

Looking beyond ANA as an entry criterion for the classification of SLE

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ANA as a cornerstone in diagnostic autoimmunity

- Valuable screening lab markers for various systemic rheumatic diseases
 - ✓ to support a diagnostic suspect
 - ✓ to confirm a diagnosis



A practical algorithm for test ordering

Wiik A. Scand J Rheumatol 2005

Tentative diagnosis	ANA-IIF	a-dsDNA	a-Sm	a-U1RNP	a-SSA/SSB	a-Scl-70	a-Jo-1	a-ribonRNP	ANCA-IIF	MPO-ANCA	PR3-ANCA	a-Cardiolipin	a-β ₂ GPI	IgM RF
SLE	■	▲	▲	●	▲			▲				▲	●	●
1° SS	■	●			▲			●				●		●
SSc	■			▲		▲						●		
MCTD	■	▲	▲	▲				▲				●		●
PM/DM	■			▲			▲					●		
APS	■											■	▲	
1° SVV									■	▲	▲			
CDT?	■	●	●	●	▲	●	▲		■	●		■	▲	■

■ Primary screen test
 ▲ Secondary test
 ● Additional test (optional)

a) Ideally should include SSc panel testing for anti-RNA polymerase I and III, anti-U3RNP and anti-To/Th RNP antibodies.

b) Ideally should include PM/DM panel testing for anti-aminoacyl-tRNA synthetase, anti-SRP, anti-Mi-2, anti-PM/Scl, (and anti-Ku antibodies in oriental patients).

c) Should always include lupus anticoagulant as well.

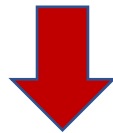
2019 SLE classification criteria

ANA positivity as new entry criterion for SLE classification

- Immunofluorescence on HEp-2 cells with titre $\geq 1:80$
(Se 97.8 - Sp 74.7; *Leuchten N, Arthritis Care Res 2018*)

or

- A solid phase ANA screening immunoassay with at least equivalent performance



High impact on inclusion in clinical trials

Ideas Underpinning the New Criteria

Pisestky D, Bossuyt X & Meroni PL (*Autoimmunity Rev* 1'18)

- ANA expression is essentially invariable in SLE and, to the extent that it may occur, ANA negative lupus is rare.
- SLE expression persists over time; as such, ANA testing can be used for disease classification at any point in the disease course.
- HEp-2-IFA or more recent equivalent solid phase assays are the gold standard for ANA detection, with different assays or kits providing similar results.

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ANA neg SLE

- 6.2 % of newly diagnosed SLE are IIF HEp2 -2000 neg
- 1.5 % display isolated **CytoplasmicMitoticPattern**

Choi et al *Arthr Res Care* 2018

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Demographic, clinical and laboratory baseline characteristics of the 240 patients suffering from systemic lupus erythematosus (SLE).

Demographics	Renal (n = 121)	Non-renal (n = 119)
Gender, %F (n)	93 (112)	87 (104)
Age in years, mean ± SD	41.6 ± 12.6	41.2 ± 16.0
Clinical features		
Age at SLE onset in years, mean ± SD	28.0 ± 11.9	35.4 ± 15.9
SLE duration in years, mean ± SD	13.8 ± 9.5	5.7 ± 7.0
SLEDAI-2K, median (min-max)	4 (0-27)	5 (0-21)
Median follow-up duration in years median (IQR; min-max)	8.8 (6.4-9.4; 2.9-11.1)	6.4 (4.0-9.0; 2.8-22.1)
Laboratory profile		
Serum creatinine mg/dL, median (min-max)	0.8 (0.4-2)	0.4 (0.4-1.2)
24 h urine protein g/24 h, median (min-max)	0.5 (0-13.8)	0.2 (0-0.5)
ANA, % pos (n)	93 (112)	97 (116)
Anti-ENA, % pos (n)	49 (59)	61 (72)
Anti-dsDNA, % pos (n)	42 (51)	35 (42)

ANA anti-nuclear antibodies, dsDNA double-strand DNA, ENA extractable nuclear antigens, F female, IQR inter-quartile range, SD standard deviation, SLE systemic lupus erythematosus, SLEDAI-2K Systemic Lupus Erythematosus Disease Activity Index 2000.

Are IF-ANA the guiding light for SLE classification in the real-world setting?

Gualtierotti et al *Autoimmun Rev* doi: 10.1016/j.autrev.2022.103138

Longitudinal analysis of ANA in the Systemic Lupus International Collaborating Clinics (SLICC) Inception Cohort

Choi MY et al. *Ann Rheum Dis* 2022;**81**:1143–1150.

Almost all SLE patients (n. 805) early in disease had highly positive ANAs and no patients had tested within the normal range over 5 years of follow-up with all three assays. As the disease evolved over 5 years of follow-up, there was decreased frequency of positive ANAs (above the normal range) and decreased ANA titres or absorbance units by some assays.

False negative and false positive ANA: two sides of the same coin

- **Assay variation in ANA detection in patients with established SLE**

Pisetsky PS et al. ARD 2018

ANA negativity occurs in patients with established SLE though the frequency varies depending on the assay kit. Given the range of negativity with well-validated assays, these findings raise question about whether ANA positivity should be employed to determine eligibility for clinical trials.

- **The clinical utility of a positive ANA test result**

Abeles AM and Abeles M. Am J Med 2013

In this retrospective study, more than 90% of patients who were referred to a tertiary rheumatology clinic for a positive ANA test result had no evidence for an ANA-associated rheumatic disease. The poor predictive value of a positive ANA in this cohort was largely attributable to unnecessary testing in patients with low pretest probabilities for ANA-associated rheumatic disease.



ANA usefulness for AARDs: to be questioned?

Table 1 Antinuclear antibodies (ANA) in systemic lupus erythematosus (SLE) by immunofluorescence (IFA) and solid phase immunoassay

Reference	No of SLE patients	IFA (% SLE patients positive)	Solid phase assay (% SLE patients positive)	Solid phase assay method
9	55	91% (1:80)*	87% 89% 78%	Radim SpA EIA Zeus EIA VarElisa ReCombi (Pharmacia)
11	53	91% (> 1:50)	49%	Athena Multilyte
16	71 (including SLE, DLE, drug-induced)	98% (1:40)	91%	RADIAS (Bio-Rad)
17	34	76% (> 1:160)	62%	ELIA Pharmacia
19	202	87%	75%	VarElisa ELISA
30	50	84% at 1:50 80% at 1:100 76% at 1:200	40% 56%	AntiNucleosomes GmbH QUANTA Lite (INOVA)
28	38	92%	79%	QUANTA Lite (INOVA)
32	192	99% (81%)†	75.5%	Bioplex
33	35	97% (> 1:160)	100% 94% 100% 60% 62%	Quanta Life Bio-Rad Relisa VarElisa UniCap

*Indicates the dilution used if reported in the paper.

†Two different percentages are reported in the paper.

Task Force: EULAR recommendations for the use and interpretation of laboratory diagnostic tests for the management of systemic autoimmune rheumatic diseases

Chairpersons: P.L. Meroni (Italy), J. Rönnelid (Sweden)

Members: X. Mariette (France), D. Boumpas (Greece), P. Migliorini (Italy), Y. Shoenfeld (Israel), T. Dorner (Germany), G. Steiner (Austria), I. Damoiseaux (The Netherlands), X. Bossuyt (Belgium), I. Sheldon (UK), P.

**Executive Committee: PLM, JR, CBC, MB, FB & librarians of the Univ
Milan evaluated 5,438 records from the literature (2010- spring 2024)**

Health Professional Representative : D. Quarry (Italy),

Fellows for the metanalysis : (C. Chighizola, Italy —M. Brink, Sweden).

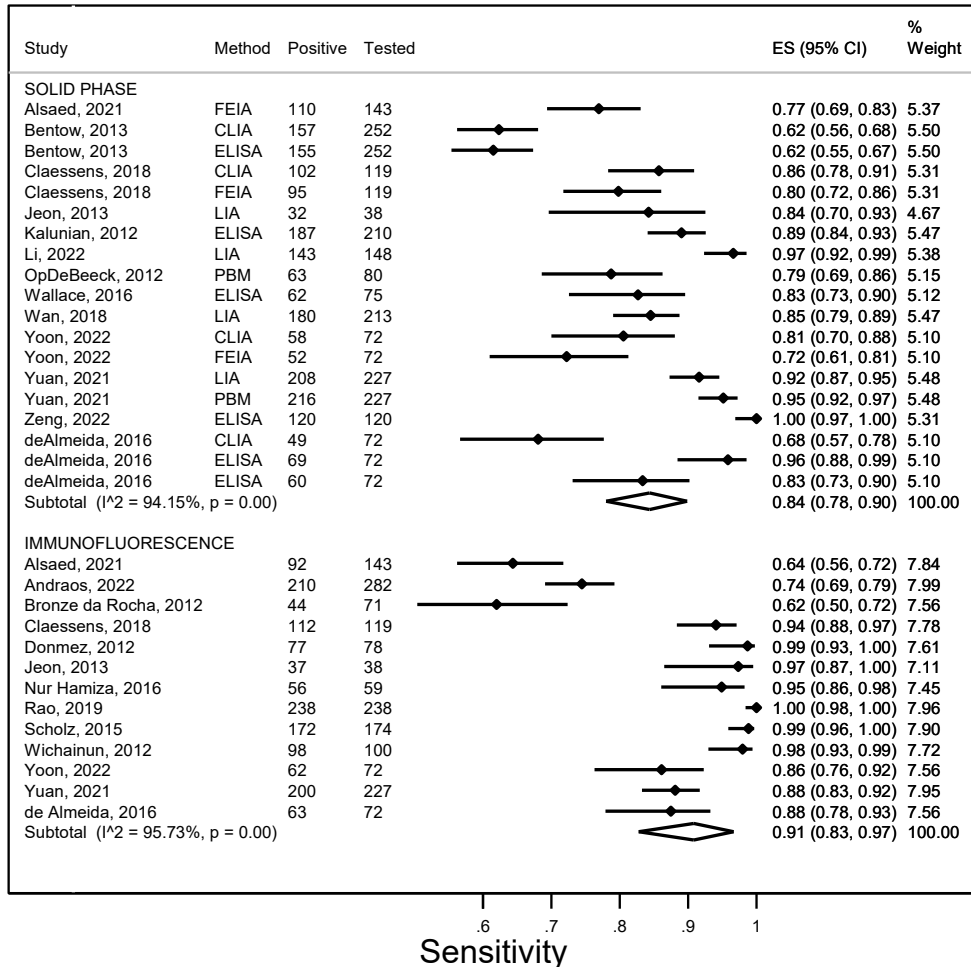
EMEUNET representatives : JD. Pauling (UK) k. Lakota (Slovenia) .

Epidemiologist: P. Machado (UK).

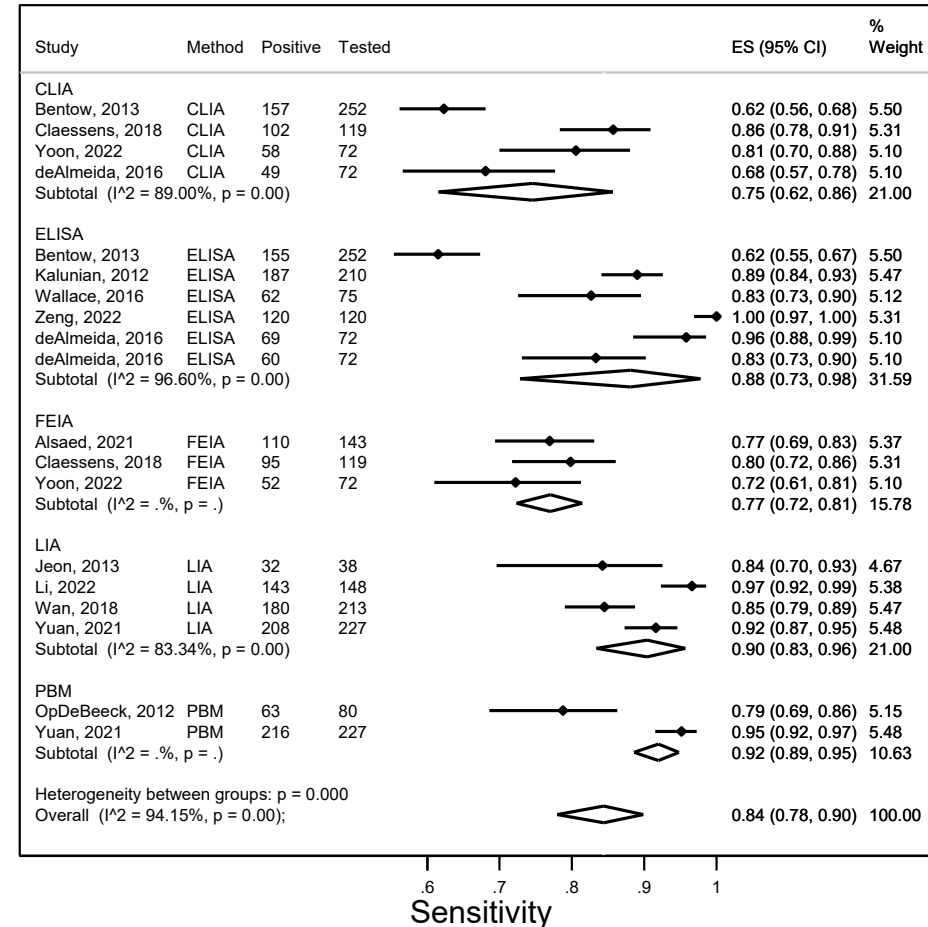
Statisticians: C. La Vecchia (Italy), F. Bravi (Italy).

Forest plots – ANA in SLE

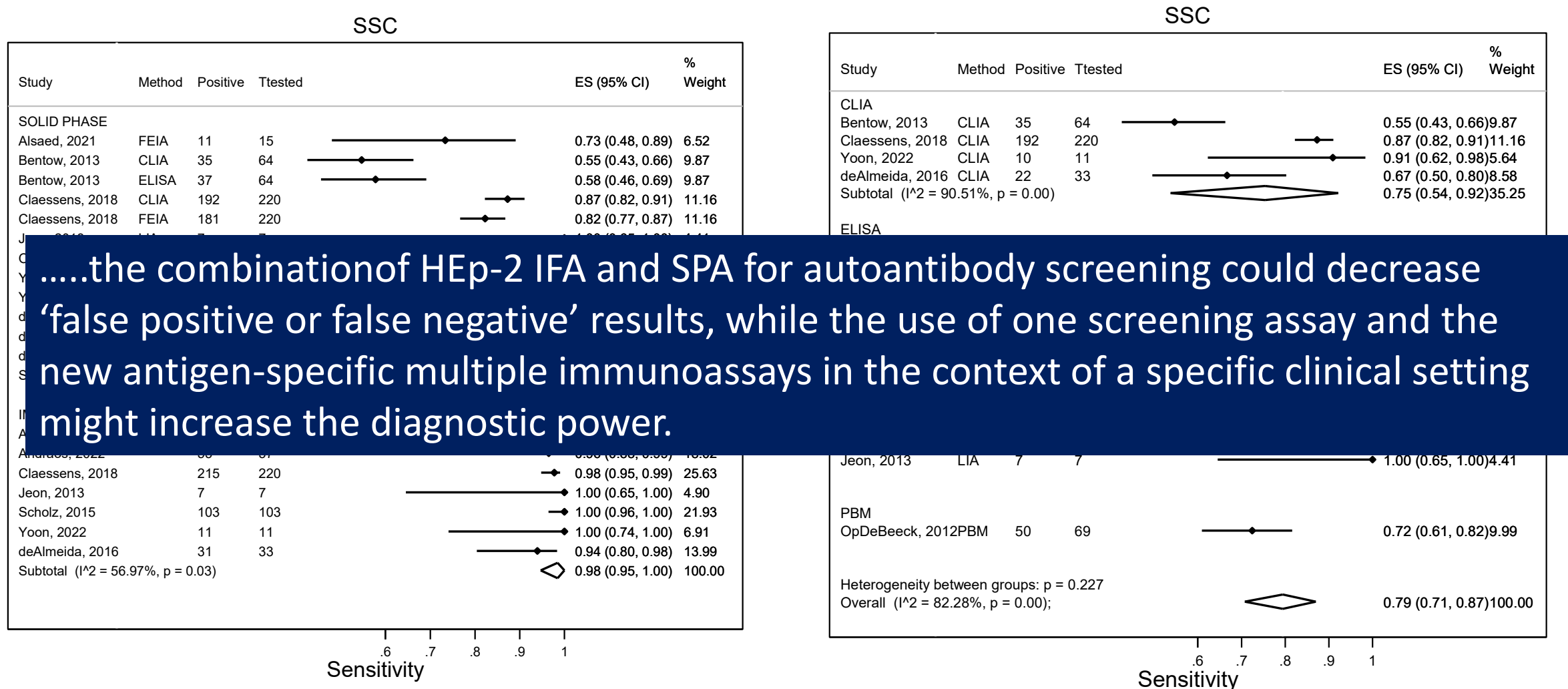
Systemic lupus erythematosus



Systemic lupus erythematosus



Forest plots – ANA in Systemic sclerosis



Antinuclear antibodies interpretation: utmost questions

HEp-2 IIF: i) is a single cut-off value informative enough to interpret test results?

ii) can the staining pattern inform about diagnosis?

iii) is there any relationship between titre and pattern?

SPAs: i) how to choose the best cut-off for ANA screening? (specificity vs sensitivity)

ii) are the different SPAs comparable in term of test performance variability?

iii) do all the available SPAs clinically perform similarly in the screening of individual ANA-associated rheumatic disease?

HEp-2 IIF vs SPAs: is the clinical performance of one method superior to the other one?

HEp-2 IIF Performance

ANA titre

The higher the ANA titre, the higher likelihood of having disease

Dicothomous interpretation (pos/neg) with a single cut-off >>>>> loss of information

Effect on positive predictive value (PPV) of the test for ANA-associated rheumatic diseases

Titer-specific likelihood ratio increases the accuracy
in interpreting HEp-2 IIF test results

HEp-2 IIF: Take-home messages

- When interpreting HEp-2 IIF test results, clinicians should consider
 - ✓ both antibody titre (or LIU for automated IIF), including titre-specific likelihood ratio
 - ✓ and the fluorescence pattern

[International Consensus on ANA Patterns (ICAP) www.anapatterns.org]
- A positive HEp-2 IIF test result should be confirmed by an antigen-specific assay
- A negative HEp-2 IIF test result should be considered with caution, owing to the low abundance of some antigens (e.g. Ro60, Ro52) and/or variability of the substrate preparation
- An alternative diagnostic assay should be required if a disease associated with these antibodies is suspected

Methods for ANA detection

HEp-2 Indirect Immunofluorescence (IIF)



Screening solid phase assays (SPAs)

- All cell components as potential antigens
- Evaluation by fluorescence microscopy
- High variability/Low specificity
- Interpreting results
 - ✓ Titre: 1:160 cutoff 95th percentile
(*Tan et al., Arthritis Rheum 1997*)
 - ! neg result does not exclude ANA-associated SARD
 - 1:80 sensitive enough for SLE classification
low specificity
- ✓ Pattern: nuclear, cytoplasmic, mitotic
(ICAP nomenclature AC-0 > AC-29 *being updated*)
- Clinically relevant patterns may not be detected
(e.g. Jo-1, ribosomal P, Ro60, Ro52, Ku, ThTo)
- «Gold standard» - ACR recommendations
(*Meroni & Schur, ARD 2010*)

- Mixture of relevant antigens
- Different (automated) technologies
 - ✓ ELISA colorimetry
 - ✓ FEIA fluorescence
 - ✓ CLIA chemiluminescence

} Screening
Ag specific follow up

 - ✓ Line/Dot IA blot/enzyme
 - ✓ ALBIA beads/flow cytometry
 - ✓ PMAT particles/light-emitting diode

} Multiplexed
Specific Abs identifiable
- High reproducibility/High specificity
- Interpreting results
 - ✓ Cutoff points determined by manufactures

Unending story of the indirect immunofluorescence assay on HEp-2 cells: old problems and new solutions?
(*Meroni PL et al, ARD 2018*)

SPA Performance

- Differences of performance between the assays is often due to the choice of the cut-off
- ELISAs show the highest variability due to the difference in the coating antigens (e.g. cell extracts or a mixture of different antigens) and in the kit set-up manufacturer

- For screening for ANA-associated rheumatic diseases

SPA (FEIA, CLIA and ALBIA) vs HEp-2 IIF lower sensitivity/higher specificity

- For screening for individual ANA-associated rheumatic disease

SLE
SPA **less sensitive**
than IIF

SSc
SPA **less sensitive**
than IIF

SS
SPA **more sensitive**
than IIF

Controls
SPA **more specific**
than IIF

Low sensitivity for IIM (IIM-specific autoabs not included in screening SPA)

SPA: Take-home messages

- When interpreting ANA screening test results, clinicians should consider that SPAs are
 - ✓ assay-dependent
 - ✓ disease-dependent
- As different assays contains different autoantigens, clinicians should be aware of which antigens are included in the SPA used
- A screening assay should be followed by an antibody-specific assay
- No single cut-off is associated with a good sensitivity and a good specificity
- Test-result specific likelihood ratio might help to interpret results

Which assay for which disease?

- Neither HEp-2 IIF nor SPA is superior to each other
- A test with a high sensitivity (e.g. IIF with cut-off 1:80) helps to exclude disease
- A test with a high specificity (e.g. FEIA or IIF with a high cut-off/threshold) helps to confirm disease
- Neither Hep-2 IIF nor SPA by itself can identify all the AARD patients
- IIF might detect relevant antibodies that are undetected by SPA, and viceversa
- IIF is largely variable among laboratories
- Test result-specific LRs can allow better interpretation and harmonization of test results
- Combining IIF with SPA has the highest clinical value

Optimal test strategy

SLE

IIF

Nuclear homogeneous

Nuclear speckled

Cytoplasmic fine speckled
PCNA-like

High sensitivity

SPA

dsDNA

Sm
SSA
U1-RNP
Rib-P
PCNA

IIF has a high sensitivity, but low specificity.
Combining IIF with SPA is the optimal strategy.

SS

IIF

Nuclear fine speckled

Cave, might miss some anti-SSA antibodies

SPA

SSA
SSB

SPA for SSA (and SSB) has a good sensitivity.

MCTD

IIF

Nuclear large speckled

SPA

U1-RNP

SSc

IIF

Centromere
TOPO-like
Nuclear speckled

Nucleolar

High sensitivity

SPA

Centromere
Topoisomerase I
Polymerase III

Fibrillarin
Th/To
NOR90
PM/Scl (overlap)

U11/U12 RNP
BICD2

High sensitivity

IIF is a good screening assay.
Specific antibodies cover majority of SSc-specific antibodies, but not all.
Check whether specific antibody fits with IIF pattern.

IIM

IIF

Cytoplasmic
(dense) fine speckled

Nuclear speckled

Nuclear multiple dots

Cave:
limited sensitivity

SPA

Anti-synthetase
Jo-1, PL-7, PL-12, OJ, EJ, ..
SRP

HMGR

Mi-2
TIF-γ
Ku (overlap)

NXP-2

MDA-5
SAE

Cave:
False positives with some assays.
Does not represent full spectrum
Check whether antibody fits with IIF pattern.

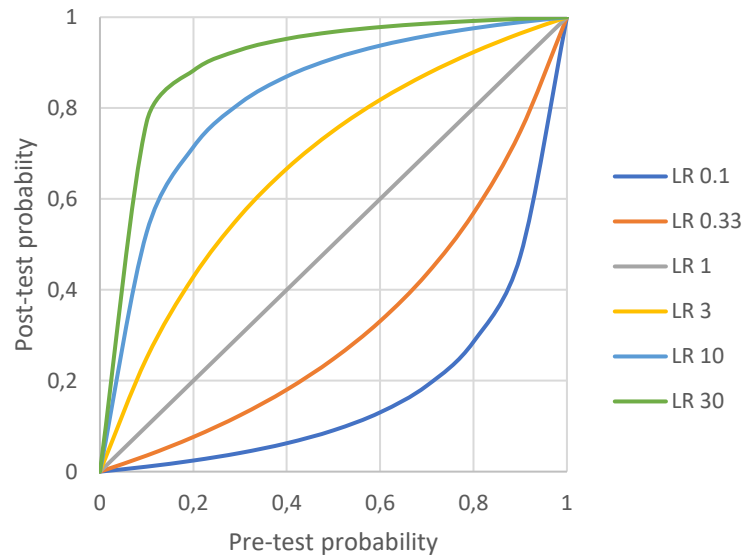
Optimal strategy: in case of clinical suspicion: SPA, but beware of weak false positives with some assays.
Available assays do not cover full antibody spectrum.
Check whether specific antibody fits with IIF pattern.

Conclusions

When interpreting ANA screening results, clinicians should be aware that:

- different methods are available to detect ANA in AARDs
- none of the methods is superior to the others
- none of the methods can identify all AARD patients
- IIF and SPAs display clinically relevant differences in performance
 - ✓ their combination has the highest value
- the performance of IIF and SPAs is disease-dependent
 - ✓ the optimal test strategy depends on the target disease and the pre-test probability
- the differences among the methods have to be kept in mind when the tests are used for classifying rather than diagnosing patients
- A dichotomous interpretation of the test results loses information
- Reporting the test result-specific LR might help both to better interpret and to harmonize the results

- ✓ Pre-test probability: probability of having a particular disease for an individual before the test
- ✓ Post-test probability: probability of having a disease in a patient with a positive test result
- ✓ Odds: probability of having a particular disease / probability of not having the disease



$$\text{Pre-test odds} = \frac{\text{pre-test-probability}}{(1 - \text{pre-test probability})}$$

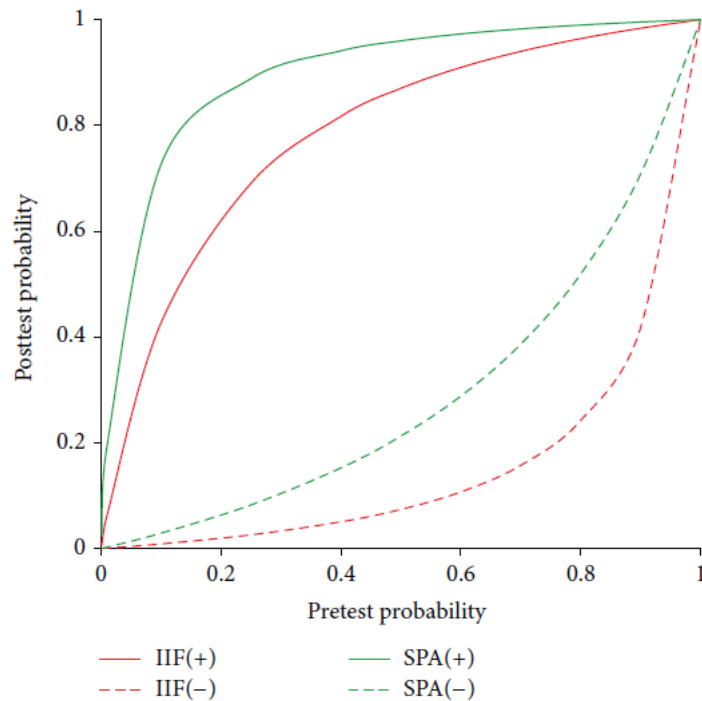
$$\text{Post-test odds} = \text{pre-test odds} * \text{LR}$$

$$\text{Post-test probability} = \frac{\text{post-test odds}}{(1 + \text{post-test odds})}$$

1) Young lady with hair loss and polyarthralgias pre-test probability for SLE 1% (= 0.01)

2) Young lady with photosensitivity and mild Leucopenia (3000-3500 WBC/mm³) pre-test probability for SLE 10% (= 0.1)

3) Young lady with photosensitivity, malar rash and simmetrical polyarthritis pre-test probability for SLE 50% (= 0.5)



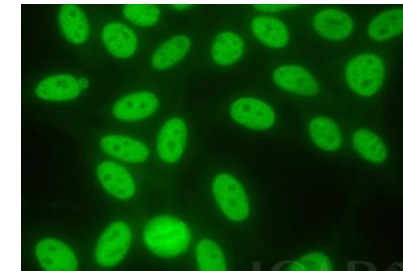
	LR+	LR-
IIF	7	0.03
FEIA	24	0.27

[Bossuyt & Fiewu, ARD 2014]

$$\begin{aligned} \text{IIF+} & \quad \frac{0.01}{(1 - 0.01)} * 7 = 0.07 > \frac{0.07}{(1 + 0.07)} = 0.066 \\ \text{FEIA+} & \quad \frac{0.01}{(1 - 0.01)} * 24 = 0.24 > \frac{0.24}{(1 + 0.24)} = 0.19 \end{aligned}$$

	Post-test probability			
Pre-test probability	IIF+*	IIF-	FEIA+	FEIA-
1%	6%	0.08%	19%	0.20%

*HEp-2 nuclear pattern fine speckled (ICAP AC-4) suggestive of anti-Ro abs



1) Young lady with hair loss and polyarthralgias

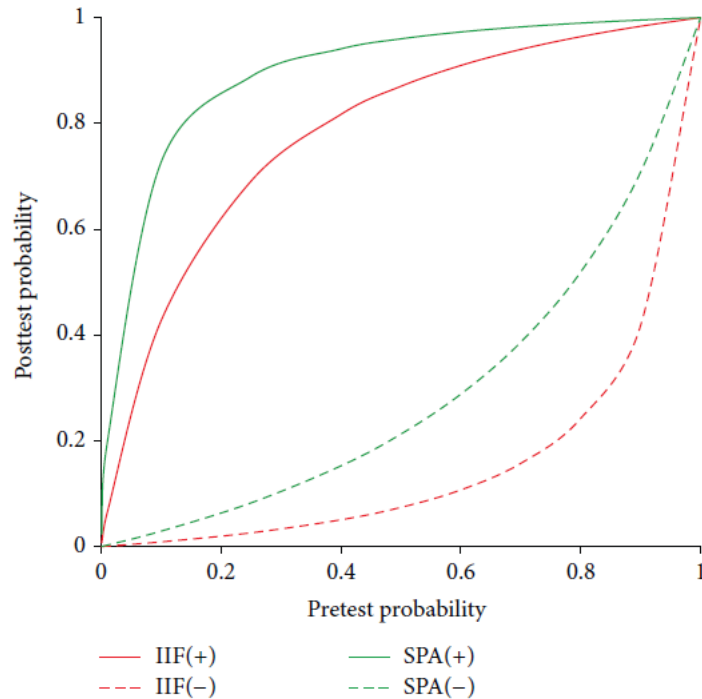
pre-test probability for SLE 1% (= 0.01)

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3) Young lady with photosensitivity, malar rash and simmetrical polyarthritis

pre-test probability for SLE 50% (= 0.5)



	LR+	LR-
IIF	7	0.03
FEIA	24	0.27

$$\text{IIF+} \quad \frac{0.1}{(1 - 0.1)} * 7 = 0.77 > \frac{0.77}{(1 + 0.7)} = 0.43$$

$$\text{FEIA+} \quad \frac{0.1}{(1 - 0.1)} * 24 = 2,6 > \frac{2,6}{(1 + 2,6)} = 0.72$$

	Post-test probability			
Pre-test probability	IIF+	IIF-	FEIA+	FEIA-
1%	6%	0.08%	19%	0.20%
10%	43%	0.80%	72%	3%

*HEp-2 nuclear pattern FS (ICAP AC-4) suggestive of anti-Ro

1) Young lady with hair loss and polyarthralgias

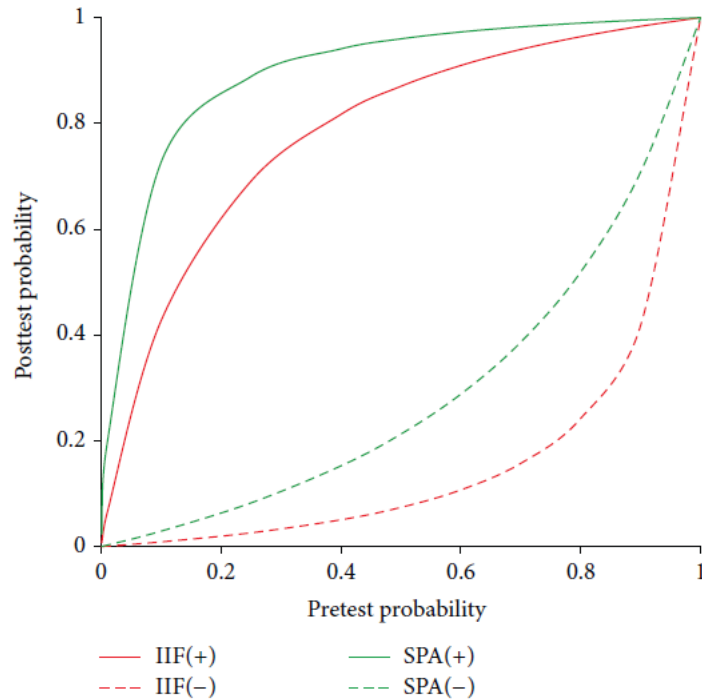
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pre-test probability for SLE 50% (= 0.5)



	LR+	LR-
IIF	7	0.03
FEIA	24	0.27

$$\text{IIF+} \quad \frac{0.5}{(1 - 0.5)} * 7 = 3.5 > \frac{3.5}{(1 + 3.5)} = 0.78$$

$$\text{FEIA+} \quad \frac{0.5}{(1 - 0.5)} * 24 = 12 > \frac{12}{(1 + 12)} = 0.92$$

	Post-test probability			
Pre-test probability	IIF+	IIF-	FEIA+	FEIA-
1%	6.6%	0.08%	19%	0.20%
10%	43%	0.80%	72%	3%
50%	78%	7%	92%	21%

*HEp-2 nuclear pattern FS (ICAP AC-4) suggestive of anti-Ro

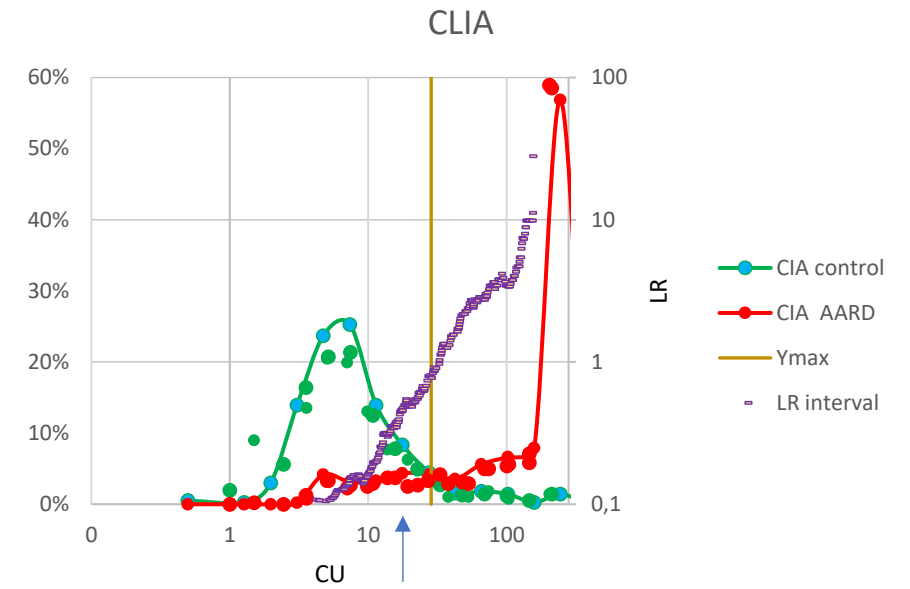
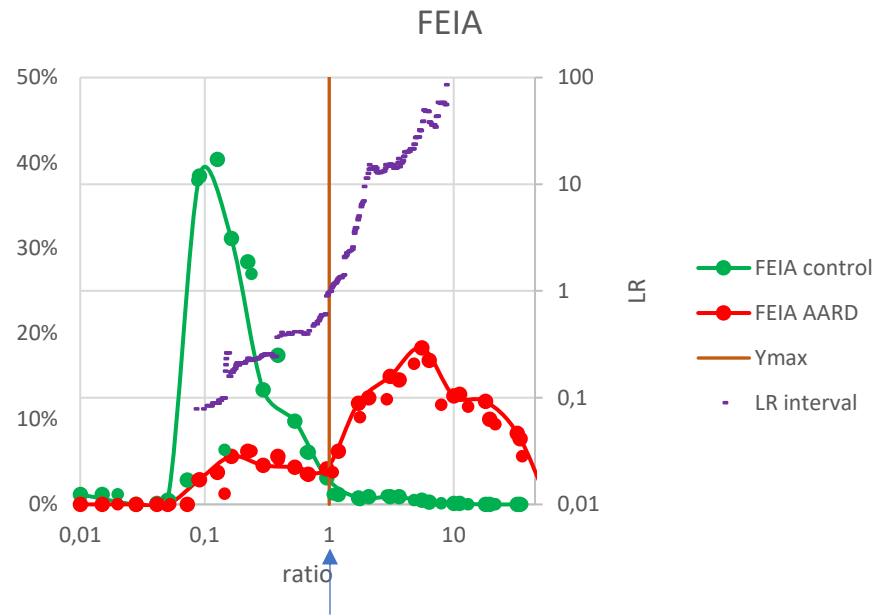
Likelihood ratio

$$\text{Likelihood ratio} = \frac{\text{fraction of patients with certain result}}{\text{fraction of controls with certain result}}$$

$$\text{Likelihood ratio} = \frac{2\% \text{ of SLE patients have titer 1:80}}{11,5 \% \text{ of controls have titer 1:80}} = \mathbf{0.17} \text{ (1:80 titer-specific LR)}$$

$$\text{Likelihood ratio} = \frac{9,8 \% \text{ of SLE patients have titer 1:160}}{10,4\% \text{ of controls have titer 1:160}} = \mathbf{0.94} \text{ (1:160 titer-specific LR)}$$

$$\text{Likelihood ratio} = \frac{86\% \text{ of SLE patients have titer 1:320 or higher}}{3.4 \% \text{ of controls have titer 1:320 or higher}} = \mathbf{25.3}$$

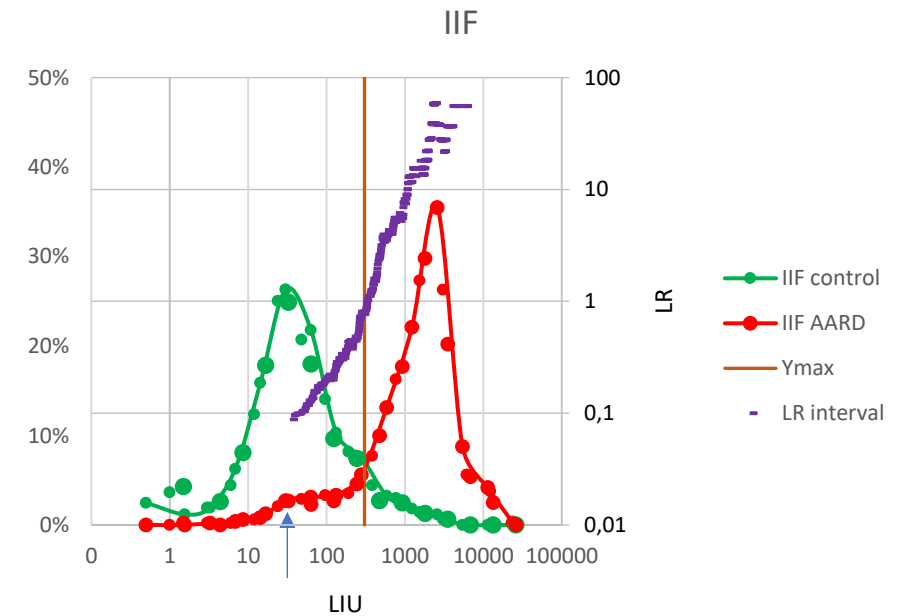


Test result-specific LR helps with interpretation of automated IIF, FEIA and CLIA

High positive LR (>10) best for confirming a diagnosis

Low negative LR (<0.1) best for excluding the disease

LR = 1 test results help neither to exclude nor to confirm the disease



the combination of HEp-2 IFA and SPA for autoantibody screening could decrease 'false positive or false negative' results, while the use of one screening assay and the new antigen-specific multiplex immunoassays in the context of a specific clinical setting might increase the diagnostic power.

- **Unending story of the indirect immunofluorescence assay on HEp-2 cells: old problems and new solutions?**

Meroni PL et al, ARD 2018

- **Are IF-ANA the guiding light for SLE classification in the real-world setting?**

Gualtierotti R et al. Autoimmun Rev 2022

IF-ANA detected by HEp-2 cells appear a reliable and stable marker of the disease in a real-world cohort of SLE patients with and without renal involvement and during a long follow-up.

Analysis ANA

Systemic lupus erythematosus

	<u>Sensitivity</u>	<u>Specificity</u>	Positive likelihood ratio	Negative likelihood ratio		<u>Specificity</u>	Positive likelihood ratio	Negative likelihood ratio		<u>Specificity</u>	Positive likelihood ratio	Negative likelihood ratio
			vs healthy controls				vs NAD				vs other AD	
	Estimate (95% confidence interval) n of studies											
<u>Immunofluorescence</u>	0.91 (0.83-0.97) n=13	0.88 (0.84-0.92) n=12	7.58* - -	0.10 ⁺ - -		0.80 (0.75-0.85) n=8	4.55* - -	0.11 ⁺ - -		0.60 (0.44-0.74) n=8	2.28* - -	0.15 ⁺ - -
Solid phase	0.84 (0.78-0.90) n=19	0.93 (0.91-0.96) n=13	13.47 (9.41-19.3) n=13	0.16 (0.10-0.25) n=13		0.89 (0.85-0.93) n=12	8.40 (5.76-12.25) n=12	0.18 (0.12-0.27) n=12		0.76 (0.64-0.86) n=14	4.44 (3.15-6.26) n=12	0.25 (0.20-0.32) n=12
CLIA	0.75 (0.62-0.86) n=4	0.94 (0.92-0.95) n=3	12.5* - -	0.27 ⁺ - -		0.94 (0.87-0.98) n=3	12.5* - -	0.27 ⁺ - -		0.90 (0.86-0.94) n=4	6.0 (4.14-8.74) n=4	0.28 (0.20-0.41) n=4
ELISA	0.88 (0.73-0.98) n=6	0.94 (0.90-0.97) n=3	14.67* - -	0.13 ⁺ - -		0.83 (0.78-0.88) n=2	5.18* - -	0.14 ⁺ - -		0.74 (0.44-0.95) n=4	3.38* - -	0.16 ⁺ - -
FEIA	0.77 (0.72-0.81) n=3	0.95 (0.87-0.99) n=3	15.4* - -	0.24 ⁺ - -		0.96 (0.92-0.98) n=2	19.25* - -	0.24 ⁺ - -		0.93 (0.81-1.00) n=2	11.0* - -	0.25 ⁺ - -
LIA	0.90 (0.83-0.96) n=4	0.87 (0.82-0.92) n=2	6.92* - -	0.11 ⁺ - -		0.85 (0.75-0.94) n=3	6.0* - -	0.12 ⁺ - -		0.67 (0.62-0.72) n=2	2.73* - -	0.15 ⁺ - -
<u>PBM</u>	0.92 (0.89-0.95) n=2	0.93 (0.89-0.95) n=2	13.14* - -	0.09 ⁺ - -		0.88 (0.85-0.91) n=2	7.67* - -	0.09 ⁺ - -		0.53 (0.46-0.59) n=2	2.0* - -	0.15 ⁺ - -
Solid phase – LIA, PBM	0.82 (0.73-0.89) n=13	0.94 (0.91-0.97) n=9	14.76 (9.81-22.21) n=9	0.18 (0.10-0.34) n=9		0.91 (0.85-0.96) n=7	9.62 (5.33-17.37) n=7	0.27 (0.20-0.35) n=7		0.83 (0.69-0.93) n=10	4.82* - -	0.22 ⁺ - -
Solid phase – LIA	0.83 (0.75-0.89) n=15	0.94 (0.91-0.96) n=11	13.99 (10.13-19.31) n=11	0.17 (0.10-0.29) n=11		0.90 (0.86-0.94) n=9	9.04 (5.86-13.94) n=9	0.22 (0.16-0.32) n=9		0.78 (0.64-0.90) n=12	5.42 (3.95-7.44) n=10	0.25 (0.19-0.34) n=10

*not directly estimable because there were <4 studies reporting both sensitivity and specificity; calculated as raw ratio between sensitivity and (1-specificity); ⁺ not directly estimable because there were <4 studies reporting both sensitivity and specificity; calculated as raw ratio between (1-sensitivity) and specificity

Systemic lupus erythematosus vs all other diseases

	<u>Sensitivity</u>	<u>Specificity</u>	Positive likelihood ratio	Negative likelihood ratio
	Estimate (95% confidence interval) n of studies			
Anti-SSA/Ro60	0.38 (0.26-0.51) n=5	0.88 (0.47-0.99) n=5	3.28 (0.64-16.70) n=5	0.70 (0.63-0.79) n=5
Anti-SSA/Ro52	0.29 (0.19-0.41) n=6	0.69 (0.56-0.80) n=6	0.93 (0.72-1.20) n=6	1.03 (0.92-1.15) n=6
Anti-SSA60_52	0.46 (0.38-0.55) n=5	0.66 (0.38-0.89) n=4	1.35* - -	0.82+ - -
Anti-SSB/La	0.18 (0.12-0.25) n=5	0.84 (0.79-0.88) n=5	1.11 (0.78-1.56) n=5	0.98 (0.91-1.05) n=5
Anti-U1snRNP	0.28 (0.22-0.34) n=2	0.83 (0.78-0.88) n=1	1.65* - -	0.87+ - -
Anti-Sm	0.18 (0.14-0.23) n=6	0.98 (0.95-0.99) n=6	9.11 (3.16-26.23) n=6	0.84 (0.78-0.90) n=6

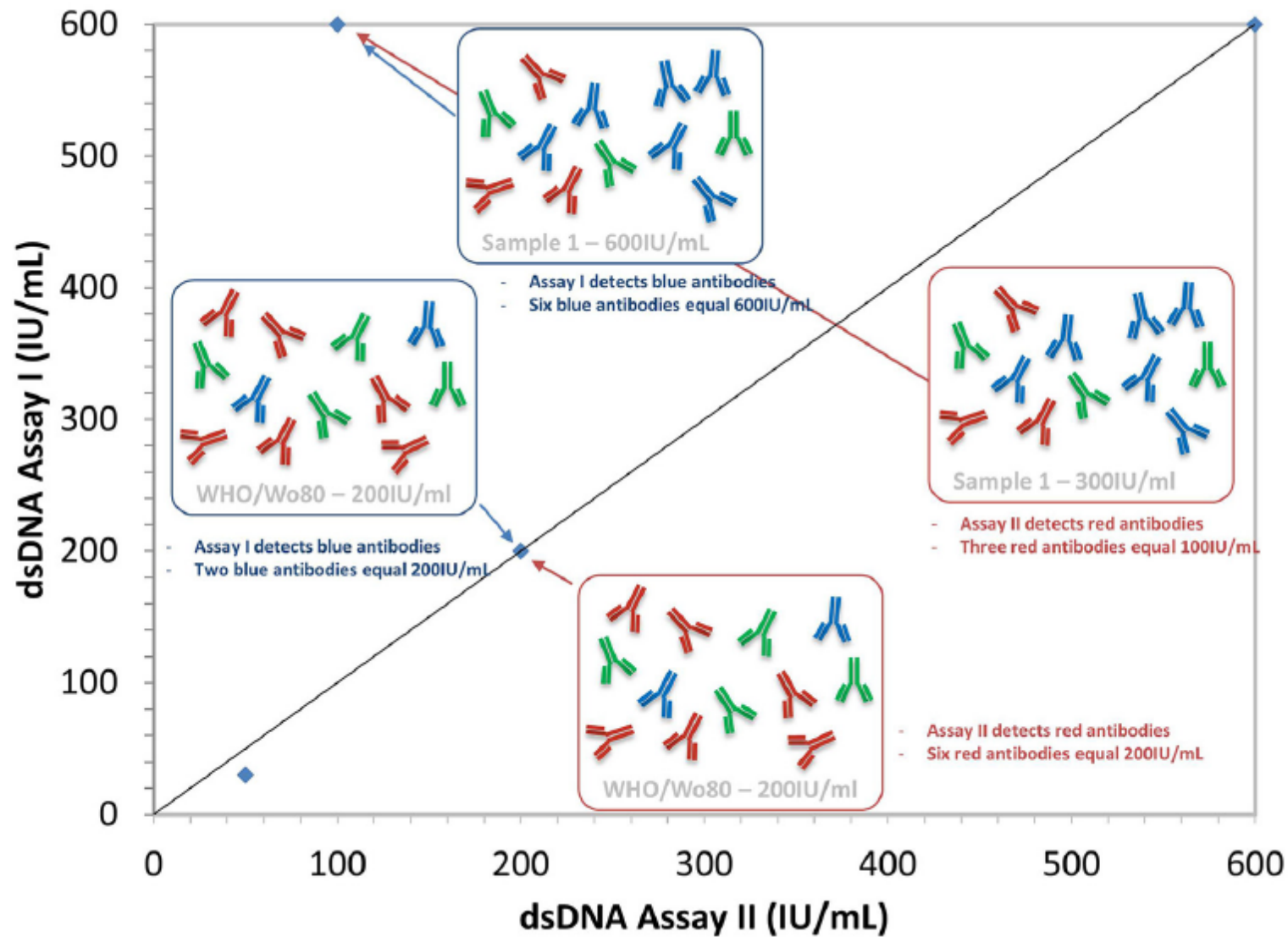
*not directly estimable because there were <4 studies reporting both sensitivity and specificity; calculated as raw ratio between sensitivity and (1-specificity); + not directly estimable because there were <4 studies reporting both sensitivity and specificity; calculated as raw ratio between (1-sensitivity) and specificity

Autoantibody Snapshot

SLE	Systemic Sclerosis	Sjögren's Syndrome	AI Myopathy	Vasculitis
dsDNA chromatin Ribosomal P, C1q	CENP-A,-B (25-40) Topo I (15-42) RNA Pol (5-31)	SS-A/Ro 60 SS-B/La M3 receptor	Jo-1 + other aa tRNA synthetases	PR3 MPO

Several nuclear Ag > ANA few SLE specific :
anti-Sm>> Anti-RibP > anti-dsDNA

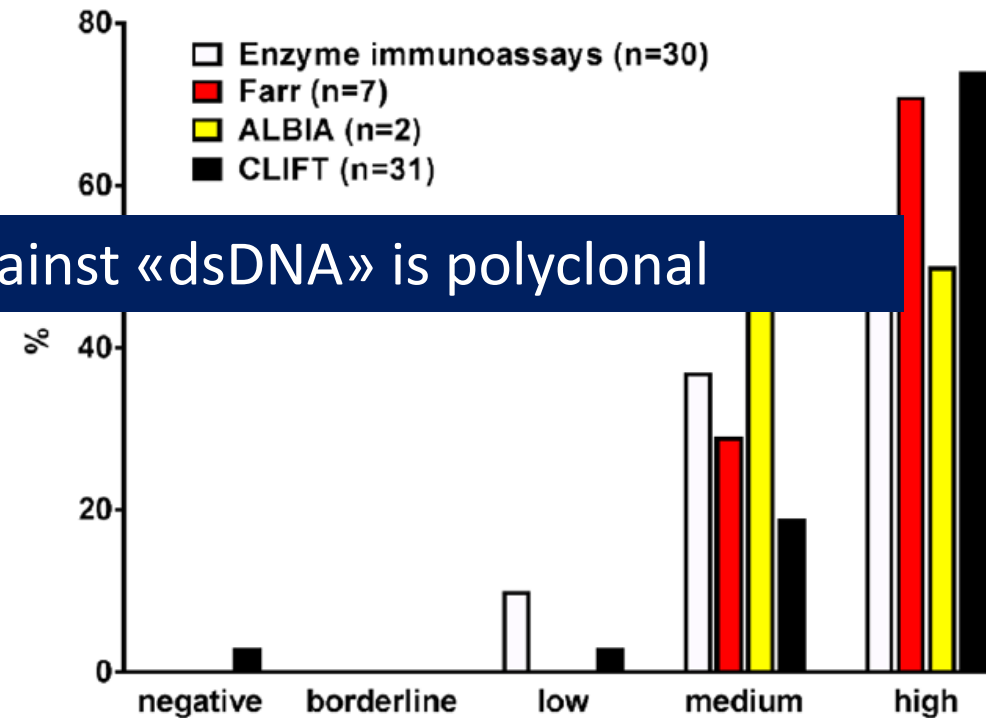
SS-A/Ro 60 Cardiolipin β-2 GP1	U11/U12 RNP (3-4) Ku (2-4)	α-fodrin ?	HMGCR	Azurocidin Lactoferrin
NMDA/NR 2 receptor Phospholipase A2R	NOR90 (<5) B23/numatrin (?) Exosome (?)	NuMA TS-1 RNA	SRP MDA-5	Defensins EEA-1
TRIM21/Ro52	TRIM 21/Ro52 (20)	TRIM21/Ro52	TRIM21/Ro52	? — ?
 DFS70	 DFS70 (1-3)	 DFS70	 DFS70	 DFS70



Lack of correlation of anti-dsDNA antibody assays. The different a-dsDNA populations in the Wo/80 and patient sample are depicted as red, green and blue antibodies. Assay 1 detects the blue antibody population while assay 2 detects the red antibody population. Both assays give 200 IU/ml for the Wo/80 but very different results for the patient sample. doi.org/10.1016/j.jim.2018.05.014

Table 1 Laboratory methods	
Laboratory code	Method
1,4,5,6,8,9,11,13,14,16,18,20,3,20,4,21,22,26,28,30,33,35,36	<i>Crithidia luciliae</i> immunofluorescence test (CLIFT)
Enzyme-linked immunosorbent assays (ELISA), chemiluminescence immunoassays (CIA) and fluoroenzyme immunoassays (FEIA)	
2,4,6,7,10,11,22,23,25,28,30	Phadia ELiA FEIA
3	Eurodiagnostica ELISA
9.2	Trinity Biotech Captia ELISA
9.3	Immco ELISA
12,17, 33	Orgentec ELISA
13	Immunoconcepts ELISA
14	
16	
18.1	Theradiag ELISA
19, 29.4	Innova Quanta Flash CIA 701178
20.1	Euroimmun anti-dsDNA ELISA
20.2	Euroimmun anti-dsDNA NcX ELISA
26	Orgentec Alegria ELISA
27.2	Bio-Rad Kallestad EIA
27.3, 34.1	Bio-Rad ELISA
29.1	Inova Quanta Lite ELISA 708510
29.2	Inova Quanta Lite HA ELISA 704615
29.3	Inova Quanta Lite dsDNAC ELISA 704650
31	Alpha Diagnostica ELISA
Addressable laser bead immunoassays	
1, 8, 27.1, 32, 35	Bioplex 2200
18.2	Theradiag FIDIS dsDNA MX005
18.3, 21	Theradiag FIDIS Connective Profile MX117
24	Zeus Athena
Farr immunoassays	
15	IBL International dsDNA Farr RIA
9.1, 34.2, 36	Trinity Farr RIA

The response against «dsDNA» is polyclonal



Fox BJ et al Ann Rheum Dis 2019;78:1677–1680

What the clinicians ask about when they have to do with ANA test results?

The key question:

“What is the probability of a patient having a particular disease
when the laboratory test is positive

or

not having the disease when the laboratory test is negative?”

The key issue: how to best interpret ANA test results?



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From autoantibody test results to decision making: Incorporating likelihood ratios within medical practice

Kevin D. Deane^{a,1}, Lieve Van Hoovels^{b,c,1}, Veena E. Joy^d, Nina Olschowka^d,
Xavier Bossuyt^{c,e,*}

The likelihood ratio

- ✓ gives information on how much more/less likely a particular test result is in patients compared to individuals without the disease
- ✓ incorporates information on antibody level (higher the level > higher the association with the disease)
- ✓ is unit-independent and helps to harmonize test result interpretation
- ✓ combining LR with pre-test probability, post-test probability can be evaluated

LR challenges for clinicians

- ✓ pre-test probability not often developed
- ✓ primary care physicians may be less confident of forming an initial diagnosis than trained rheumatologists
- ✓ LR is not often reported on a lab test result
- ✓ different lab test may have different LRs because of the cutoff and the features of the test

ANA interpreting, beyhond the basis

- LR can aid Rheumatologists in diagnosing a disease
- Large-scaled multicenter studies are needed to determine LRs
- Individual laboratories cannot deal with LR calculation
- A collaboration among clinical researchers, laboratory people and diagnostics companies is essential

ANA interpreting, looking forward

Machine Learning Identifies Clusters of Longitudinal Autoantibody Profiles Predictive of Systemic Lupus Erythematosus Disease Outcomes

Choi et al. Ann Rheum Dis. 2023

A novel longitudinal clustering technique was applied to comprehensive autoantibody data from a large, well-characterized, multinational inception systemic lupus erythematosus (SLE) cohort to determine profiles predictive of clinical outcomes.

Could machine-learning be the future?

Outline

- ANA usefulness
- ANA detection methods
- Which assay for which disease
- Likelihood ratio
- Clinical examples
- Conclusions

False negative and false positive ANA

Should usefulness for AARDs be questioned?



- **ANA negativity in patients with established SLE**
Should ANA positivity be employed to determine eligibility for clinical trials?
[Pisetsky et al. ARD 2018]
- **>90% of patients with an ANA positive test had no evidence of AARD**
Unnecessary testing in patients with low pre-test probability of AARD?
[Abeles AM and Abeles M. Am J Med 2013]

- **IIF on HEp-2: old problem and new solutions?**
Combining methods for ANA screening could decrease false positive or false negative test results
[Meroni et al. ARD 2018]
- **ANA detection by IIF and SPA**
Combining methods adds clinical value to the routine investigation of patients with suspected AARD
[Bossuyt et al. ARD 2020]
- **IIF-ANA as the guiding light for SLE classification?**
IIF HEp-2 ANA appear a reliable and stable disease marker in a real-world cohort of SLE patients and during follow-up
[Gualtierotti et al. Autoimmun Rev 2022]
- **ANA test results should be interpreted in the context of the whole laboratory and clinical scenario**
ANA positivity is a significant risk factor for SLE. Avoid ANA testing can lead to the risk of missing AI patients
[Meroni et al. Am J Med 2013]

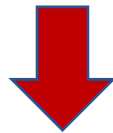
2019 SLE classification criteria

ANA positivity as new entry criterion for SLE classification

- Immunofluorescence on HEp-2 cells with titre $\geq 1:80$
(Se 97.8 - Sp 74.7; *Leuchten N. Arthrtis Care Res 2018*)

or

- A solid phase ANA screening immunoassay with at least equivalent performance



High impact on inclusion in clinical trials

ANA in classification criteria

ANA-associated disease	Autoandibody specificity
Systemic Lupus Erithematosus	dsDNA, Smith (Sm) [Aringer et al. <i>Ann Rheum Dis.</i> 2019]
Sjögren's syndrome	SSA/Ro [Shiboski et al. <i>Ann Rheum Dis.</i> 2017]
Systemic sclerosis	CENPB, topoisomerase I (Scl70), RNA polymerase III [van den Hoogen et al. <i>Ann Rheum Dis.</i> 2013]
Idiopathic Inflammatory Myopathies	Jo-1 (amino-acyl tRNA synthetase) [Lundberg et al. <i>Ann Rheum Dis.</i> 2017]
Juvenile Idiopathic Arthritis(early-onset)	ANA pos $\geq 1:160$ [Martini et al. <i>J Rheumatol.</i> 2019]
<i>Mixed Connective Tissue Disease</i>	<i>U1RNP [Ferrara et al. <i>Immunotargets Ther.</i> 2023]*</i>

**Specific classification criteria for MCTD not yet provided by ACR and EULAR*

Methods for ANA detection

HEp-2 Indirect Immunofluorescence (IIF)

- All cell components as potential antigens
- Evaluation by fluorescence microscopy
- High variability/Low specificity
- Interpreting results
 - ✓ Titre: 1:160 cutoff 95th percentile
(*Tan et al., Arthritis Rheum 1997*)
 - neg result does not exclude ANA-associated SARD
 - 1:80 sensitive enough for SLE classification
low specificity
 - ✓ Pattern: nuclear, cytoplasmic, mitotic
(ICAP nomenclature AC-0 > AC-29 *being updated*)
 - ✓ Computer-aided interpretation
- Clinically relevant patterns may not be detected
(e.g. Jo-1, ribosomal P, Ro60, Ro52, Ku, ThTo)
- «Gold standard» - ACR recommendations
(*Meroni & Schur, ARD 2010*)



Screening solid phase assays (SPAs)

- Mixture of relevant antigens
- Different (automated) technologies
 - ✓ ELISA colorimetry
 - ✓ FEIA fluorescence
 - ✓ CLIA chemiluminescence

} Screening
Ag specific follow up
- ✓ Line/Dot IA blot/enzyme
 - ✓ ALBIA beads/flow cytometry
 - ✓ PMAT particles/light-emitting diode

} Multiplexed
Specific Abs identifiable
- High reproducibility/High specificity
- Interpreting results
 - ✓ Cutoff points determined by manufactures



Should the clinical performance of one method
be superior to the other ones?

HEp-2 IIF performance: ANA titre

The higher the ANA titre, the higher likelihood of having disease

Dicothomous interpretation (pos/neg) with a single cut-off >>>>> loss of information

Effect on positive predictive value (PPV) of the test for ANA-associated rheumatic diseases

Titer-specific likelihood ratio can increase the accuracy
in interpreting HEp-2 IIF test results

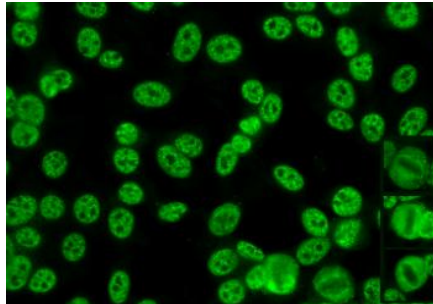
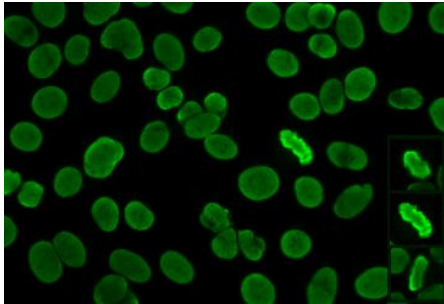
HEp-2 IIF performance: ANA pattern

Different patterns >>>> different clinical value

Classical nuclear patterns

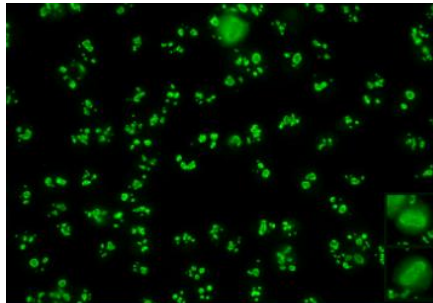
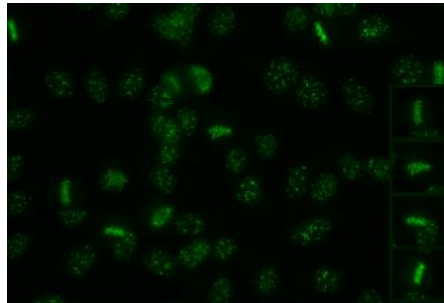
Homogeneous (anti-dsDNA)

Speckled (anti-Ro60, -U1RNP, -Sm)



Centromere (anti-CENPB)

Nucleolar (anti-PM/Scl, -U3RNP)



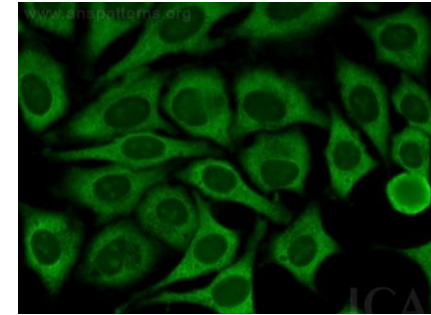
PPV for AARD increases with ANA titre

Centromere - specific for anti-CENPB

- strong association with SSc

Nuclear dense fine speckled

Anti-DFS70

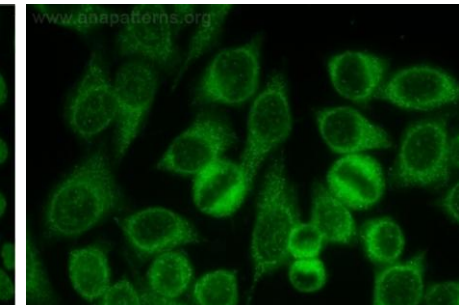
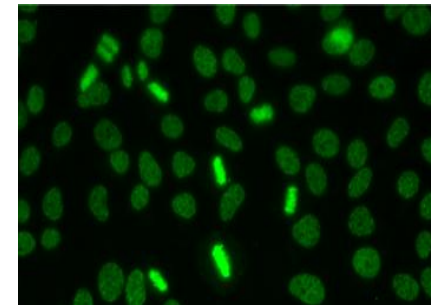


Not associated with AARDs
even at high titre
(as monospecificity)

Cytoplasmic speckled patterns (anti-amino-acyl tRNA synthetase antibodies)

Dense fine speckled (anti-Jo1)

Fine speckled (anti-PL7, -PL12)



Associated with IIM
Detected at low titre
Can be missed

HEp-2 IIF: Take-home messages

- When interpreting HEp-2 IIF test results, clinicians should consider
 - ✓ both antibody titre (or LIU for automated IIF), possibly including titre-specific likelihood ratio
 - ✓ and the fluorescence pattern

[International Consensus on ANA Patterns (ICAP) www.anapatterns.org]
- A positive HEp-2 IIF test result should be confirmed by an antigen-specific assay
- A negative HEp-2 IIF test result should be considered with caution, owing to the low abundance of some antigens (e.g. Ro60, Ro52) and/or variability of the substrate preparation
- An alternative diagnostic assay should be required if a disease associated with these antibodies is suspected

SPA Performance

- Differences of performance between the assays is often due to the choice of the cut-off
- ELISAs show the highest variability due to the difference in the coating antigens (e.g. cell extracts or a mixture of different antigens) and in the kit set-up manufacturer
- For screening for ANA-associated rheumatic diseases as a group
SPA (FEIA, CLIA and ALBIA) vs HEp-2 IIF lower sensitivity/higher specificity
- For screening for individual ANA-associated rheumatic disease

SLE <i>SPA less sensitive than IIF</i>	SSc <i>SPA less sensitive than IIF</i>	SS <i>SPA more sensitive than IIF</i>	Controls <i>SPA more specific than IIF</i>
---	---	--	---

Low sensitivity for IIM (IIM-specific autoabs not included in screening SPA)

SPA: Take-home messages

- When interpreting ANA screening test results, clinicians should consider that SPAs are
 - ✓ assay-dependent
 - ✓ disease-dependent
- As different assays contains different autoantigens, clinicians should be aware of which antigens are included in the SPA used
- A screening assay should be followed by an antibody-specific assay
- No single cut-off is associated with a good sensitivity and a good specificity
- Test-result specific likelihood ratio might help to interpret results

What the clinicians ask about when they have to do with ANA test results?

The key question:

“What is the probability of a patient having a particular disease
when the laboratory test is positive

or

not having the disease when the laboratory test is negative?”

The key issue: how to best interpret ANA test results?

Which assay for which disease?

- Neither HEp-2 IIF nor SPA is superior to each other
- A test with a high sensitivity (e.g. IIF with cut-off 1:80) helps to exclude disease
- A test with a high specificity (e.g. FEIA or IIF with a high cut-off/threshold) helps to confirm disease
- Neither HEp-2 IIF nor SPA by itself can identify all the AARD patients
- IIF might detect relevant antibodies that are undetected by SPA, and viceversa
- IIF is largely variable among laboratories
- The differences among the methods have to be kept in mind when the tests are used for classifying rather than diagnosing patients
- Test result-specific LR_s can allow better interpretation and harmonization of test results
- Combining IIF with SPA has the highest clinical value

Optimal test strategy

SLE

IIF

Nuclear homogeneous

Nuclear speckled

Cytoplasmic fine speckled
PCNA-like

High sensitivity

SPA

dsDNA

Sm
SSA
U1-RNP
Rib-P
PCNA

IIF has a high sensitivity, but low specificity.
Combining IIF with SPA is the optimal strategy.

SS

IIF

Nuclear fine speckled

Cave, might miss some anti-SSA antibodies

SPA

SSA
SSB

SPA for SSA (and SSB) has a good sensitivity.

MCTD

IIF

Nuclear large speckled

SPA

U1-RNP

SSc

IIF

Centromere
TOPO-like
Nuclear speckled

Nucleolar

High sensitivity

SPA

Centromere
Topoisomerase I
Polymerase III

Fibrillarin
Th/To
NOR90
PM/Scl (overlap)

U11/U12 RNP
BICD2

High sensitivity

IIF is a good screening assay.
Specific antibodies cover majority of SSc-specific antibodies, but not all.
Check whether specific antibody fits with IIF pattern.

IIM

IIF

Cytoplasmic
(dense) fine speckled

Nuclear speckled

Nuclear multiple dots

Cave:
limited sensitivity

SPA

Anti-synthetase
Jo-1, PL-7, PL-12, OJ, EJ, ..
SRP

HMGR

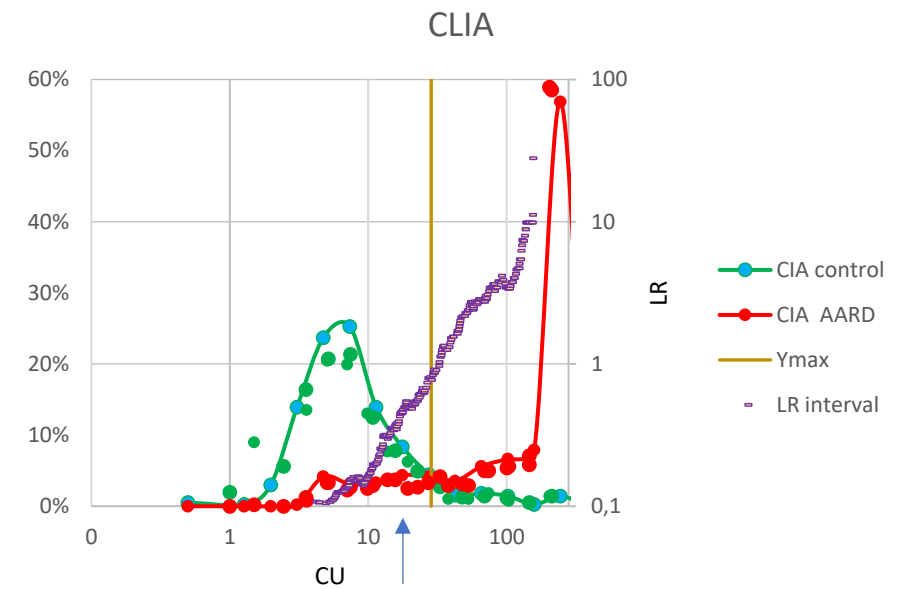
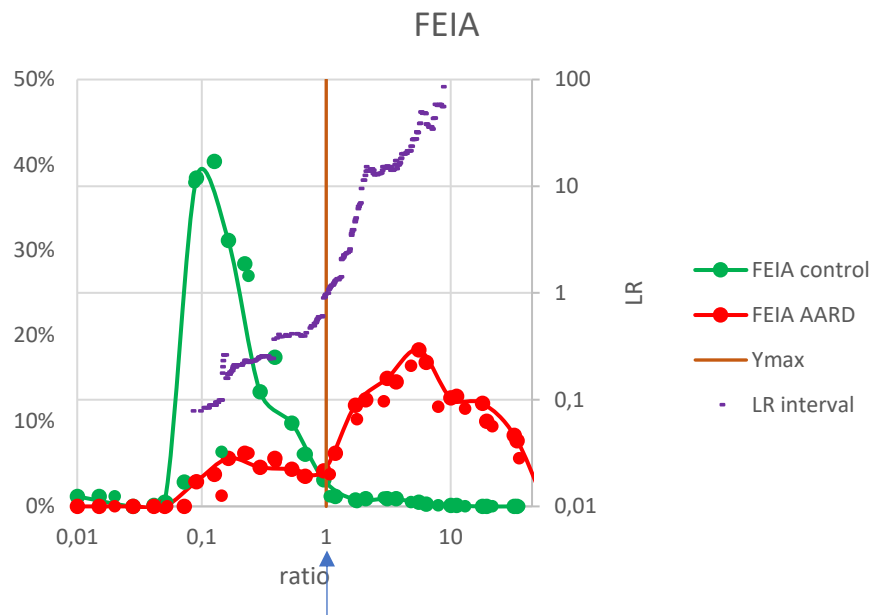
Mi-2
TIF- γ
Ku (overlap)

NXP-2

MDA-5
SAE

Cave:
False positives with some assays.
Does not represent full spectrum
Check whether antibody fits with IIF pattern.

Optimal strategy: in case of clinical suspicion: SPA, but beware of weak false positives with some assays.
Available assays do not cover full antibody spectrum.
Check whether specific antibody fits with IIF pattern.



fraction of patients with certain result

Likelihood ratio = -----

fraction of controls with certain result

86% of SLE patients have titer 1:320

Ex. LR = ----- = 25.3

3.4 % of controls have titer 1:320

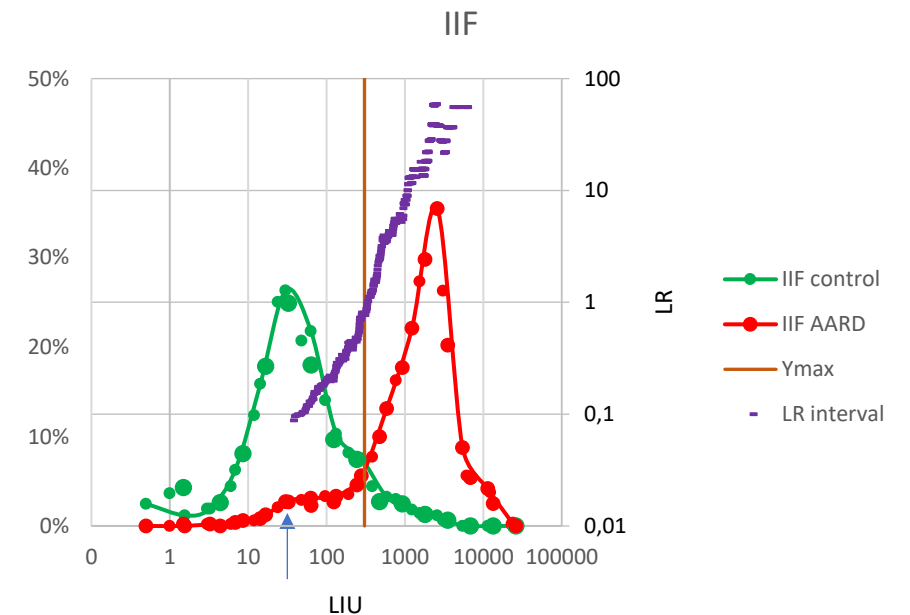
LR depends on the control population used

Test result-specific LR helps with interpretation of (automated) IIF, FEIA and CLIA

High positive LR (>10) best for confirming a diagnosis

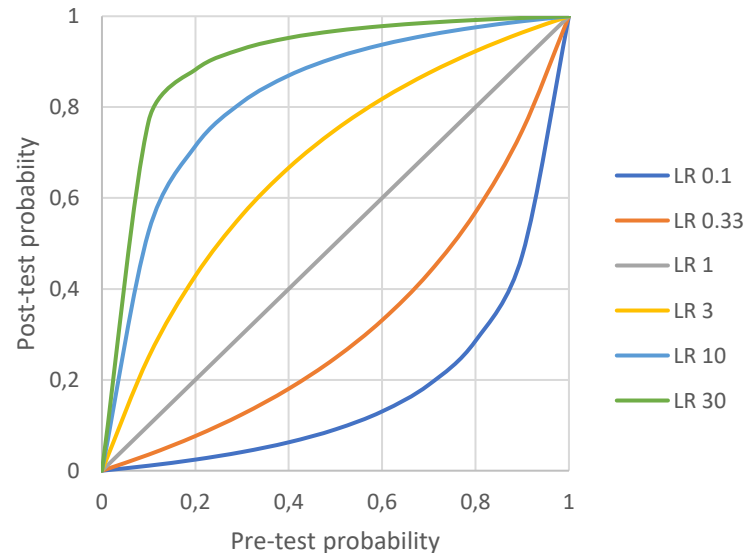
Low negative LR (<0.1) best for excluding the disease

LR = 1 test results help neither to exclude not to confirm the disease



From Likelihood ratio to post-test probability

- ✓ Pre-test probability: probability of having a particular disease for an individual before the test
- ✓ Post-test probability: probability of having a disease in a patients with a positive test result
- ✓ Odds: probability of having a particular disease / probability of not having the disease



$$\text{Pre-test odds} = \frac{\text{pre-test-probability}}{(1 - \text{pre-test probability})}$$

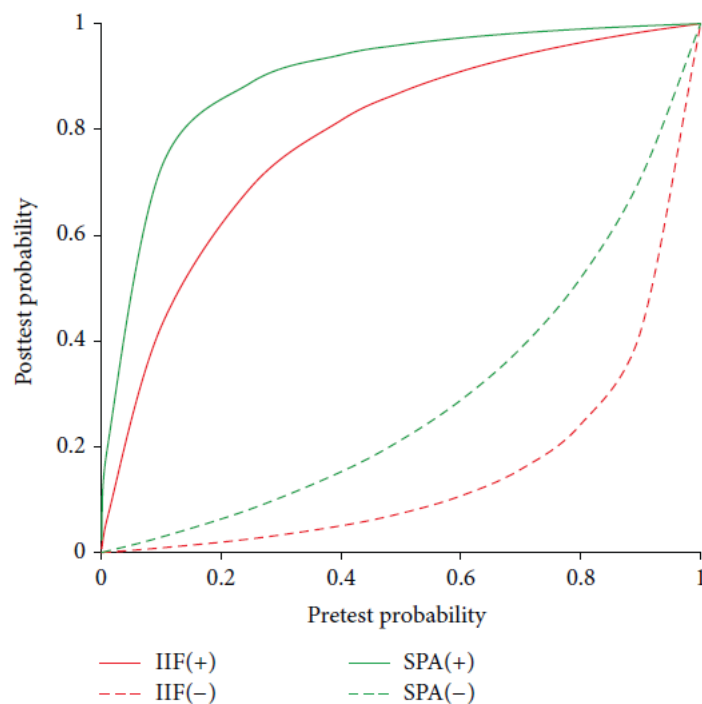
$$\text{Post-test odds} = \text{pre-test odds} * \text{LR}$$

$$\text{Post-test probability} = \frac{\text{post-test odds}}{(1 + \text{post-test odds})}$$

1) Young lady with hair loss and polyarthralgias pre-test probability for SLE 1% (= 0.01)

2) Young lady with photosensitivity and mild Leucopenia (3000-3500 WBC/mm³) pre-test probability for SLE 10% (= 0.1)

3) Young lady with photosensitivity, malar rash and simmetrical polyarthritis pre-test probability for SLE 50% (= 0.5)



	LR+	LR-
IIF	7	0.03
FEIA	24	0.27

[Bossuyt & Fiewu, ARD 2014]

$$\begin{aligned} \text{IIF+} & \frac{0.01}{(1 - 0.01)} * 7 = 0.07 > \frac{0.07}{(1 + 0.07)} = 0.066 \\ \text{FEIA+} & \frac{0.01}{(1 - 0.01)} * 24 = 0.24 > \frac{0.24}{(1 + 0.24)} = 0.19 \end{aligned}$$

	Post-test probability			
Pre-test probability	IIF+*	IIF-	FEIA+	FEIA-
1%	6%	0.08%	19%	0.20%

*HEp-2 nuclear pattern fine speckled (ICAP AC-4) suggestive of anti-Ro abs



1) Young lady with hair loss and polyarthralgias

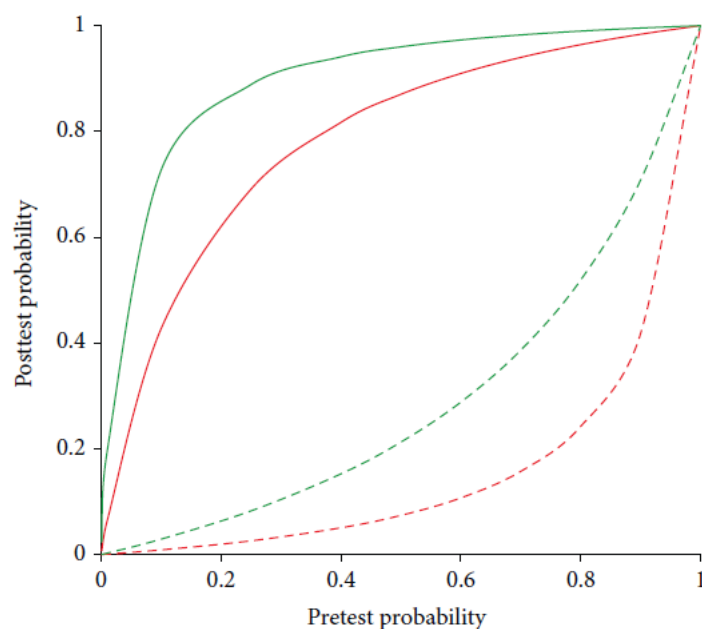
pre-test probability for SLE 1% (= 0.01)

2) Young lady with photosensitivity and mild Leucopenia (3000-3500 WBC/mm³)

pre-test probability for SLE 10% (= 0.1)

3) Young lady with photosensitivity, malar rash and simmetrical polyarthritis

pre-test probability for SLE 50% (= 0.5)



	LR+	LR-
IIF	7	0.03
FEIA	24	0.27

$$\text{IIF+} \quad \frac{0.1}{(1 - 0.1)} * 7 = 0.77 > \frac{0.77}{(1 + 0.7)} = 0.43$$

$$\text{FEIA+} \quad \frac{0.1}{(1 - 0.1)} * 24 = 2.6 > \frac{2.6}{(1 + 2.6)} = 0.72$$

	Post-test probability			
Pre-test probability	IIF+	IIF-	FEIA+	FEIA-
1%	6%	0.08%	19%	0.20%
10%	43%	0.80%	72%	3%

*HEp-2 nuclear pattern FS (ICAP AC-4) suggestive of anti-Ro

1) Young lady with hair loss and polyarthralgias

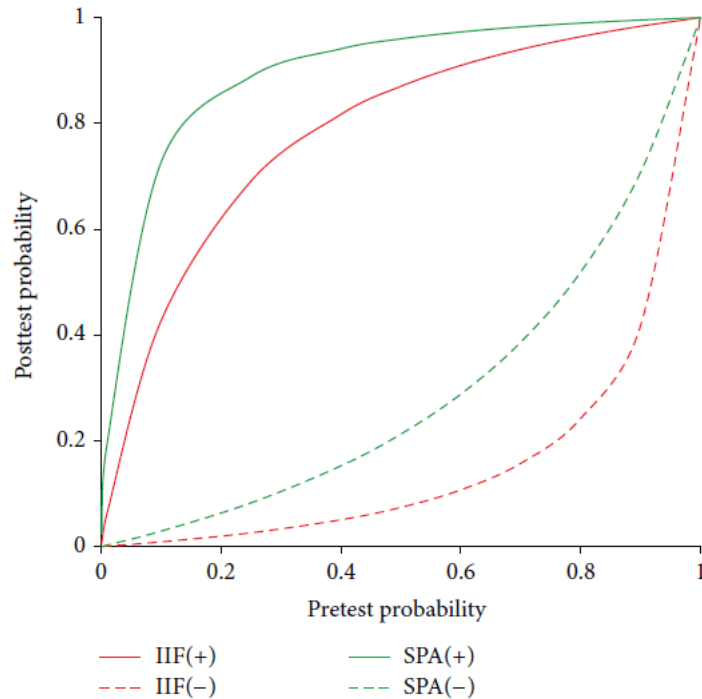
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Leucopenia (3000-3500 WBC/mm³)

pre-test probability for SLE 10% (= 0.1)

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and simmetrical polyarthritis

pre-test probability for SLE 50% (= 0.5)



	LR+	LR-
IIF	7	0.03
FEIA	24	0.27

$$\text{IIF+} \quad \frac{0.5}{(1 - 0.5)} * 7 = 3.5 > \frac{3.5}{(1 + 3.5)} = 0.78$$

$$\text{FEIA+} \quad \frac{0.5}{(1 - 0.5)} * 24 = 12 > \frac{12}{(1 + 12)} = 0.92$$

	Post-test probability			
Pre-test probability	IIF+	IIF-	FEIA+	FEIA-
1%	6.6%	0.08%	19%	0.20%
10%	43%	0.80%	72%	3%
50%	78%	7%	92%	21%

*HEp-2 nuclear pattern FS (ICAP AC-4) suggestive of anti-Ro



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From autoantibody test results to decision making: Incorporating likelihood ratios within medical practice

Kevin D. Deane^{a,1}, Lieve Van Hoovels^{b,c,1}, Veena E. Joy^d, Nina Olschowka^d,
Xavier Bossuyt^{c,e,*}

The likelihood ratio

- ✓ gives information on how much more/less likely a particular test result is in patients compared to individuals without the disease
- ✓ incorporates information on antibody level (higher the level > higher the association with the disease)
- ✓ is unit-independent and helps to harmonize test result interpretation
- ✓ combining LR with pre-test probability, post-test probability can be evaluated

LR challenges for clinicians

- ✓ pre-test probability not often developed
- ✓ primary care physicians may be less confident of forming an initial diagnosis than trained rheumatologists
- ✓ LR is not often reported on a lab test result
- ✓ different lab test may have different LRs because of the cutoff and the features of the test

ANA interpreting, beyhond the basis

- LR can aid Rheumatologists in diagnosing a disease
- Large-scaled multicenter studies are needed to determine LRs
- Individual laboratories cannot deal with LR calculation
- A collaboration among clinical researchers, laboratory people and diagnostics companies is essential

Conclusions

When interpreting ANA screening results, clinicians should be aware that:

- IIF and SPAs display clinically relevant differences in performance
 - none of the methods can identify all AARD patients
 - none of the methods is superior to the others
- } their combination has the highest value
- the performance of IIF and SPAs is disease-dependent
 ↳ the optimal test strategy depends on the target disease and the pre-test probability
 - Reporting the test result-specific LR might help both to better interpret and to harmonize the results

ANA interpreting, looking forward

Machine Learning Identifies Clusters of Longitudinal Autoantibody Profiles Predictive of Systemic Lupus Erythematosus Disease Outcomes

Choi et al. Ann Rheum Dis. 2023

A novel longitudinal clustering technique was applied to comprehensive autoantibody data from a large, well-characterized, multinational inception systemic lupus erythematosus (SLE) cohort to determine profiles predictive of clinical outcomes.

Could machine-learning be the future?