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A comparison of a fluorescence enzyme immunoassay versus indirect immunofluorescence for initial screening of connective tissue diseases: Systematic literature review and meta-analysis of diagnostic test accuracy studies

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A B S T R A C T

The aim was to compare indirect immunofluorescence (IIF) and fluorescence enzyme immunoassay (FEIA) for initial screening of connective tissue diseases (CTDs) and to evaluate whether combining IIF with FEIA adds value.

A comprehensive systematic literature review was conducted to identify fully paired, cross-sectional or case–control studies on ANA screening of CTD reporting results for IIF and FEIA. Study quality was assessed using the QUADAS-2 checklist. The reference standard was assessed against established classification criteria. The meta-analysis used hierarchical, bivariate and mixed-effects models to allow test results to vary within and across studies.

Eighteen studies of good to fair quality were included in the review. IIF had a higher sensitivity than FEIA [cut-off 1:160, 7 studies, 3251 patients, 0.83 (95% CI 0.75–0.89) versus 0.73 (95% CI 0.64–0.80); cut-off 1:80, 7 studies, 12,311 patients, 0.89 (95% CI 0.84–0.93) versus 0.78 (95% CI 0.71–0.84)] but lower specificity [1:160, 0.81 (95% CI 0.73–0.87) versus 0.94 (95% CI 0.91–0.95); 1:80, 0.72

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(95% CI 0.62–0.81) versus 0.94 (95% CI 0.90–0.96)]. A double-positive test had a higher likelihood ratio (LR) for CTD (26.2 (95% CI 23.0–29.9)) than a single positive test (14.4 (95% CI 13.1–15.9) FEIA+, 5.1 (95% CI 4.8–5.4) IIF+). A double-negative test result had more clinical value for ruling out CTD than a single negative test (LR 0.15 (95% CI 0.12–0.18) versus 0.21 (95% CI 0.18–0.25) IIF; 0.33 (95% CI 0.29–0.37) FEIA-). A FEIA+/IIF- discordant result had a higher LR than an IIF+/FEIA- discordant result (LR 2.4 (95% CI 1.7–3.4) versus 1.4 (95% CI 1.2–1.7)).

Because of the comparatively higher specificity of FEIA and higher sensitivity of IIF, the combination of FEIA and IIF increases the diagnostic value. Clinicians should be acquainted with the clinical presentation of CTD and aware of the advantages and disadvantages of FEIA and IIF to avoid misinterpretation.

Introduction

The presence of antinuclear antibodies (ANAs) is associated with connective tissue diseases (CTDs) and can predict the development of autoimmune disease before clinical onset [1]. Tests for ANAs are performed in routine practice to screen for CTD and to support the clinical diagnosis [2]. However, these antibodies can be present in sera from patients with other rheumatic diseases and, to some extent, in patients with non-rheumatic disorders, e.g. infection [3,4], and in healthy individuals [5–9].

The indirect immunofluorescence (IIF) test on human epidermoid laryngeal carcinoma cells (HEp-2 or HEp-2000 cells) is considered the 'gold standard' for the detection of ANA [2]. However, this technique is time-consuming, requires skilled operators, has a high inter-observer variability and lacks standardisation. Solid phase assays have been developed to screen for specific analytes and can be fully automated to overcome some of the limitations of a manual IIF. For example, one such automated solid phase fluorescence enzyme immunoassay (FEIA, EliA CTD Screen) is coated with 15 antigens that are associated with CTDs (dsDNAs, SSA/Ro 60 kDa, SSA/Ro 52 kDa, SSB/La, U1-RNP (RNP-70, A,C), Sm, Jo-1, Scl-70, centromere B, fibrillarin, RNA Pol III, PM-Scl, Mi-2, Rib-P and PCNA).

To date, there has been no quantitative assessment across diagnostic test accuracy studies to compare solid phase assays and IIF for ANA screening in the diagnosis of CTD and a range of CTD conditions. To this end, a comprehensive systematic literature review was conducted to identify and assess the quality of published studies evaluating FEIA and IIF. Study data were then combined in a meta-analysis using a hierarchical bivariate model to provide a direct comparison of the sensitivity and specificity of FEIA versus IIF for screening CTD.

In addition, we also evaluated whether combining IIF with FEIA adds value.

Patients and methods

Systematic literature review process

The systematic literature review followed the process recommended by The Cochrane Collaboration for diagnostic test accuracy studies [10] with reporting as per the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement [11]. A structured search strategy was developed to combine search filters for CTD, index tests and diagnostic accuracy test studies using Emtree/MeSH terms and free text strings. An electronic search using these filters was conducted using MEDLINE, Embase and Cochrane databases (from 2000 to March 2018) along with handsearching to identify fully

Abbreviations:

ACR	American College of Rheumatology
ANA	Anti-nuclear Antibody
CI	Confidence Interval
CTD	Connective Tissue Disease
DC	Diseased Control
DM	Dermatomyositis
DOR	Diagnostic Odds Ratio
EULAR	European League Against Rheumatism
EQV	Equivocal
FEIA	Fluorescence Enzyme Immunoassay
FN	False Negative
FP	False Positive
HC	Healthy Control
HEp-2	Human Epithelial type 2 cells
HEp-2000	Human Epithelial type 2 cells – Human Epithelial type 2 cells –transfected with Ro60 cDNA
HSROC	Hierarchical Summary Receiver Operating Characteristic
IIF	Indirect Immunofluorescence
Lim SD	Limited Scleroderma
LR	Likelihood Ratio
MCTD	Mixed Connective Tissue Disease
MeSH	Medical Subject Headings
MLE	Maximum Likelihood Estimation
NA	Not Applicable
NLR	Negative Likelihood Ratio
NR	Not Recorded
PLR	Positive Likelihood Ratio
PM	Polymyositis
PRISMA	Preferred Reporting Items for Systematic reviews and Meta-Analyses
QUADAS-2	Quality Assessment Tool for Diagnostic Accuracy Studies – version 2
SjS	Sjögren's Syndrome
SLE	Systemic Lupus Erythematosus
SSc	Systemic Sclerosis
TN	True Negative
TP	True Positive
UCTD	Undifferentiated Connective Tissue Disease

paired, cross-sectional or case-control studies on ANA screening of CTD, where the study reported diagnostic test accuracy for both FEIA and IIF. The study needs to include a reference standard to verify the ANA test result and confirm or rule out a diagnosis of CTD. The conditions on the CTD spectrum that are associated with the presence of ANA include systemic lupus erythematosus (SLE) incorporating subacute cutaneous lupus erythematosus (ScLE); Sjögren's syndrome (SjS); systemic sclerosis (SSc); dermatomyositis and polymyositis (DM/PM); mixed connective tissue disease (MCTD); and undifferentiated connective tissue disease (UCTD).

All citations retrieved from the electronic search and by handsearching were imported into a reference manager (EndNote X8) for screening by two reviewers (MEO, MDO). The initial screening was based on abstract and title, then a second screen using full-text papers confirmed the eligibility of the study for inclusion in the systematic review.

Quality assessment

The study quality assessment was adapted from the QUADAS-2 checklist [12] to assess the quality of each study in relation to patient selection, attrition, flow and timing of the tests, and potential bias arising from the conduct and interpretation of the index tests and reference standard. As part of the assessment, the reference standard was compared to the most recent clinically accepted diagnostic criteria for CTD classification as follows: SLE: 1997 ACR criteria [13] or 2012 Systemic Lupus International Collaborating Clinics criteria [14]; SjS: 2016 ACR/EULAR criteria [15] or 2012 ACR/EULAR criteria [16]; SSc: 2013 ACR/EULAR criteria [17]; DM/PM: Bohan and Peter 1975 [18,19], Dalakas and Hohlfeld's criteria 2003 [20], or European Neuromuscular Centre criteria 2004 [21]; and MCTD: Alarcon-Segovia and Villarreal 1987 [22], Kasukawa et al., 1987 [23] or Sharp and Anderson 1987 [24]. The reference standard was graded A-E, whereby the reference standard is graded A when the diagnosis/classification of CTD is based on the most recent disease-specific guidelines or classification criteria as listed above, B for classification criteria that were relevant at the time of the study, C if some clinical criteria and most relevant immunological criteria were used (e.g. authors indicated that disease-specific classification criteria were used but did not provide references), D if some relevant clinical criteria were used (e.g. authors indicated that they used some consensus criteria but did not provide references for the criteria) and with E referring to a reference standard that is not described in sufficient detail in the publication.

Data cleaning

For each study, the average sensitivity and specificity of FEIA and IIF were calculated from the test count data, i.e. the number of true positives (TPs), true negatives (TNs), false positives (FPs) and false negatives (FNs) reported for each test. Prior to the analysis, the data reported in the studies were accounted for as follows:

- CTD cohort: the total TP is the total reported number of positive tests in this cohort, and the total FN is the total reported number of negative tests in this cohort. If a study included other types of systemic autoimmune rheumatic diseases in the CTD cohort (e.g. rheumatoid arthritis), then data were adjusted to account for these patients in the diseased control (DC) group instead, i.e. positive tests are recorded as an FP and negative tests are recorded as a TN.
- DC cohort: the total FP is the total reported number of positives test in this cohort, and the total TN is the total reported number of negative tests in this cohort. If a study included healthy controls as part of the DC, then these patients were excluded from the analysis.

Study data summary estimates

The 95% confidence interval around the study estimates was calculated using the exact method, and forest plots summarising these estimates were plotted using the *metan* package in STATA MP v14.2 [25].

A hierarchical summary receiver operating characteristic (HSROC) curve for FEIA and IIF was produced using the *metandi* package in STATA MP v14.2 [26]. This plot includes an overall estimate of sensitivity and (1-specificity) across all studies, a 95% confidence region around this estimate and a 95% prediction region taking into account heterogeneity: the 'true' sensitivity and specificity of a new study will lie within this region with a 95% confidence level [26].

Meta-analysis methods

The meta-analysis was conducted using hierarchical, bivariate, mixed-effect models as recommended in the Cochrane Handbook for Systematic Reviews of Diagnostic Test Accuracy [10]. The meta-analysis was conducted in STATA MP v14.2 using the *meqrlogit* function [27]. The bivariate method estimates sensitivity and specificity directly from the TP, TN, FP and FN counts, by assuming that sensitivity and specificity follow a binomial distribution [28,29]. This method accounts for the

correlation between sensitivity and specificity modelled as a single bivariate normal distribution. A mixed model allows for variability in test results within a study due to study sampling error (fixed effects) as well as across studies due to study heterogeneity (random effects) [27]. We tested a range of models that used different assumptions relating to the variance of sensitivity and specificity (see Supplementary materials Table S-9). The base model (Model 0) was a fixed effects model only (results vary because of sampling errors only and it is assumed that test results do not vary across studies). Model B included both fixed and random effects (observations vary because of sampling errors within the study (fixed effects), and in addition, test results vary because of differences across the studies (random effects)). Model E is the same as model B except with test-specific random effects (variance across studies and variance differs by test). Maximum likelihood estimation (MLE) methods were used to estimate the probability of observing the test results, given the model variables and the model. To determine the model that fitted the data the best, we conducted a post-estimation test in STATA through the *lrtest* command, which uses a chi-squared test to detect significant differences between the MLE from different models. The statistical significance of differences between tests is based on the p-value estimated from a two-sided *t*-test.

The cut-off for the IIF test varies across studies, and for some studies, IIF results are reported for more than one cut-off. The main analysis was conducted using data for IIF at a cut-off of 1:160 (Analysis 1) as per international recommendations [2] and, separately, an analysis using IIF data at 1:80, which is the entry criterion for SLE classification [30] (Analysis 2), and at 1:320 (Analysis 3), excluding studies not reporting IIF results at these cut-offs. For the meta-analysis, the cut-off for FEIA is > 1 in the main analysis as per the manufacturer's recommendations.

The sensitivity of a test is defined as the probability that the index test result will be positive in a diseased case. The specificity of a test is defined as the probability that the index test result will be negative in a non-diseased case. The likelihood ratio (LR) is the probability that a given test result is obtained in the patients with CTD compared to the probability of the same results in the controls. The positive likelihood ratio (PLR) describes how many times more likely positive index test results were in the CTD group than the disease control group. The negative likelihood ratio (NLR) summarises how many times less likely negative index test results were in the CTD group than in the DC group. The diagnostic odds ratio (DOR) is a summarised estimate of how many times higher the odds are of obtaining a test positive result in a patient with CTD rather than a patient with DC.

Results

Study summary

The literature review (up to March 2018) identified 18 studies that could be included in the meta-analysis [31–48] (see PRISMA diagram in Supplementary Materials, Figure S-1). A summary of the QUADAS-2 quality assessment is shown in Supplementary Materials, Figure S-2. Overall, the quality assessment indicated that the studies were of good to fair quality in terms of patient selection and participant flow (see Supplementary Materials Table S-3), and index test and reference standard conduct (see Supplementary Materials Table S-4). Twelve out of the 18 studies used a case-control design, which is judged as a high risk of bias [31–36,38–41,44,47]; though for this, review was deemed appropriate to increase the study sample size, given the low rate of CTD in clinical practice. The main issue was that some of the publications reported limited information on study methodology such that the quality could not be assessed (judged as an unclear risk of bias). In particular, there was a lack of information regarding the reference standard, with 12 out of the 18 studies graded E [31–33, 35, 37, 39, 42–44, 46, 47]. Six of the studies used sera samples taken before or at the time of diagnosis [34,38,40,41,43,45], seven studies did not report this information [31,35,39,42,44,46,47]. One study [33] only included SSc in the CTD group, one included SSc, MCTD, and DM/PM [37], one study included SLE, SjS, and DM/PM plus other CTD (unspecified) [39], 12 studies included at least SLE, SjS, and SSc (+/− (MCTD and DM/PM) +/− UCTD) in the CTD cohort [34–36,38,40,41,43–48] and three studies did not report the type of patients with CTD included [31,32,42].

Sixteen studies included test results for a representative cohort of patients with CTD and a DC group allowing for a meta-analysis of sensitivity with specificity. Figure S-5 is a forest plot of the sensitivity and specificity for each of these 16 studies for IIF by cut-off, with corresponding data for FEIA in Figure S-6.

Two studies did not include a representative DC group [35,37], but instead, these studies reported test sensitivity for different CTD subtypes. Figure S-7 and Figure S-8 are forest plots of the sensitivity of FEIA and IIF, respectively, by CTD subtypes for a total of 13 studies that reported this data.

Meta-analysis results of IIF versus FEIA

Three meta-analyses were conducted using subsets of studies that reported direct comparisons of FEIA and IIF at a cut-off of 1:160 (Analysis 1: 7 studies, 3251 tests, 20.2% CTD, HSROC graph Fig. 1 top panel), 1:80 (Analysis 2: 7 studies, 12,311 tests, 10.1% CTD, HSROC graph Fig. 1 middle panel), and 1:320 (Analysis 3: 5 studies, 2588 tests, 38.4% CTD, HSROC graph Fig. 1 bottom panel). Across all analyses, model E, the model with separate fixed and random effects by test for sensitivity and specificity, had the best fit, i.e., the model that is most likely to produce the observed data (see model comparison in Supplementary Materials Table S-9). The chi-squared test detected a significant difference ($p < 0.05$) between the MLE for model B (variance across studies is independent of test) compared to model E (variance across studies is dependent upon test). The inference is that the variance in results across studies is of a different magnitude for IIF compared to FEIA and that heterogeneity is more likely to be caused by differences in the IIF methodology rather than underlying patient risk factors. Table 1 shows the sensitivity, specificity, DOR, PLR and NLR estimated from model E.

The DOR was higher with FEIA than with IIF across all analyses. For FEIA versus IIF at a cut-off of 1:80, this difference was significant ($p < 0.001$). The sensitivity of FEIA was lower than that of IIF at a cut-off of 1:80 ($p = 0.005$) and 1:160 ($p = 0.051$). FEIA had a significantly higher specificity than IIF at a cut-off of 1:80 ($p < 0.001$) and 1:160 ($p < 0.001$).

Meta-analysis results by CTD sub-type

Figure S-7 and Figure S-8 show the study-level sensitivity of FEIA and IIF, respectively, by CTD subtypes for 13 studies that reported these data (SLE 11 studies, 692 patients, SSc 12 studies, 856 patients, SjS 10 studies, 296 patients, DM/PM 11 studies, 183 patients and MCTD 12 studies, 274 patients). However, there were insufficient data to conduct a meta-analysis for different IIF dilutions.

In most cases, the best model (model with highest MLE) was the model that allowed for separate random effects by test for sensitivity and specificity (Model E). The exception was the analysis of MCTD where the model with separate random effects by test could not be estimated because of extreme values. Instead, model B (exchangeable random effects) was the best model. Table S-10 shows the estimated sensitivity by CTD subtype from the best fitting model. There was no significant difference in sensitivity between the tests for detecting SLE, SjS, DM/PM and MCTD ($p > 0.05$). The sensitivity of IIF was significantly higher than FEIA in the SSc subgroup of patients ($p < 0.001$). These results should be viewed with some caution given the small number of patients included in the CTD subgroups in the studies.

Analysis of single versus double test strategy

Twelve studies [32,36–38,41–48] reported data on the concordance between the IIF and FEIA tests (17,239 tests, see Table S-11) with an average concordance of 83.5% across the studies (9.0% of tests were double positive, 74.0% were double negative, 13.8% IIF positive/FEIA negative and 3.1% of tests were IIF negative/FEIA positive).

Data for concordant and discordant test results by CTD status were reported for four studies [32,38,43,48] (see Table S-12) from which we calculated the conditional probability of a test result given the CTD status (Table 2). Two of the studies were large cross-sectional studies covering 11,564 patients in total [43,48], whilst the other two studies were smaller studies of a case-control design (602 patients in total). The probability of CTD following a double negative IIF/FEIA test is low (<1%).

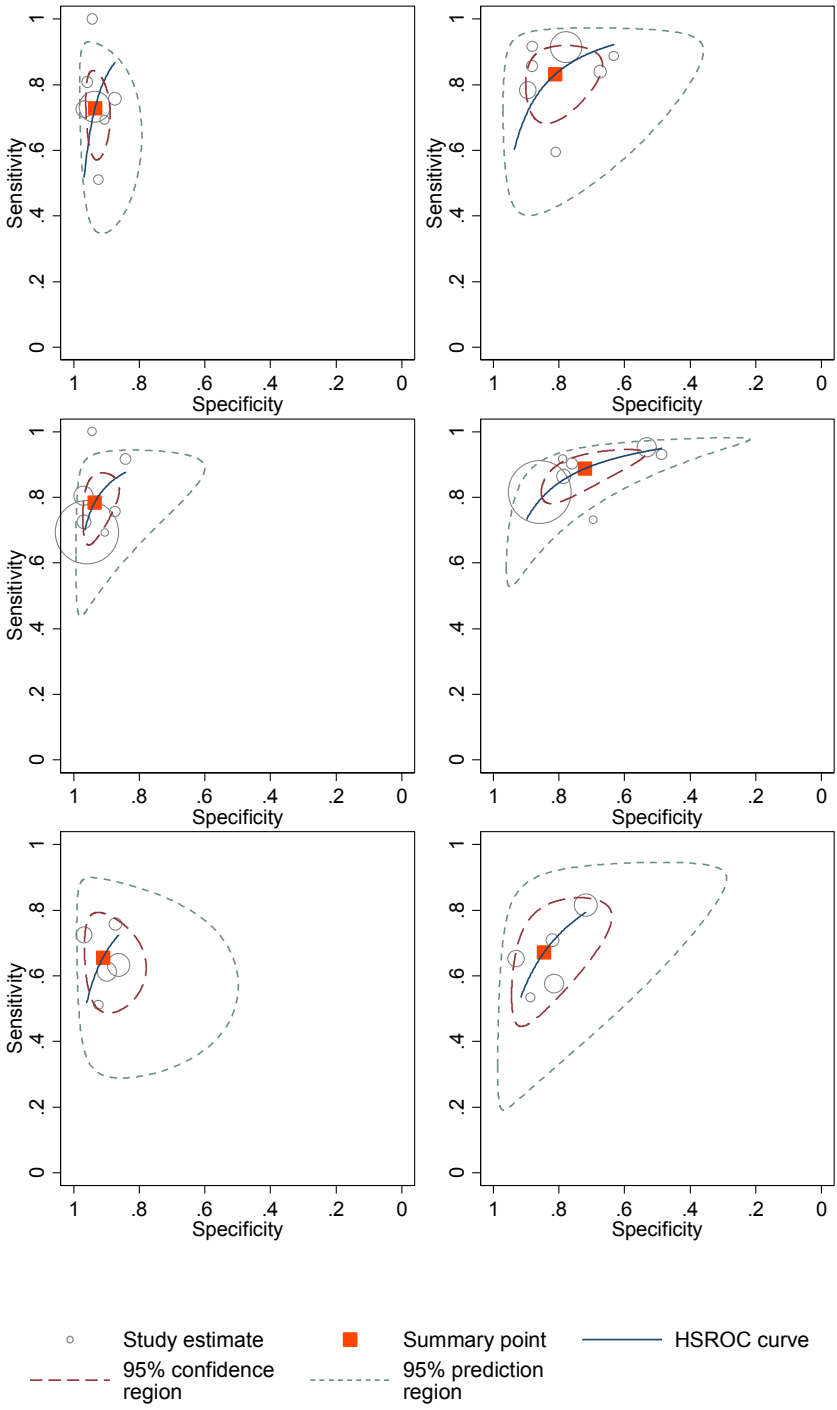

Fig. 1. HSROC graph with 95% prediction/confidence region: Analysis 1 (Top panel: FEIA (left) and IIF 1:160 (right)); Analysis 2 (Middle panel: FEIA (left) and IIF 1:80 (right)); and Analysis 3 (Bottom panel: FEIA (left) and IIF 1:320 (right)). The size of circle corresponds to the size of study cohort.

Table 1
Meta-analysis results from studies reporting direct comparisons of FEIA (>1) and IIF (1:160, 1:80, 1:320): Model E – mixed model with different random-effects by test.

		FEIA Estimate (95% CI)	IIF Estimate (95% CI)	p-value
Analysis 1 (7 studies, n = 3,251): IIF 1:160	Sensitivity	0.73 (0.64, 0.80)	0.83 (0.75, 0.89)	0.051
	Specificity	0.94 (0.91, 0.95)	0.81 (0.73, 0.87)	<0.001
	DOR	38.61 (21.89, 68.09)	21.23 (12.11, 37.20)	0.51
	PLR	11.22 (7.88, 15.96)	4.39 (3.12, 6.19)	
	NLR	0.29 (0.22, 0.39)	0.21 (0.14, 0.31)	
Analysis 2 (7 studies, n = 12,311): IIF 1:80	Sensitivity	0.78 (0.71, 0.84)	0.89 (0.84, 0.93)	0.005
	Specificity	0.94 (0.90, 0.96)	0.72 (0.62, 0.81)	<0.001
	DOR	53.14 (32.66, 86.46)	21.44 (17.12, 26.85)	<0.001
	PLR	12.23 (7.90, 18.95)	3.22 (2.39, 4.34)	
	NLR	0.23 (0.17, 0.31)	0.15 (0.12, 0.20)	
Analysis 3 (5 studies, n = 2,588): IIF 1:320	Sensitivity	0.66 (0.58, 0.72)	0.67 (0.57, 0.76)	0.80
	Specificity	0.91 (0.86, 0.94)	0.85 (0.77, 0.90)	0.1
	DOR	19.39 (10.46, 35.94)	11.16 (7.31, 17.04)	0.15
	PLR	7.33 (4.60, 11.67)	4.34 (3.02, 6.24)	
	NLR	0.38 (0.30, 0.47)	0.39 (0.31, 0.50)	

CI, Confidence Interval; DOR, Diagnostic Odds Ratio; FEIA, Fluorescence Enzyme Immunoassay; IIF, Indirect Immunofluorescence; NLR, Negative Likelihood Ratio; PLR, Positive Likelihood Ratio.

We considered the utility of single test results for ruling in or ruling out CTD against concordant double test results and discordant double test results. The LRs by test result are shown in Table 3. A double positive test has a higher LR for CTD than a single positive test (LR 26.2 (95% CI 23.0, 29.9) for a double positive test versus 14.4 (95% CI 13.1, 15.9) FEIA+ only or 5.1 (95% CI 4.8, 5.4) IIF+ only). A double negative test result has more clinical value for ruling out CTD than a single negative value (LR 0.15 (95% 0.12, 0.18) versus 0.21 (95% CI 0.18, 0.25) for IIF- only; 0.33 (95% CI 0.29, 0.37) for FEIA- only). For discordant test results, a positive FEIA/negative IIF result has a higher LR than a positive IIF/negative FEIA result (LR 2.4 (95% CI 1.7, 3.4) versus 1.4 (95% CI 1.2, 1.7)).

Discussion

The sensitivity of IIF at a cut-off of 1:160 was higher than the sensitivity of FEIA across the main meta-analyses (83% versus 73% from Analysis 1 model E), but the specificity was lower (81% versus 94% Analysis 1 model E). The difference between FEIA and IIF in specificity, estimated by this analysis, is clinically meaningful. The meta-analysis indicates that IIF has a low specificity at a cut-off of 1:80 (72% Analysis 2 model E), which is the entry criterion for the new classification system for SLE [30].

An IIF or FEIA test result, when taken in isolation, does not provide a definitive picture, and as standalone tests, they both have disadvantages. A positive ANA screening test will be followed by an additional laboratory workup that may include a second ANA test and tests for specific antibodies [2]. FP results, which are more likely to occur with IIF, may lead to wrong diagnoses and inappropriate treatments by physicians not familiar with systemic connective tissue diseases [8,9,49]. Inappropriate

Table 2
Conditional probabilities calculated from four studies reporting test concordance/discordance by CTD status: 12,166 tests, CTD prevalence 5%.

All results	IIF+ & FEIA +	IIF- & FEIA-	IIF+ & FEIA -	IIF- & FEIA+
% of all tests	5.4%	78.1%	14.0%	2.5%
Prob of CTD| test results	58.1%	0.8%	7.0%	11.4%
Prob of No CTD| test results	41.9%	99.2%	93.0%	88.6%
% with CTD	3.2%	0.6%	1.0%	0.3%
% with no CTD	2.3%	77.5%	13.0%	2.2%
Prob of test results| CTD	62.8%	11.8%	19.6%	5.7%
Prob of test results| no CTD	2.4%	81.6%	13.7%	2.4%

Table 3
Likelihood ratio of the probability of a test result in the patient with CTD cohort compared to the probability of result in the control group: single or double-testing strategy using projected data from four studies (12,166 tests).

Test	Result	% of patients with CTD	% of controls	LR (95% CI)[a]
Single test	FEIA +	68.5%	4.8%	14.4 (13.1, 15.9)
	FEIA -	31.5%	95.2%	0.33 (0.29, 0.37)
	IIF +	82.4%	16.1%	5.12 (4.85, 5.42)
	IIF -	17.6%	83.9%	0.21 (0.18, 0.25)
Double test: concordance	FEIA + IIF+	62.8%	2.4%	26.2 (23.0, 29.9)
	FEIA- IIF-	11.8%	81.6%	0.15 (0.12, 0.18)
Double test: discordance	FEIA - IIF+	19.6%	13.7%	1.43 (1.21, 1.69)
	FEIA + IIF-	5.7%	2.4%	2.42 (1.72, 3.42)

[a] Likelihood ratio, LR; LR estimated as Willems 2018 data is extrapolated to full set of 9856 samples.

treatment with glucocorticoids should be avoided because of the risks of adverse effects (infections, metabolic disorder and osteoporosis) [50]. FN test results are more likely with FEIA. The current recommendation is that if a FEIA test is negative and there is a high clinical suspicion of a CTD, then an IIF should also be performed [2]. Similarly, for a negative IIF test where the physician strongly suspects CTD, further tests are recommended to identify specific antibodies such as anti-Jo-1 antibodies for clinically suspected DM/PM and anti-ribosomal P for SLE or anti-SS-A/Ro antibodies for SjS or subacute cutaneous lupus [2].

Recently, it has been proposed that combining IIF with FEIA could increase diagnostic accuracy overall [34,48,49,51–53]. The analysis presented here using data from four studies suggests that a double testing strategy does have more clinical value than single test results alone. The following example illustrates the expected number of test results for a single test or double test strategy, based on a CTD prevalence rate of 2.7% (the estimated prevalence of CTD from the largest prospective cross-sectional study included in this review [48]). Using an IIF cut-off of 1:160, for every 1,000 patients screened in practice, on average, three additional patients with CTD will have an FN test with FEIA compared to IIF (7 FN versus 4 FN), but 121 more patients without CTD will have an FP test with IIF compared to FEIA (184 FP versus 63 FP). Based on data from four studies reporting concordance between IIF and FEIA and a CTD prevalence of 2.7%, on average, for every 1,000 tests, 40 tests will be double positive (with 23 FP) and 797 will be double negative (with 3 FN). FEIA alone will, on average, correctly classify 94.5% of patients compared to 83.9% correctly classified with IIF alone at a cut-off of 1:160. A double positive or double negative test correctly classifies 96.8% of patients with concordant results. When there is a discrepancy in the test results, a positive FEIA/negative IIF result is more likely to occur in a patient with CTD than a negative FEIA/positive IIF result (LR 2.4 versus 1.4). Even in the case of a double negative IIF/FEIA test, it is important that medical doctors are acquainted with the clinical presentation of systemic rheumatic disorders and do not solely rely on the laboratory tests.

The presence of autoantibodies can predict the development of autoimmune disease before clinical onset [1,54,55]. For example, SLE-related autoantibodies could be found in 88% of patients with SLE prior to diagnosis [1]. Pérez et al. [56] showed recently that a multiplexed assay (that simultaneously detects antibodies to Ro60, Ro52, RNP-A, La, chromatin, centromere B, Sm-RNP, dsDNA, Topo I, Sm, Rib-P, RNP-68 and Jo-1) can detect antibodies in patients that were ANA negative by IIF. A substantial proportion of these patients (76% = 312 out of 411) became ANA positive by IIF over a 3-year follow-up period: the majority of the patients (87% = 358 out of 411) were diagnosed with systemic rheumatic disease [56]. This illustrates that measurement of specific antibodies has the potential to identify individuals that are at risk of developing an autoimmune disease. Early identification of risk factors (e.g. autoantibodies) is important for disease prediction, prevention, and early start of effective therapy [57]. As such, reliable (multiplexed) antibody detection can play a role in precision health [57]. Early diagnosis and correct treatment have been shown to improve the clinical outcomes, prognosis, remission rate and survival and quality of life of patients with SLE [58–61], and furthermore, the costs to the health care system [62]. For patients with SjS, the University of Toronto estimated an average diagnosis time of 5 years (range 0–28 years) from the onset of the symptoms [63,64] and that earlier

diagnosis and correct treatment could improve the symptoms and reduce the complications and comorbidities associated with SjS.

Although the early diagnosis and correct management of autoimmune diseases is important to reduce complications and improve the quality of life, there is insufficient evidence for initiating treatment in asymptomatic patients [65]. Moreover, routine testing for ANA in patients without symptoms suggestive of autoimmune diseases is inappropriate [66]. This is because of the fact that ANAs can be present in healthy individuals and non-affected relatives of patients with autoimmune diseases, and in non-autoimmune conditions such as infections, malignancy, viral hepatitis, the elderly and even healthy children [3–6,67]. Because of its low predictive value and specificity, there is a limited value in a positive ANA result in patients without signs and symptoms suggestive of autoimmune disease. Positive ANA results without clinical suspicions can lead to misdiagnosis, wrong treatment, anxiety and unnecessary healthcare costs. In one study [7], 90% of patients referred to a clinic with a positive ANA test had no evidence of an ANA-associated rheumatic disease. The positivity of ANA screening tests should be confirmed with more specific tests to identify and quantify which autoantibodies are causing the positivity in the screening and be able to perform a differential diagnosis.

This review identified some studies reporting diagnostic test data for specific CTD subtypes, but an analysis at specific IIF cut-offs could not be conducted. No significant differences between the sensitivity of IIF versus FEIA were found for the most common CTD subtypes of SjS and SLE, though FEIA may perform better for SjS, given that the FEIA included anti-SS-A/Ro. Both tests had a low sensitivity for DM/PM and a high sensitivity for MCTD. The average rate of ANA-IIF positivity in patients with SLE was lower in this study than the expected prevalence rate of 95% reported in the international recommendations [2]. Pisetsky et al. [68,69] have suggested that ANA negativity in patients with SLE with established disease occurs more frequently than previously thought, with an expected range of ANA-positivity in patients with SLE of 80–95% depending on the assay kit used and the demographic features of the population under study. A recent publication by EULAR and ACR states that IIF with 1:80 will be used as an entry criterion for the novel classification of SLE [30]. Three studies included in our review reported diagnostic outcomes for patients with SLE tested before diagnosis with IIF at a cut-off of 1:80: the average sensitivity was 90% [48], 93% [45], and 94% [34]. A negative ANA test does not definitely rule out SLE without consideration of other clinical characteristics.

In this comprehensive systematic literature review, all relevant published studies were identified and subject to a quality review to assess potential bias arising from the study design, conduct, and interpretation of results. One common quality issue is that some studies used a case-control design, which is most likely because of the practicality of conducting a cross-sectional study using unselected patients referred for ANA testing in the clinic. For a case-control design, the mix of CTD sub-types in the study cohorts may not be representative of the real prevalence of the diseases in a clinical setting. However, most of the studies included a range of CTD sub-types (SLE, SjS and SSc with MCTD and DM/PM). Specificity was calculated using a cohort of diseased patients excluding healthy controls, which is more representative of practice. A more important limitation is the lack of information reported in some of the studies to whether the sera were sampled before diagnosis or after treatment had been initiated. The ANA tests are intended to be used as screening tests to support diagnosis, and ANA levels can change with treatment. There was also a lack of information reported in some of the studies regarding the reference standard used to confirm the diagnosis of CTD. There were too few studies to perform a sub-group analysis using studies that reported a good quality reference standard and used the same IIF cut-off. As the analysis included studies of fully paired design and the hierarchical meta-analysis grouped test data by study, the potential biases mentioned above would impact on the estimates for both types of tests.

The main meta-analysis includes 16 studies covering 18,889 test results, and all available data were used in the meta-analysis wherever data were reported. The meta-analysis provides a robust method to synthesise data across studies: larger studies are given more weight in the analysis and the use of fully paired direct comparisons allows for correlations within studies. The meta-analysis models include separate random-effects by test, allowing for variation within and across studies and for the magnitude of the variation to differ for IIF compared to FEIA. The HSROC curves indicate a large variance in IIF test results across the studies (Fig. 1 right panels), particularly for specificity.

Heterogeneity across studies may be due in part to differences in the study designs but also due to variations in practice. Given that the analysis uses fully paired studies and the variance is not seen to the same extent with FEIA (Fig. 1 left panels), it could be hypothesised that the variance is due to differences in the conduct and interpretation of the IIF tests within and/or across studies. The 95% prediction region is the area within which the sensitivity and specificity of a future study are predicted to lie with a 95% level of confidence. It is noted that the prediction region for IIF is quite large at all three cut-offs used in the analysis. For three of the studies [33,34,47], the IIF results were interpreted using an automated system. The sensitivity and specificity using the automated IIF test did not seem to differ from manual IIF (see Figure S-5) though the number of studies is too small to draw a robust conclusion. Further work could be done to investigate reasons for this variation.

This review compares the current gold standard IIF versus one type of technology that includes specific antigens covering the most relevant CTDs, FEIA. A larger review and meta-analysis covering additional studies of IIF versus other immunoassays, such as multiplex technology, is currently in development. Multiplex technologies in the diagnosis of autoimmune diseases allow for the detection of multiple autoantibodies using fewer tests. Limitations of multiplex technologies are that cross-reactivity limits the number of proteins that can be used, and there can be high background signals and cross-binding that could lead to FP reactions [70–72]. A review by Satoh et al. published in 2015 [72] highlighted that the main concern relating the quality of the multiplex assays was a lack of validation. Satoh et al. [72] reported a study where sera from patients with SLE were analysed by three multiplex technologies with large discrepancies in the results. Seven per cent of the patients with SLE were positive for anti-Scl-70 with one of the multiplex technologies tested, and this could be related to cross-binding between anti-dsDNA antibodies and anti-Scl-70. However, harmonisation and standardisation of multiplexed antibody testing are needed, as recently illustrated for the detection of myositis-specific antibodies [73].

In conclusion, in the main meta-analysis, FEIA has a high specificity for CTD. In the four studies where FEIA and IIF were combined, LR of double positivity was higher than that of positivity with the single tests alone, and the LR of double negativity was lower than that of negativity with the single tests alone. Whilst a concordant test result will correctly classify the majority of patients, it is important that medical doctors are acquainted with the clinical presentation of systemic rheumatic disorders to avoid misinterpretation of results.

Practice points

- ANA testing is used in routine practice for the early detection of autoantibodies and the diagnosis of CTD.
- IIF on HEp-2 cells is considered the current gold standard given its high sensitivity for several autoimmune diseases.
- IIF on HEp-2 has a low specificity and is limited by the variability and the subjectivity of the test.
- Specificity can be improved by using an immunoassay such as FEIA that detects antibodies to a selection of CTD-associated autoantigens.
- A double-testing strategy using IIF and FEIA has more clinical value than IIF or FEIA alone.

Research agenda

- The clinical guidelines should be updated to reflect the value of a double-testing strategy using IIF and an immunoassay such as FEIA.
- More research is required to determine the value of combining IIF with other multidetector technologies for the diagnosis of CTD.

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Conflicts of interest

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Appendix A. Supplementary data

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