Algorithm for antinuclear antibodies in subjects with clinical suspicion of autoimmune diseases

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Abstract

Objective

Antinuclear antibodies (ANA) are fundamental in the diagnosis of systemic autoimmune rheumatic diseases (SARDs). Different assays for ANA screening are available, such as indirect immunofluorescence (IIF) on HEp-2 cells and Multiplex fluorescent immunoassay (MFI). This study aimed to clarify the importance of ANA detected only by IIF in the future development of SARDs and to recommend a laboratory algorithm that integrates the available diagnostic approaches to optimise the diagnosis of ANA IIF+MFI- subjects.

Methods

A total of 9,291 subjects with clinical suspicion of SARDs were evaluated for ANA by IIF and MFI. One hundred and ninety-eight subjects (2.1%) were ANA IIF+MFI-, who were followed up for 2 years. ANA were evaluated using IIF on HEp-2 cells and MFI on the BioPlex 2200.

Results

The ANA IIF+MFI- cohort included 106 subjects with SARDs, 26 subject with other autoimmune diseases (not-SARDs) and 66 subjects with minor symptoms or ANA requested in check-ups. Only 94 subjects underwent re-evaluation. After a 2-year follow-up, most re-evaluated subjects (51 patients) became ANA negative for both assays (mainly rheumatoid arthritis, polymyalgia and inflammatory bowel disease patients) and 35 subjects remained ANA IIF+MFI- (principally systemic sclerosis and systemic lupus erythematosus patients). A new algorithm for ANA evaluation was suggested.

Conclusion

According to the proposed algorithm, ANA IIF+MFI- subjects should be screened by an alternative solid-phase assay such as line-immunoassay or ELISA.

Key words

algorithms, antibodies, antinuclear, fluorescent antibody technique, rheumatic diseases, autoimmune diseases

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Introduction

Antinuclear antibodies (ANA) are a group of autoantibodies that react with nuclear constituents of cells, such as the nuclear membrane, nucleoplasm and nuclear organelles. ANA can be detected in the sera of subjects with systemic autoimmune rheumatic diseases (SARDs), such as systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), systemic sclerosis (SSc), idiopathic inflammatory myopathies (IIMs) and rheumatoid arthritis (RA) (1). Therefore, ANA are essential in the diagnosis of SARDs (2).

In recent decades, several studies have reported that ANA could appear years before the clinical onset of the disease and the relevance of these autoantibodies as predictors of autoimmunity has been demonstrated (3-15). Thus, it may help to predict the future development of SARD in subjects with arthralgia/arthritis, photosensitive rash, muscle weakness, sicca symptoms or Raynaud's phenomenon (8, 12, 14, 15).

The first method used to detect ANA was the indirect immunofluorescence (IIF), described in 1950 by Coons and Kaplan (16). Nowadays, IIF assay is still one of the widely used methods for ANA detection (17). The ability to detect a large number of nuclear and cytoplasmic antigens is the main advantage of IIF (18). However, it has some significant limitations. A major drawback is subjectivity in the interpretation of the results of this ANA assay (19-21). Moreover, the visual evaluation is time consuming and personnel require training. Furthermore, the results are often influenced by the variability of the morphology of HEp-2 cells, which depend on the different manufacturing procedures (22-26). The lack of specificity is another limitation of IIF, given that up to 25% of healthy individuals may be ANA positive by IIF depending on the titre used for primary screening (17, 27-29).

These limitations and the increased demand for ANA detection have led to the development of new, more efficient and automated ANA screening techniques (30, 31). In parallel to traditional Enzyme-Linked Immunosorbent Assay (ELISA), alternative solid-phase assays (SPAs) became widespread (32, 33). These include Multiplex Fluorescent Immunoassay (MFI), mostly referred to as addressable laser bead assays (ALBIA) and line immunoassays (LIA) (34). The MFI is a multi-antigen technique that allows the simultaneous detection of different autoantibodies in a single tube using an array of beads that incorporate purified or recombinant antigens (35).

Numerous studies have shown discrepancies in the results obtained by the different ANA assays (9, 11, 35) and the optimal method for ANA screening remains a hot topic of debate. However, it has been reported that the association of IIF and SPAs increased the sensitivity (from 89.2% to 97.4%) and the specificity (from 64.6% to 98.4%) of serological tests for ANA screening (36).

In a previous study, Pérez *et al*. evaluated the clinical significance of ANA detected only by MFI (with ANA negative by IIF), and a higher sensitivity of MFI in comparison with IIF has been reported. After a 3-year follow-up of the subjects from routine tests without autoimmune diseases, the study showed that 76% became ANA positive by IIF and MFI and 87% were finally diagnosed with an autoimmune disease. Besides, the positive predictive value of MFI when there are more than one autoantibody was reported. After a 3-year follow-up, 96.8% of subjects with three or more positive autoantibodies detected by MFI developed autoimmune diseases (8). The above data indicate that these methods can complement each other leading to an increase in both sensitivity and specificity of ANA determination depending on each clinical situation.

This study has two aims. The first is to clarify the importance of ANA detected only by IIF in the future development of SARDs. The second aim is to recommend a laboratory algorithm that integrates the available diagnostic approaches to optimise the diagnosis of ANA IIF+ MFI- subjects with suspected SARDs.

Material and methods

Subjects

A total of 26,390 subjects were referred to the Autoimmunity Laboratory of Hospital 12 de Octubre (Madrid, Spain) to perform an ANA screening during

a 4-year period (from January 2012 to December 2015). For this study, 9,291 subjects (70.3% women and 29.7% men, mean age 54.7±17.8 years) were selected according to the following selection criteria: subjects not previously studied in the Autoimmunity Laboratory of Hospital 12 de Octubre and subjects not evaluated for ANA for at least 1 year before they were selected. More than 95% of the included patients were Mediterranean Caucasian. Subjects under study during the last year with an active autoimmune disease were excluded. The presence of ANA was evaluated simultaneously by two techniques: IIF and MFI. SARD diagnoses were determined according to internationally validated disease criteria (37-42).

Methods

Autoantibody detection by IIF assay on HEp-2 cells

ANA detection was performed by IIF assay on HEp-2 cells (Inova Diagnostics, Inc, San Diego, CA, USA). The results were analysed using a Nikon Eclipse fluorescence microscope with magnification ×400. The detection of any nuclear pattern (nuclear homogeneous AC-1, centromere AC-3, fine speckled AC-4, coarse speckled AC-5, nuclear dots AC-6/AC-7, nucleolar AC-8/AC-9, etc.) at a dilution of 1:160 or higher was considered a positive result (IIF+) (17, 43). New provisional classification criteria for SLE have been proposed, suggesting an ANA titre of at least 1:80 on HEp-2 cells or an equivalent positive test (44, 45). In this work, a dilution of 1:160 was used, according to the recommendation of Agmon-Levi *et al*. for SARDs evaluation. MFI was performed in all patients as MFI is an equivalent test (and more sensitive), so the protocol meets the new EULAR/ACR proposed recommendations.

Autoantibody detection by MFI

ANA screening was performed using the BioPlex 2200 ANA Screen kit (Bio-Rad Laboratories, Hercules, CA, USA). It employs fluorescently dyed magnetic beads for the simultaneous detection of the levels of 13 autoantibodies (anti-double-strand DNA (dsDNA), anti-chromatin, anti-ribosomal P, anti-Ro52 (SS-A), anti-Ro60 (SS-A), anti-La (SS-B), anti-Sm, anti-Sm-RNP complex, anti-ribonucleoprotein 68 (RNP 68), anti-topoisomerase I (Scl-70), anti-centromere B and anti-Jo-1) within a single serum sample. The BioPlex 2200 ANA Screen is run on the BioPlex 2200 System, a fully-automated, random access multiplex testing platform. IgG is the primary antibody isotype detected by this assay. The cut-off values were established based on the 99th percentile for a non-disease population in Spain (blood donors). The cut-off values for autoantibodies detected by BioPlex coincide with the manufacturer's recommended values (1.0 AI), except for anti-RNP 68 and anti-dsDNA antibodies (2.0 AI and 20 AI, respectively). BioPlex ANA Screen positive results (MFI+) were considered when at least one autoantibody was detected by this assay.

Ethical approval

The study was approved by the Ethics Committee for Clinical Research of Hospital 12 de Octubre (ref. no. CEIC-16/383). No informed consent was required.

Table I. Results of ANA screening by IIF and MFI in the studied population.

n=9,291 subjects		ANA IIF	
		Negative	Positive
ANA MFI	Negative	5,717 (61.6%)	**198 (2.1%)**
	Positive	2,455 (26.4%)	921 (9.9%)

ANA: antinuclear antibodies; IIF: Indirect Immunofluorescence; MFI: multiplex fluorescent immunoassay.

Table II. Clinical manifestations of 198 ANA IIF+MFI- subjects and 94 followed-up subjects.

Pathology	ANA IIF+ MFI- subjects n=198		ANA IIF+ MFI- followed-up subjects n=94 (47%)	
	n	% of total subjects	n	% of total subjects with this pathology
Group 1: SARDs				
SLE	18	9%	12	67%
SSc	14	7%	10	71%
Fibromyalgia	4	2%	2	50%
RA	48	24%	21	44%
Inflammatory myopathy	2	1%	2	100%
Polymyalgia	8	4%	5	63%
Other SARDs	12	6%	5	42%
Total	**106**	**54%**	**57**	**54%**
Group 2: Other autoimmune diseases (not-SARDs)				
Inflammatory bowel disease	13	7%	9	69%
Psoriasis	7	4%	2	29%
Hepatopathy	6	3%	3	50%
Total	**26**	**13%**	**14**	**54%**
Group 3: Minor symptoms or health check-ups				
Polyarthritis-Osteoarthritis	13	7%	5	38%
Non autoimmune diseases found during health check-ups	39	20 %	12	31 %
No rheumatic diseases found during health check-ups	14	7 %	6	43 %
Total	**66**	**33 %**	**23**	**35 %**

The total 198 ANA IIF+ MFI- subjects are shown in the second column, while the 94 ANA IIF+ MFI- subjects who were followed-up for two years are indicated in the third column. The table indicates the number and percentage that subjects represent over the total number of ANA IIF+ MIF- subjects (second column) or over the total number of these subjects who presented the pathology in the first evaluation (third column).
ANA: antinuclear antibodies; IIF: indirect immunofluorescence; MFI: multiplex fluorescent immunoassay; SARD: systemic autoimmune rheumatic diseases; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; RA: rheumatoid arthritis.

Statistical analysis

Data from subjects and ANA results were included in randomised databases that were processed and analysed using MedCalc for Windows v. 14.12 (MedCalc Software, Ostend, Belgium). Association between qualitative variables was determined with Pearson's Chi-square test (or Fisher's exact test when appropriate). In scaled variables with two categories, the comparisons were performed using Mann-Whitney U-test. *p*-values less than 0.05 were considered significant.

Results

Percentage of ANA IIF+ MFI- subjects in the studied population

Among the 9,291 subjects included in the study, 921 (9.9%) were ANA IIF+ MFI+, 5,717 (61.6%) were ANA IIF- MFI-, 2,455 (26.4%) were ANA IFI- MFI+ and 198 (2.1%) were ANA IIF+ MFI- (Table I).

Subjects in the ANA IIF+ MFI- group were slightly older in comparison with the ANA IIF+ MFI+ group (56.4 *vs*. 53.1 years, *p*=0.018), and the proportion of women was significantly lower (77.8% *vs*. 85.5%, p = 0.007).

The 198 ANA IIF+ MFI- subjects were classified into three different groups according to their clinical manifestations (Table II): 106 (54%) were subjects with high clinical suspicion of SARDs (group 1); 26 (13%) were subjects with other autoimmune diseases (not-SARDs) that were referred to laboratory requesting systemic autoimmunity studies (group 2); and 66 (33%) were subjects with minor symptoms, mostly polyarthritis and osteoarthritis, that may be related to SARDs but without a final diagnosis of these disorders, or with this ANA evaluation requested in the context of health check-ups (group 3).

Within group 1, almost half of the subjects had RA (48 patients) and 80% were seropositive for anti-citrullinated cyclic peptide or rheumatoid factor.

Follow-up of ANA IIF+ MFI- subjects

Out of the 198 ANA IIF+ MFI- subjects, only 94 (47%) were re-evaluated and followed-up for two years. In the context of the above-mentioned clinical groups, this re-evaluation included 57 subjects (54%) in group 1, 14 subjects (54%) in group 2 and 23 subjects (35%) in group 3 (Table II).

After the re-evaluation, most subjects (51, 54%) were ANA negative by both assays (ANA IIF- MFI-); 8 subjects (9%) changed to ANA positive by MFI, becoming positive for both assays (ANA IIF+ MFI+); and 35 subjects (37%) preserved the positivity of ANA IIF with negative MFI.

The majority of patients with SSc and SLE maintained their status of ANA IIF+ MFI-. In contrast, most RA patients and patients with polymyalgia and inflammatory bowel disease become ANA negative by IIF and MFI. For a large proportion of patients with psoriasis and inflammatory myopathy as well as for some patients with RA, ANA MFI became positive (maintaining IIF positivity) (Table III).

Furthermore, the conversion of ANA IIF+ MFI- subjects to ANA-negative by both assays is not dependent of the titre of ANA by IIF (Table IV).

Table III. Evolution of 94 ANA IIF+MFI- followed-up subjects after ANA re-evaluation.

Pathology	ANA IIF+ MFI- followed-up subjects			
	n total (n=94)	Remain IIF+ MFI- (n=35)	Became IIF+ MFI+ (n=8)	Became IIF- MFI- (n=51)
Group 1: SARDs				
SLE	12	7	-	5
SSc	10	6	1	3
Fibromyalgia	2	1	-	1
RA	21	5	3	13
Inflammatory myopathy	2	1	1	-
Polymyalgia	5	1	-	4
Other SARDs	5	3	1	1
Total	**57**	**24**	**6**	**27**
Group 2: Other autoimmune diseases (not-SARDs)				
Inflammatory bowel disease	9	2	-	7
Psoriasis	2	1	1	-
Hepatopathy	3	-	-	3
Total	**14**	**3**	**1**	**10**
Group 3: Minor symptoms or health check-ups				
Polyarthritis-Osteoarthritis	5	1	-	4
Non autoimmune diseases found during health check-ups	12	4	1	7
No rheumatic diseases found during health check-ups	6	3	-	3
Total	**23**	**8**	**1**	**14**

After the 2-year follow-up, most patients with RA, polymyalgia and inflammatory bowel disease change to negative ANA IIF (blue), becoming negative for both assays. Among the subjects who maintain the state of ANA IIF+ MFI- (orange) those who suffer from SSc and SLE stand out. For a considerable proportion of patients with psoriasis, inflammatory myopathy and for some patients with RA, ANA MFI became positive (yellow), with both ANA assays positive.

IIF: indirect immunofluorescence; MFI: multiplex fluorescent immunoassay; SARD: systemic autoimmune rheumatic diseases; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; RA: rheumatoid arthritis.

Laboratory algorithm

A new laboratory algorithm (Fig. 1) is proposed for ANA screening in subjects with clinical suspicion of SARDs. Initially, performing both assays (MFI and IIF) is suggested. The clinical suspicion may be confirmed in subjects with autoantibodies detected by MFI (ANA MFI+), having either negative or positive ANA IIF. In subjects without ANA detected by MFI (ANA MFI-), an alternative method of SPAs should be performed where appropriate antigens for the diagnosis of suspected diseases are represented, especially in patients with suspected SSc, SLE or inflammatory myopathy. A positive result by the alternative method will support the diagnosis of SARD. If a negative result by the alternative method is obtained, it can help to exclude the diagnosis of SARDs.

Table IV. Conversion of ANA IIF+MFI- subjects after re-evaluation according to the titre of ANA IIF.

Conversion of ANA IIF+ MFI- subjects	Titre of ANA IIF 1:160		Titre of ANA IIF 1:320		Titre of ANA IIF ≥1:640	
	n (n=147)	%	n (n=33)	%	n (n=18)	%
Remain IIF+ MFI-	24	16.3	6	18.2	5	27.8
SLE	1		2		3	
SSc	3		4		0	
Fibromyalgia	1		0		0	
RA	5		0		0	
Inflammatory myopathy	1		0		0	
Polymyalgia	2		0		0	
Other SARDs	3		0		0	
Inflammatory bowel disease	2		0		0	
Psoriasis	1		0		0	
Polyarthritis-Osteoarthritis	1		0		0	
Non-autoimmune diseases found during health check-ups	2		0		2	
No rheumatic diseases found during health check-ups	2		0		0	
Became IIF+ MFI+	5	3.4	2	6.1	1	5.6
SSc	1		0		0	
RA	2		1		0	
Inflammatory myopathy	0		1		0	
Other SARDs	0		0		1	
Psoriasis	1		0		0	
Non-autoimmune diseases found during health check-ups	1		0		0	
Became IIF- MFI-	38	25.9	8	24.2	5	27.8
SLE	5		0		1	
SSc	2		0		0	
Fibromyalgia	1		0		0	
RA	8		2		3	
Polymyalgia	3		0		0	
Other SARDs	1		0		0	
Inflammatory bowel disease	4		2		1	
Hepatopathy	2		1		0	
Polyarthritis-Osteoarthritis	3		1		0	
Non-autoimmune diseases found during health check-ups	6		1		0	
No rheumatic diseases found during health check-ups	3		1		0	
Not re-evaluated	80	54.5	17	51.5	7	38.8

The number and percentage that subjects represent over the total number of subjects with a determined titre are shown. ANA: antinuclear antibodies; IIF: indirect immunofluorescence; MFI: multiplex fluorescent immunoassay.

Discussion

In the present study, 2.1% of subjects with suspected SARDs were IIF positive and MFI negative. This data is similar to our previous study carried out in 2011 with a similar cohort of patients in the same hospital (46), which reported a rate of 1.7%.

Of the re-evaluated ANA IFI+ MFI- subjects, 54% became negative by both assays (ANA IIF- MFI-) and most subjects who remained ANA IFI+ MFI- were patients with SSc and SLE. In the recommended algorithm, these patients would be diagnosed by the alternative method since MFI is not an appropriate assay for ANA evaluation in these diseases.

The percentage of ANA IIF+ MFI- subjects who became positive for both assays (ANA IIF+ MFI+) in the follow-up evaluation is clearly lower (9%) than that previously described for ANA IIF- MFI+ subjects (76%) (8). These data suggest that the positive predictive value of MFI is greater than IIF.

In the group of subjects with minor symptoms that could be related to SARDs, 61% became negative for ANA IIF, which can be explained by the fluctuations of ANA determinations or may represent the percentage of the general population that is ANA positive by IIF.

Several studies have investigated the diagnostic utility of IIF *versus* other systems such as SPAs (34, 36, 47) and recently we reported a higher sensitivity of MFI for ANA detection in comparison with IIF on HEp-2 cells (8). Furthermore, it has been reported that the combination of IIF and SPAs adds value to ANA detection in subjects with suspicion of SARDs (36, 47-52). Bizzaro *et al*. compared IIF *vs*. SPAs in the detection of ANA and described that the association of both methods increased the sensitivity (from 89.2% to 97.4%) and the specificity (from 64.6% to 98.4%) of serological test for ANA screening. This combination was also cost-effective, reducing global costs for the serological diagnosis of SARD by 22% (36).

The choice of the best possible assay for ANA screening depends on the disease to be diagnosed. The inability to detect several autoantibodies related to SSc or IIM has been described as the major limitation of autoantibody detection by MFI (8, 53). Anti-centromere B, anti-topoisomerase I (Scl70) and anti-Jo1 autoantibodies are included in the BioPlex ANA Screen. However, other important autoantibodies such as anti-RNApol-III, anti-fibrilarin, anti-SRP, anti-Mi2, anti-MDA5 and anti-TIF1-γ (54, 55) are missed by this screen (56). Due to this low representation of autoantigens associated with IIM and SSc in the MFI system, the proposed algorithm uses an alternative method when SSc or IIM are suspected. In addition, SLE patients should also be confirmed by an alternative method since many of them are not detected by MFI according to our results.

An important limitation is that only 94 of the 194 patients were followed up for 2 years, and therefore the results need to be confirmed by larger studies. An additional limitation of MFI is the high prevalence of anti-RNP-A antibodies in healthy subjects and its low positive predictive value (8, 11). Since a

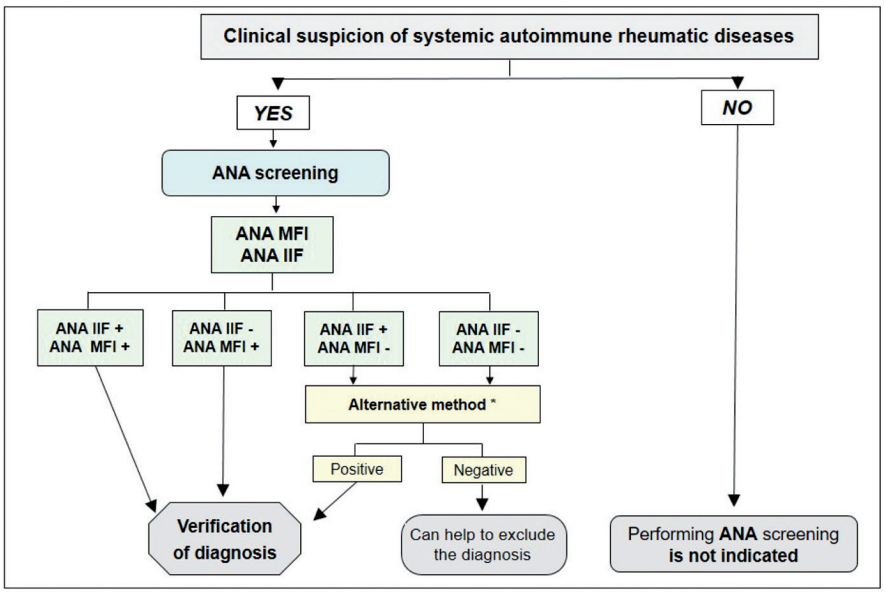

Fig. 1. Recommended algorithm for antinuclear antibodies screening in subjects with clinical suspicion of systemic autoimmune rheumatic diseases.
*An alternative method of solid-phase assay should be performed, where appropriate antigens for the diagnosis of suspected diseases are represented. The algorithm needs to be related to the degree of the clinical suspicion (low or high). ANA: antinuclear antibodies; IIF: indirect immunofluorescence; MFI: multiplex fluorescent immunoassay; SPAs: solid-phase assays.

consensus about their clear association with SARDs is still missing, anti-RNP-A antibodies were not evaluated and are not considered when the recommended algorithm is performed.

The proposed algorithm suggests that ANA IIF+ MFI- subjects should be screened by an alternative SPAs such as line immunoassay or ELISA. Because a higher MFI sensitivity compared to IIF was reported for ANA detection, an early diagnosis of autoimmune diseases can be done, treating subjects immediately upon first clinical manifestations and limiting the clinical impact of the disease (2, 57). Since an individualised approach of autoantibody measurement in patients with ANA-associated autoimmune rheumatic diseases suspicion is cost-effective, the algorithm needs to be related to the degree of the clinical suspicion (low or high). In summary, as laboratory techniques for detecting autoantibodies are improving, laboratory diagnosis algorithms should be adjusted according to the method and the antibodies panel used for a more effective diagnosis and treatment of patients.

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References

1. SUR LM, FLOCA E, SUR DG, COLCERIU MC, SAMASCA G, SUR G: Antinuclear antibodies: marker of diagnosis and evolution in autoimmune diseases. *Lab Med* 2018; 49: 62-73.
2. DAMOISEAUX J, ANDRADE LE, FRITZLER MJ, SHOENFELD Y: Autoantibodies 2015: From diagnostic biomarkers toward prediction, prognosis and prevention. *Autoimmun Rev* 2015; 14: 555-63.
3. 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.
4. ISRAELI E, GROTTO I, GILBURD B *et al*.: Anti-Saccharomyces cerevisiae and antineutrophil cytoplasmic antibodies as predictors of inflammatory bowel disease. *Gut* 2005; 54: 1232-36.
5. SHOENFELD Y, CARP HJ, MOLINA V *et al*.: Autoantibodies and prediction of reproductive failure. *Am J Reprod Immunol* 2006; 56: 337-44.
6. MERONI PL, SHOENFELD Y: Predictive, protective, orphan autoantibodies: the example of anti-phospholipid antibodies. Autoimmun Rev 2008; 7: 585-87.
7. AGMON-LEVIN N, SHOENFELD Y: Prediction and prevention of autoimmune skin disorders. *Arch Dermatol Res* 2009; 301: 57-64.
8. PEREZ D, GILBURD B, CABRERA-MARANTE O *et al*.: Predictive autoimmunity using autoantibodies: screening for anti-nuclear antibodies. *Clin Chem Lab Med* 2018; 56: 1771-7.
9. OP DE BEECK K, VERMEERSCH P, VERSCHUEREN P *et al*.: Detection of antinuclear antibodies by indirect immunofluorescence and by solid phase assay. *Autoimmun Rev* 2011; 10: 801-8.
10. FRANKE B, GALLOWAY TS, WILKIN TJ: Developments in the prediction of type 1 diabetes mellitus, with special reference to insulin autoantibodies. *Diabetes Metab Res Rev* 2005; 21: 395-415.
11. SHOVMAN O, GILBURD B, BARZILAI O *et al*.: Evaluation of the BioPlex 2200 ANA screen: analysis of 510 healthy subjects: incidence of natural/predictive autoantibodies. *Ann N Y Acad Sci* 2005; 1050: 380-8.
12. SHEPSHELOVICH D, SHOENFELD Y: Prediction and prevention of autoimmune diseases: additional aspects of the mosaic of autoimmunity. *Lupus* 2006; 15: 183-90.
13. NANCY AL, YEHUDA S: Prediction and prevention of autoimmune skin disorders. *Arch Dermatol Res* 2009; 301: 57-64.
14. ROSE NR: Prediction and Prevention of Autoimmune Disease in the 21st Century: A Review and Preview. *Am J Epidemiol* 2016; 183: 403-6.
15. HAREL M, SHOENFELD Y: Predicting and preventing autoimmunity, myth or reality?. *Ann N Y Acad Sci* 2006; 1069: 322-45.
16. COONS AH, KAPLAN MH: Localization of antigen in tissue cells; improvements in a method for the detection of antigen by means of fluorescent antibody. *J Exp Med* 1950; 91: 1-13.
17. 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.
18. BURLINGAME RW, PEEBLES C: Detection of antibodies. *In*: POLLARD KM (Ed.): *Autoantibodies and Autoimmunity: Molecular Mechanisms in Health and Disease*. Weinheim, Wiley-VCH 2006: 159-188.
19. TAN EM: Autoantibodies to nuclear antigens (ANA): their immunobiology and medicine. *Adv Immunol* 1982; 33: 167-240.
20. INFANTINO M, MEACCI F, GROSSI *et al*.: The burden of the variability introduced by the HEp-2 assay kit and the CAD system in ANA indirect immunofluorescence test. *Immunol Res* 2017; 65: 345-54.
21. RIGON A, INFANTINO M, MERONE M *et al*.: The inter-observer reading variability in antinuclear antibodies indirect (ANA) immunofluorescence test: A multicenter evaluation and a review of the literature. *Autoimmun Rev* 2017; 16: 1224-29.
22. PHAM BN, ALBAREDE S, GUYARD A, BURG E, MAISONNEUVE P: Impact of external quality assessment on antinuclear antibody detection performance. *Lupus* 2005; 14: 113-19.
23. BIZZARO N, TOZZOLI R, TONUTTI E *et al*.: Variability between methods to determine ANA, anti-dsDNA and anti-ENA autoantibodies: a collaborative study with the biomedical industry. *J Immunol Methods* 1998; 219: 99-107.
24. SONG L, HENNINK EJ, YOUNG IT, TANKE HJ: Photobleaching kinetics of fluorescein in quantitative fluorescence microscopy. *Biophys J* 1995; 68: 2588-600.

25. HAHM D, ANDERER U: Establishment of HEp-2 cell preparation for automated analysis of ANA fluorescence pattern. *Cytometry A* 2006; 69: 178-81.
26. COPPLE SS, GILES SR, JASKOWSKI TD, GARDINER AE, WILSON AM, HILL HR: Screening for IgG antinuclear autoantibodies by HEp-2 indirect fluorescent antibody assays and the need for standardization. *Am J Clin Pathol* 2012; 137: 825-30.
27. SATOH M, CHAN EK, SOBEL ES *et al*.: Clinical implication of autoantibodies in patients with systemic rheumatic diseases. *Expert Rev Clin Immunol* 2007; 3: 721-38.
28. AKMATOV MK, ROBER N, AHRENS W *et al*:. Anti-nuclear autoantibodies in the general German population: prevalence and lack of association with selected cardiovascular and metabolic disorders-findings of a multicenter population-based study. *Arthritis Res Ther* 2017; 19: 127.
29. PISETSKY DS: Antinuclear antibody testing - misunderstood or misbegotten?. *Nat Rev Rheumatol* 2017; 13: 495-502.
30. MERONI PL, BIZZARO N, CAVAZZANA I, BORGHI MO, TINCANI A: Automated tests of ANA immunofluorescence as throughput autoantibody detection technology: strengths and limitations. *BMC Med* 2014; 12:38.
31. RIGON A, BUZZULINI F, SODA P *et al*.: Novel opportunities in automated classification of antinuclear antibodies on HEp-2 cells. *Autoimmun Rev* 2011; 10: 647-52.
32. TEBO AE: Recent approaches to optimize laboratory assessment of antinuclear antibodies. *Clin Vaccine Immunol* 2017; 24: e00270-17.
33. AU EY, IP WK, LAU CS, CHAN YT: Evaluation of a multiplex flow immunoassay versus conventional assays in detecting autoantibodies in systemic lupus erythematosus. *Hong Kong Med J* 2018; 24: 261-69.
34. PEREZ D, GILBURD B, AZOULAY D, SHOVMAN O, BIZZARO N, SHOENFELD Y: Antinuclear antibodies: Is the indirect immunofluorescence still the gold standard or should be replaced by solid phase assays?. *Autoimmun Rev* 2018; 17: 548-52.
35. DESPLAT-JEGO S, BARDIN N, LARIDA B, SANMARCO M: Evaluation of the BioPlex 2200 ANA screen for the detection of antinuclear antibodies and comparison with conventional methods. *Ann N Y Acad Sci* 2007; 1109: 245-55.
36. BIZZARO N, BRUSCA I, PREVITALI G *et al*.: The association of solid-phase assays to immunofluorescence increases the diagnostic accuracy for ANA screening in patients with autoimmune rheumatic diseases. *Autoimmun Rev* 2018; 17: 541-47.
37. ALARCON-SEGOVIA D, CARDIEL MH: Comparison between 3 diagnostic criteria for mixed connective tissue disease. Study of 593 patients. *J Rheumatol* 1989; 16: 328-34.
38. ALETAHA D, NEOGI T, SILMAN AJ *et al*.: 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Ann Rheum Dis* 2010; 69: 1580-88.
39. 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.
40. LUNDBERG IE, TJARNLUND A, BOTTAI M *et al*.: 2017 European League Against Rheumatism/American College of Rheumatology Classification Criteria for Adult and Juvenile Idiopathic Inflammatory Myopathies and Their Major Subgroups. *Arthritis Rheumatol* 2017; 69: 2271-82.
41. SHIBOSKI CH, SHIBOSKI SC, SEROR R *et al*.: 2016 American College of Rheumatology/European League Against Rheumatism Classification Criteria for Primary Sjogren's Syndrome: a consensus and data-driven methodology involving three international patient cohorts. *Arthritis Rheumatol* 2017; 69: 35-45.
42. PETRI M, ORBAI AM, ALARCON GS *et al*.: Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. *Arthritis Rheum* 2012; 64: 2677-86.
43. WILLEMS P, DE LANGHE E, WESTHOVENS R, VANDERSCHUEREN S, BLOCKMANS D, BOSSUYT X: Antinuclear antibody as entry criterion for classification of systemic lupus erythematosus: pitfalls and opportunities. *Ann Rheum Dis* 2019; 78: e76.
44. CHOI MY, FRITZLER MJ: Challenges and advances in SLE autoantibody detection and interpretation. *Curr Treat Opt Rheumatol* 2019; 5: 147-67.
45. TEDESCHI SK, JOHNSON SR, BOUMPAS DT *et al*.: Multicriteria decision analysis process to develop new classification criteria for systemic lupus erythematosus. *Ann Rheum Dis* 2019; 78: 634-40
46. LERMO S, TALISE M, TALAYERO *et al*.: Evaluation of the sensitivity of two antinuclear antibodies screening methods: multiplex technology vs indirect immunofluorescence. 8th International Congress on Autoimmunity. Granada (Spain). 2012.
47. MERONI PL, BORGHI MO: Diagnostic laboratory tests for systemic autoimmune rheumatic diseases: unmet needs towards harmonization. *Clin Chem Lab Med* 2018; 56: 1743-48.
48. MAHLER M, MERONI PL, ANDRADE LE *et al*.: Towards a better understanding of the clinical association of anti-DFS70 autoantibodies. *Autoimmun Rev* 2016; 15: 198-201.
49. SOWA M, HIEMANN R, SCHIERACK P, REINHOLD D, CONRAD K, ROGGENBUCK D: Next-generation autoantibody testing by combination of screening and confirmation-the CytoBead(R) technology. *Clin Rev Allergy Immunol* 2017; 53: 87-104
50. OLSEN NJ, CHOI MY, FRITZLER MJ: Emerging technologies in autoantibody testing for rheumatic diseases. *Arthritis Res Ther* 2017; 19: 172.
51. WILLEMS P, DE LANGHE E, CLAESSENS J *et al*.: Screening for connective tissue disease-associated antibodies by automated immunoassay. *Clin Chem Lab Med* 2018; 56: 909-18
52. CLAESSENS J, BELMONDO T, DE LANGHE *et al*.: Solid phase assays versus automated indirect immunofluorescence for detection of antinuclear antibodies. *Autoimmun Rev* 2018; 17: 533-40
53. SHANMUGAM VK, SWISTOWSKI DR, SADDIC N, WANG H, STEEN VD: Comparison of indirect immunofluorescence and multiplex antinuclear antibody screening in systemic sclerosis. *Clin Rheumatol* 2011; 30: 1363-68.
54. INIESTA ARANDIA N, SIMEON-AZNAR CP, GUILLEN DEL CASTILLO A *et al*.: Influence of antibody profile in clinical features and prognosis in a cohort of Spanish patients with systemic sclerosis. *Clin Exp Rheumatol* 2017; 35: 98-105.
55. SATOH M, TANAKA S, CERIBELLI A, CALISE SJ, CHAN EK: A comprehensive overview on myositis-specific antibodies: new and old biomarkers in idiopathic inflammatory myopathy. *Clin Rev Allergy Immunol* 2017; 52: 1-19.
56. HAMAGUCHI Y, KODERA M, MATSUSHITA T *et al*.: Clinical and immunologic predictors of scleroderma renal crisis in Japanese systemic sclerosis patients with anti-RNA polymerase III autoantibodies. *Arthritis Rheumatol* 2015; 67: 1045-52.
57. KONFORTE D, DIAMANDIS EP, VAN VENROOIJ WJ, LORIES R, WARD MM: Autoimmune diseases: early diagnosis and new treatment strategies. *Clin Chem* 2012; 58: 1510-14.