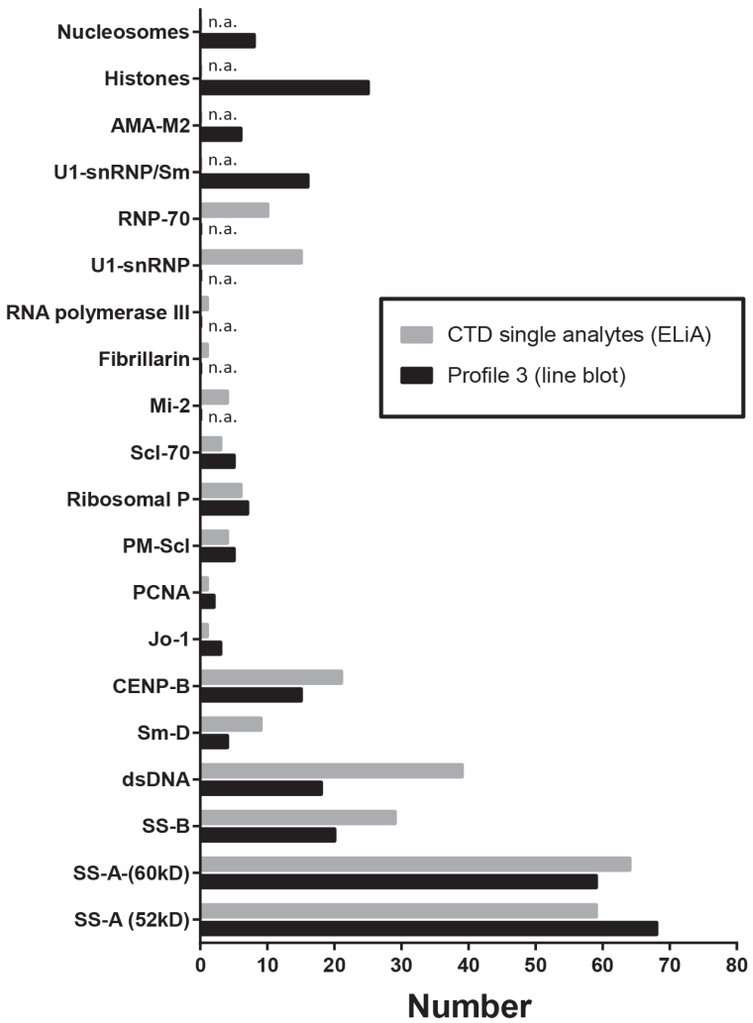

Fig. 1. Comparison of autoantibodies found by 2 techniques.
A: The numbers of autoantibody specificities found in all 1034 patient sera investigated are depicted according to the technique used. Autoantigens absent in 1 assay are displayed as not applicable ("n.a."). Results were defined by the cut-off values provided by the suppliers. For the profile 3 line blot cut-offs defined by the intensity of staining interpreted by the accompanying software was used. Cut-off values for EliA CTD single analytes used are >15 IU/mL for dsDNA and >10 U/ml for SS-A (both 52 and 60kDa), SS-B, Sm, Scl-70, CENP-B, Jo-1, ribsosomal-P, fibrillarin, RNA Polymerase III, PCNA, PM-Scl, Mi-2, U1-RNP, and RNP70. Equivocal results were considered negative in this study.

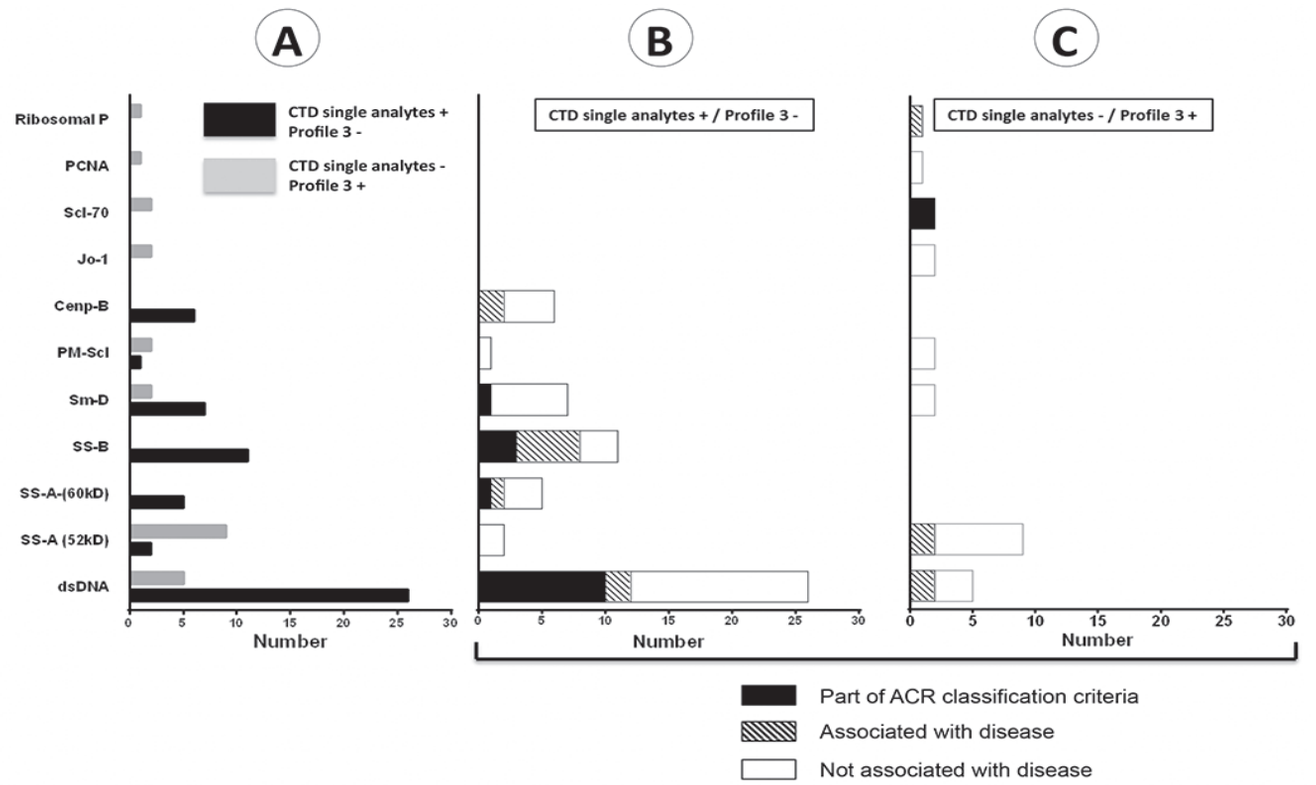

Fig. 2. Number and clinical relevance of discrepant autoantibody results.
A: The number of discrepancies between line blot and CTD single analytes are displayed by number according to autoantibody specificities found. Positive autoantibody results by CTD single analytes but negative by line blot are displayed in black histograms, whereas positive results according to the line blot but negative by single analytes are displayed in grey histograms. The clinical relevance of these discrepancies are displayed in figures **B** and **C**. Shown in black histograms are the number of discrepancies with results which were supportive for diagnosis (*i.e.* part of the ACR disease classification criteria), in hatched histograms those who were compatible with diagnosis (*i.e.* compatible with the diagnosis of an autoimmune disease or predictive for disease), and in white histograms the results which were not relevant for the diagnosis. Positive results from single analyte but negative according line blot are shown in **B**, whereas negative single analyte results but positive for line blot are depicted in **C**.

other positive results were not relevant. Antibodies to fibrillarin, Mi-2 and RNA-polymerase III were found in 1, 4 and 1 patients respectively. For fibrillarin and Mi-2 one case was compatible with disease indicating a very low prevalence of these autoantibodies and therefore limited clinical use in this cohort.

In summary, these data indicate that antibodies against autoantigens uniquely present on line blot only (n=55) were supportive of a given diagnosis in 2 patients (4%), were compatible with diagnosis in 22 patients (40%), and were not relevant in 31 patients (56%), whereas specificities unique for CTD single analytes (n=31) showed 4 diagnostically supportive results (13%), 7 compatible with diagnosis (16%), and 20 not relevant for diagnosis (65%).

Discussion

Commercial manufacturers of ANA tests provide data with regard to sensitivity and specificity of their autoantibody tests for specific AID by using test results from well-defined different CTDs, healthy and disease controls (23). However, these data are not representative for daily clinical practice, where most patients investigated do not have a definite diagnosis. In addition, commercial assays analysing ANA specificities differ amongst each other with regard to the origin of autoantigens used (native or recombinant proteins), the assay principle, and the composition of the autoantigen panel in the test (24). We have compared two different commercial ANA screening assays and two assays defining ANA fine specificities (the CTD single analytes and the profile 3 line blot) in a large unselected cohort of 1030 patients. The purpose of this study was to characterise the presence of ANA in 5 different categories of pre-test probability of CTD, and therein define the clinical relevance of discrepant results between two different commercial assays used for antigenic specificity. Our results indicate that comparable autoantigens yielded discordant results in 17% of patients investigated, with over a third of these results being clinically relevant. These findings are novel and to our knowledge derived from the largest tertiary care cohort studied for this purpose so far.

A number of autoantibodies analysed purportedly have a high specificity for a subset of rare CTD (such as RNA polymerase III antibodies in scleroderma), but have a low pre-test probability in general practice. This results in positive results not compatible with the diagnosis or clinical symptoms causing confusion with regard to interpretation of test results. Both commercial assays investigated contain autoantigens which are part of the ACR classification criteria for connective tissue diseases. Nevertheless, large differences are present in clinically relevant results for dsDNA, SS-A, SS-B and CENP-B. Analysis for dsDNA antibodies by the profile 3 line blot showed a very poor performance as only 8 out

of 23 SLE patients were positive compared to 18 patients by CTD single analytes, resulting in a large difference in sensitivity for SLE (35% versus 78%). No cases of SLE had dsDNA antibodies defined by line blot only. The ACR classification criteria for pSS do not discriminate between SS-A and SS-B antibodies. In pSS patients, antibodies against SS-A (52kDa), SS-A (60kDa) and SS-B were found, respectively, in 0, 1 and 3 patients by CTD single analytes and not with the lineblot, whereas positive results for these autoantibodies only by line blot were never found. This indicates inferior performance of line blot for detection of SS-A/SS-B antibodies. Other notable differences are found in CENP-B positive results. No patient tested positive for these antibodies only by line blot. In contrast, 6 patients tested positive for CENP-B antibodies by CTD single analytes and negative in the line blot which was irrelevant (or confusing) in 4/6 cases. This indicates that the line blot provides less false negative results for the detection of anti-CENP-B antibodies. The number of patients with the same connective tissue disease was limited in this study, precluding accurate estimation of sensitivity and specificity per specific autoantigen for the test systems used. We have analysed this for patients of which the same connective tissue disease occurred more than 10x. The sensitivity/specifity of dsDNA testing for SLE was 35%/99% for the line blot *versus* 82%/99% for the CTD single analytes. Results for pSS showed comparable numbers between both assays. When results of all autoantibodies were analysed together, the line blot and CTD single analyte show a sensitivity/specificity of 27%/97% and 31%/97% respectively, indicating that the CTD single analytes appears to be more sensitive in detecting relevant autoantibodies compared to the line blot.

The two assays used in our study differed in some autoantigens allowing analysis of autoantibodies which were known to be compatible with specific autoimmune diseases but are not part of the ACR classification criteria. Examples of the latter are histones and fibrillarin (U3-RNP), which are compatible with drug-induced lupus and systemic sclerosis, respectively (25,26). One test system (CTD single analytes) included RNA polymerase III, which has just recently been added to the disease classification criteria for SSc (17). In our cohort only one patient had anti-RNA polymerase III antibodies, and was diagnosed with JIA. The same manufacturer included autoantibody analyses for fibrillarin which was found only in one serum sample in a patient with systemic sclerosis, whereas Mi-2 autoantibodies were also found in low frequencies and in most cases present in patients without the "fitting" disease, such as dermatomyositis (27). The line blot includes analysis for AMA-M2. These antibodies have a prevalence of more than 90% in primary biliary cirrhosis (PBC) and can precede the clinical symptoms (15). Although they are found in low frequencies in the general population (less than 0.5%), the line blot test system imposes analysis on all patients tested for ANA including those without symptoms of an auto-immune liver disease. As our study shows, this policy results in positive results in patients without primary biliary cirrhosis and leads to confusion in doctors and patients and may cause unnecessary follow-up, given the reported strong association with PBC. The same test system assays for anti-histone and -nucleosome antibodies, and all SLE patients in our cohort with these antibodies were also ANA and dsDNA positive, suggesting a lack of value added of testing for these autoantibodies.

The CTD screen assay cannot replace the ANA-IIF according to a position statement of the American College of Rheumatology, as solid phase assays do not contain all clinical relevant autoantigens present in Hep-2 cells (28). Our data do not support this notion. In our study we found 131 patients tested positive by ANA-IIF which were negative according to the CTD screen. In only 1 of these cases we found antibodies compatible with disease (centromere-B antibodies in a patient with abnormal liver function tests), whereas false-negative ANA-IIF results occurred in 44 cases including 9 patients with antibody patterns supportive for or compatible with disease. These data suggest that screening by solid phase yields results are at least comparable to and probably better than ANA-IIF results. Limitations of our study are that this is a single centre study, the clinical information was retrieved by analysis of patient records and in most cases the expert panel did not order the ANAs analysed in this study, and that the patient cohort investigated was derived from daily clinical practice in our university hospital and thus may not be representative of other centres using the same type of autoantibody tests. In addition, this study was not designed to determine sensitivity or specificity of autoantibody tests in a large number of patients with the same disease (for instance 100 patients with SSc) which limits conclusions. Strengths include unbiased inclusion of all patients for which ANA testing was ordered, clinical evaluation without knowledge of autoantibody result, and the clinical relevance of discrepant laboratory results by a side-by-side comparison of different autoantibody assays in the clinical practice.

In conclusion, we found that both the CTD single analytes as well as the profile 3 line blot assays generally perform well with regard to identification of clinically relevant antibodies, except for poor detection of dsDNA autoantibodies by the profile 3 line blot. A direct comparison between the line blot and CTD single analytes shows a large number of discrepant, but clinically relevant, autoantibody results, indicating that both test system should be improved regarding quality of antigen preparations. Both systems have also included "unique" tests for autoantibodies not present in the other assay system, which appear to be of no real value to the clinic according to this study. Screening by solid phase assays followed by analysis for reactivity against all individual autoantigens provides many clinically irrelevant autoantibody results and it is costly. The material costs for 1030 ANA-IIF tests plus subsequent testing of positive sera by line blot analysis was in the order of €25.000 in this study, whereas CTD-screen testing followed by CTD single analytes was approximately €40.000. The combination of ANA-IIF and CTD single analytes would have cost approximately €50.000 for materials, as more samples were ANA-IIF positive than by

CTD screen requiring more single analyte analysis. Evaluation of a relevant selection of autoantibodies based on clinical guidance (*e.g.* suspected for SSc) is more cost-effective. This raises the question whether the combination of CTD screen and single analyte assay should be used in laboratory settings with a low pretest probability for a connective tissue disease. Positive results from antibody analysis not compatible with clinical symptoms may cause confusion, and this problem is inherently aggravated by the strategy employed by most laboratories blindly processing positive ANA screening results into a broad and nonselective analysis for all ANA specificities at hand (2,15,16). In this study 3100 out of the 3736 CTD single analyte analyses (83% of the single analyte assays which is equivalent to approximately €26.000 of their material costs), were performed in order to find specific autoantibodies in patients without the fitting clinical signs or symptoms. Thus whenever a laboratory finds a positive result for CTD screen, without a clinical guide that indicates what should be further tested for, a complete analysis for all CTD single analytes will increase material costs approximately 6-fold (100/(100-83)) without diagnostic benefit. In this study it would make a difference in total material costs between €14.000 (in case single analytes were based on clinical guidance) up to €40.000 (all analytes tested when CTD screen was positive). Therefore, a change towards ANA analysis purely based on clinical suspicion would not only be a cost-saving strategy omitting tests irrelevant for diagnosis but also limit generation of confusing results. Finally, detection of autoantibodies in patients having at least one sign/symptom that is relatively frequent in some CTD (category 2 patients) did not reveal any compatible autoantibody patterns at present, but these data should be reexamined in 5-10 years to define whether the antibodies detected had any prognostic relevance as has been shown previously in extensive studies (29, 30).

Acknowledgements

We thank the technicians of the laboratory of Serology for performing all laboratory tests analysed in this paper.

References

1. AGMON-LEVIN N, DAMOISEAUX J, KALLENBERG C *et al*.: International recommendations for the assessment of autoantibodies to cellular antigens referred to as anti-nuclear antibodies. *Ann Rheum Dis* 2014; 73: 17-23.
2. HOCHBERG MC: Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997; 40: 1725.
3. ALVAREZ F, BERG PA, BIANCHI FB *et al*.: International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999; 31: 929-38.
4. SHIBOSKI SC, SHIBOSKI CH, CRISWELL L *et al*.: American College of Rheumatology classification criteria for Sjögren's syndrome: a data-driven, expert consensus approach in the Sjögren's International Collaborative Clinical Alliance cohort. Sjögren's International Collaborative Clinical Alliance (SICCA) Research Groups. *Arthritis Care Res* Hoboken 2012; 64: 475-87.
5. ALARCÓN-SEGOVIA D, CARDIEL MH: Comparison between 3 diagnostic criteria for mixed connective tissue disease. Study of 593 patients. *J Rheumatol* 1989; 16: 328-34.
6. DE ROOIJ DJ, VAN DE PUTTE LB, HABETS WJ, VAN VENROOIJ WJ: Marker antibodies in scleroderma and polymyositis: clinical associations. *Clin Rheumatol* 1989; 8: 231-7.
7. SPENCER-GREEN G, ALTER D, WELCH HG: Test performance in systemic sclerosis: anticentromere and anti-Scl-70 antibodies. *Am J Med* 1997; 103: 242-8 (1997).
8. GLIDDON AE, DORÉ CJ, DUNPHY J, BETTERIDGE Z, MCHUGH NJ, MADDISON PJ: Antinuclear antibodies and clinical associations in a british cohort with limited cutaneous systemic sclerosis. QUINS Trial Study Group. *J Rheumatol* 2011; 38: 702-5.
9. OP DE BEÉCK K, VERMEERSCH P, VERSCHUEREN P *et al*.: Antinuclear antibody detection by automated multiplex immunoassay in untreated patients at the time of diagnosis. *Autoimmun Rev* 2012; 12: 137-43.
10. PARKER JC, BUNN CC: Sensitivity of the Phadia EliA connective tissue disease screen for less common disease-specific autoantibodies. *J Clin Pathol* 2011; 64: 631-3.
11. LÓPEZ-LONGO FJ, RODRÍGUEZ-MAHOU M, ESCALONA-MONGE M, GONZÁLEZ CM, MONTEAGUDO I, CARREÑO-PÉREZ L: Simultaneous identification of various antinuclear antibodies using an automated multiparameter line immunoassay system. *Lupus* 2003; 12: 623-38.
12. BRUNER BF, GUTHRIDGE JM, LU R *et al*.: Comparison of autoantibody specificities between traditional and bead-based assays in a large, diverse collection of patients with systemic lupus erythematosus and family members. *Arthritis Rheum* 2012; 64: 3677-86.
13. VILLALTA D, SORRENTINO MC, GIROLAMI E *et al*.; STUDY GROUP ON AUTOIMMUNE DISEASES OF THE ITALIAN SOCIETY OF LABORATORY MEDICINE: Autoantibody profiling of patients with primary biliary cirrhosis using a multiplexed line-blot assay. *Clin Chim Acta* 2015; 438: 135-8.
14. AVERY TY, VAN DE CRUYS M, AUSTEN J, STALS F, DAMOISEAUX JG: Anti-nuclear antibodies in daily clinical practice: prevalence in primary, secondary, and tertiary care. *J Immunol Res* 2014; 401739.
15. BOGDANOS DP, KOMOROWSKI L: Disease-specific autoantibodies in primary biliary cirrhosis. *Clin Chim Acta* 2011; 412: 502-12.
16. STEEN VD: The many faces of scleroderma. *Rheum Dis Clin North Am* 2008; 34: 1-15.
17. VAN DEN HOOGEN F, KHANNA D, FRANSEN J *et al*.: 2013 classification criteria for systemic sclerosis: an American college of rheumatology/European league against rheumatism collaborative initiative. *Ann Rheum Dis* 2013; 72: 1747-55.
18. ALETAHA D, NEOGI T, SILMAN AJ *et al*.: 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum* 2010; 62: 2569-81.
19. PETTY RE, SOUTHWOOD TR, MANNERS P *et al*.: International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision. *J Rheumatol* 2004; 31: 390-2.
20. TANIMOTO K, NAKANO K, KANO S *et al*.: Classification criteria for polymyositis and dermatomyositis. *J Rheumatol* 1995; 22: 668-74.
21. MOSCA M, NERI R, BOMBARDIERI S: Undifferentiated connective tissue diseases (UCTD): a review of the literature and a proposal for preliminary classification criteria. *Clin Exp Rheumatol* 1999; 17: 615-20.
22. MIYAKIS S, LOCKSHIN MD, ATSUMI T *et al*.: International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006; 4: 295-306.
23. FRITZLER MJ, WIIK A, FRITZLER ML, BARR SG: The use and abuse of commercial kits used to detect autoantibodies. *Arthritis Res Ther* 2003; 5: 192-201.
24. SATOH M, TANAKA S, CHAN EKL: The use and abuse of commercial kits used to detect autoantibodies in systemic autoimmune rheumatic diseases. *Front Immunol* 2015; 6: 181.
25. MONESTIER M, KOTZIN BL: Antibodies to histones in systemic lupus erythematosus and drug-induced lupus syndromes. *Rheum Dis Clin North Am* 1992; 18: 415-36.
26. VILLALTA D, IMBASTARO T, DI GIOVANNI S *et al*.: Diagnostic accuracy and predictive value of extended autoantibody profile in systemic sclerosis. *Autoimmun Rev* 2012; 12: 114-20.
27. TROYANOV Y, TARGOFF IN, PAYETTE MP *et al*.: Redefining dermatomyositis: a description of new diagnostic criteria that differentiate pure dermatomyositis from overlap myositis with dermatomyositis features. *Medicine* (Baltimore) 2014; 93: 318-32.
28. Position Statement on the Methodology of Testing for Antinuclear Antibodies. *American College of Rheumatology* (ACR) 2009.
29. ARBUCKLE MR, MCCLAIN MT, RUBERTONE MV *et al*.: Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *N Engl J Med* 2003; 349: 1526-33.
30. THEANDER E, JONSSON R, SJÖSTRÖM B, BROKSTAD K, OLSSON P, HENRIKSSON G: Autoantibody profiling can predict primary Sjögren's syndrome years before diagnosis and identify those with early onset and severe disease course. *Arthritis Rheumatol* 2015; 67: 2427-36.