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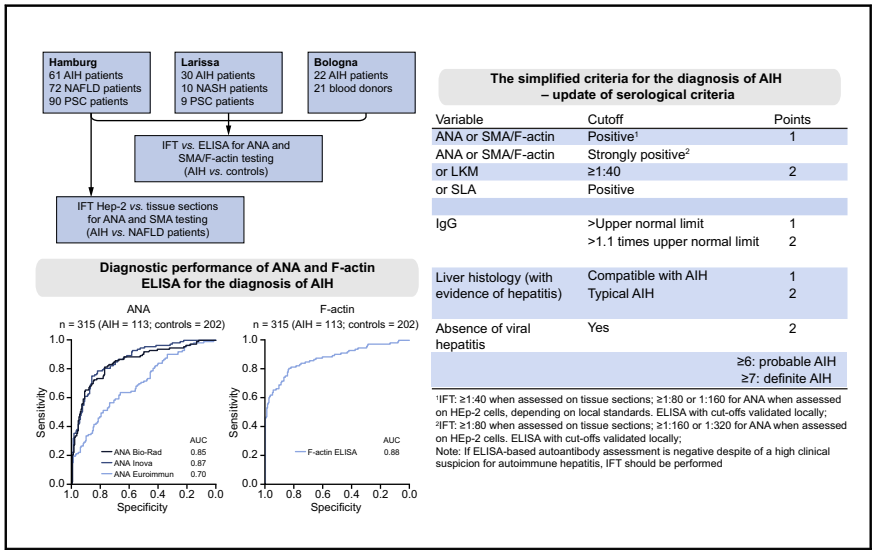
Update of the simplified criteria for autoimmune hepatitis: Evaluation of the methodology for immunoserological testing

Graphical abstract



Highlights

- IFT on HEp-2 cells is a valid alternative for the diagnosis of AIH when cut-off titers are increased.
- ANA ELISA and F-actin ELISA represent potential alternatives to IFT for the diagnosis of AIH.
- ANA ELISA kits should include HEp-2 nuclear extracts to account for unrecognized autoantigens.
- ELISA cut-offs need to be validated locally to be predictive in diagnosing AIH.

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Lay summary

Autoantibodies are a hallmark of autoimmune hepatitis and are traditionally tested for by immunofluorescence assays on rodent tissue sections. Herein, we demonstrate that human epithelioma cells can be used as a reliable substrate for immunofluorescence testing. ELISA-based testing is also a potentially reliable alternative for autoantibody assessment in autoimmune hepatitis. We propose the implementation of these testing methods into the simplified criteria for the diagnosis of autoimmune hepatitis.

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Update of the simplified criteria for autoimmune hepatitis: Evaluation of the methodology for immunoserological testing

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Background & Aims: The simplified criteria for the diagnosis of autoimmune hepatitis (AIH) include immunofluorescence testing (IFT) of antinuclear and smooth muscle autoantibodies (ANA and SMA) on rodent tissue sections. We aimed to establish scoring criteria for implementation of ANA IFT on human epithelioma-2 (HEp-2) cells and ELISA-based testing.

Methods: ANA and SMA reactivity of 61 AIH sera and 72 non-alcoholic fatty liver disease controls were separately assessed on tissue sections and HEp-2 cells to compare the diagnostic value at increasing titers. A total of 113 patients with AIH at diagnosis and 202 controls from 3 European centers were assessed by IFT as well as 3 different commercially available ANA ELISA and 1 anti-F-actin ELISA.

Results: ANA assessment by IFT on liver sections had 83.6% sensitivity and 69.4% specificity for AIH at a titer of 1:40. On HEp-2 cells, sensitivity and specificity were 75.4% and 73.6%, respectively, at an adjusted titer of 1:160. Area under the curve (AUC) values of ANA ELISA ranged from 0.70–0.87, with ELISA coated with HEp-2 extracts in addition to selected antigens performing significantly better. SMA assessment by IFT had the highest specificity for the SMA-VG/T pattern and anti-microfilament reactivity on HEp-2 cells. ELISA-based anti-F-actin evaluation was a strong predictor of AIH (AUC 0.88) and performed better than SMA assessment by IFT (AUC 0.77–0.87).

Conclusion: At adjusted cut-offs, both ANA IFT using HEp-2 cells and ELISA-based autoantibody evaluation for ANA and SMA are potential alternatives to tissue-based IFT for the diagnosis of AIH.

Lay summary: Autoantibodies are a hallmark of autoimmune hepatitis and are traditionally tested for by immunofluorescence assays on rodent tissue sections. Herein, we demonstrate that human epithelioma cells can be used as a reliable substrate for immunofluorescence testing. ELISA-based testing is also a potentially reliable alternative for autoantibody assessment in autoimmune hepatitis. We propose the implementation of these testing methods into the simplified criteria for the diagnosis of autoimmune hepatitis.

Introduction

Autoimmune hepatitis (AIH) is a chronic immune-mediated liver disease. Due to heterogeneity of the presentation, the diagnosis remains challenging. An early diagnosis is, however, critical for timely initiation of life-saving immunosuppressive therapy. To assist diagnostic evaluation, a simplified diagnostic score was established by the International Autoimmune Hepatitis Group (IAIHG) in 2008 for use in clinical practice.[1] Scoring criteria include characteristic findings on liver histology, the absence of viral hepatitis, an elevation of IgG, and circulating autoantibodies.

Autoantibodies associated with AIH include antinuclear antibodies (ANA), smooth muscle antibodies (SMA), liver kidney microsomal type 1 (LKM1) antibodies, liver cytosol type 1 (LC1) antibodies, and soluble liver antigen/liver pancreas (SLA/LP) antibodies. Screening for liver disease-associated autoantibodies is traditionally performed by immunofluorescence testing (IFT) on rodent tissue sections. Accordingly, the simplified AIH score refers to autoantibody titers as measured by IFT using tissue sections at a cut-off titer of 1:40. However, in several laboratories, there has been a shift towards performing autoantibody assessment in human epithelioma-2 (HEp-2) cells rather than tissue sections. Furthermore, ELISA, which the score does not account for, is frequently used in some countries. In order to make the simplified AIH score usable across the world, adaptation of the score to different immunoserological methods is urgently needed.

HEp-2 cells are widely used as a substrate for ANA evaluation. In addition to a higher sensitivity, characteristic staining patterns evaluated on HEp-2 cells are useful in guiding further

Keywords: Autoantibodies; Antinuclear antibodies; Smooth muscle antibodies; F-actin; Immunofluorescence; ELISA.
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confirmatory testing. However, a consensus statement by the IAIHG committee for autoimmune serology advises against the use of HEp-2 cells at a screening stage[2] because of a high positivity rate in healthy individuals at low cut-off titers.[3] If HEp-2 cells are used, the IAIHG suggests titers should be halved for the simplified score to be applicable.[1] However, this possible correction factor suggestion has never been validated by comparative studies.[4]

SMAs constitute a heterogeneous group of autoantibodies that primarily target F-actin.[5] On kidney tissue sections, Bottazzo and colleagues distinguished 3 immunofluorescence patterns: SMA-V (vessels), SMA-VG (vessels, glomeruli), and SMA-VGT (vessels, glomeruli, tubuli).[6] In contrast to the SMA-V pattern, SMA-VG/T correlates with F-actin reactivity and is more specific for AIH.[6–8] Similarly, anti-F-actin antibodies stain microfilaments (MF) on HEp-2 cells.[9] Overall, sensitivity and specificity of SMA positivity strongly depend on fluorescence patterns, which is not taken into consideration by current AIH scoring systems.

Since IFT is time-consuming, requires experienced technicians and lacks standardization, ELISA has emerged as a widely used alternative for routine autoantibody testing in many laboratories, especially in the United States. These tests were originally developed for use in the evaluation of rheumatic diseases and their diagnostic value in liver disease is unknown. ELISA testing can minimize interobserver variability inherent to IFT. However, it is unclear whether ELISA can replace IFT for the detection of the heterogeneous autoantibodies ANA and SMA with their range of antigenic specificities. To complicate matters even further, up to 30% of ANA-positive patients with AIH do not react with any known nuclear antigens[10] and might thus be missed by ELISA testing, which is based primarily on known nuclear antigens. In addition, commercially available ANA ELISA tests lack standardization – they differ in their antigenic profiles and assay-specific cut-off values.

Taken together, the AIH simplified score does not account for ANA and SMA as evaluated by IFT on HEp-2 cells or for ELISA, even though these tests are widely used. We therefore set out to study the diagnostic validity of IFT and ELISA-based autoantibody testing for the diagnosis of AIH to make these applicable in diagnosing AIH.

Patients and methods

Study population

This multicenter study included a total of 113 patients with AIH at diagnosis and 202 controls (82 patients with non-alcoholic fatty liver disease [NAFLD], 99 patients with primary sclerosing cholangitis [PSC] and 21 healthy controls) from 3 centers: Hamburg (Germany), Bologna (Italy), and Larissa (Greece). A flow-chart of patient cohorts is shown in Fig. 1. The large majority of patients with AIH (106/113, 93.8%) were treatment-naïve at the time of sampling. In addition, sera from 26 patients with primary biliary cholangitis (PBC) were tested and analyzed separately. Sera were collected between December 2006 and March 2020 and stored at −80°C until use. The study was approved by the local ethics committee (PV4081-0005, PV 4081-0008).

The diagnosis of AIH was based on clinical, serological, and histopathological criteria, consistent with the EASL clinical practice guidelines,[11] and confirmed by long-term follow-up in all patients. Patients with AIH and features of PSC or PBC were excluded from the study. Diagnoses of disease controls were based on established diagnostic criteria.[12–14] Blood donors with liver enzymes within the normal range, who were negative for HBV/HCV and for autoantibodies (by IFT), were included as healthy controls.

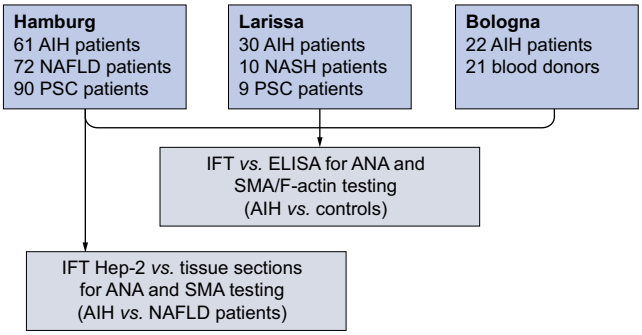

Fig. 1. Flow-chart of patient cohorts included in this study. AIH, autoimmune hepatitis; ANA, antinuclear antibodies; IFT, immunofluorescence testing; NAFLD, non-alcoholic fatty liver disease; PSC, primary sclerosing cholangitis; SMA, smooth muscle antibody.

Autoantibody assessment by IFT

IFT was performed in the respective center in which sera were collected. At the University Medical Center Hamburg-Eppendorf sera were tested using a Biochip Mosaic of primate liver, rat kidney, and rat stomach tissue sections as well as HEp-2 cells (Mosaic Basic Profile 3, Euroimmun, Germany). The assay was performed manually according to the manufacturer's instructions at a dilution of 1:40. Further dilutions up to 1:1,280 were processed by the Helios automated IFA system (Aesku Diagnostics, Wendelsheim, Germany), using the same substrates and conditions. Reactivity patterns were assessed under a fluorescence microscope (Eurostar, Euroimmun, Germany). ANA and SMA reactivity were separately evaluated on all 4 substrates. SMA reactivity on kidney sections was assessed according to Bottazzo *et al.*[6] The observers were blinded to clinical data.

Sera from the University Hospital of Bologna, Italy, were tested by IFT on both tissue sections and HEp-2 cells (Euroimmun, Germany) and were automatically processed at a starting dilution of 1:80 up to 1:640. ANA titers were mainly reported as assessed on HEp-2 cells and thus these data were used for comparison with ANA ELISA.

Sera from the University Hospital of Larissa, Greece, were tested by immunofluorescence on in-house fresh cryostat liver, kidney and stomach rat sections and HEp-2 cells (Inova Diagnostics). ANA titers were mainly reported as assessed on tissue sections and thus these data were used for comparison with ANA ELISA. Sera were processed manually at a starting dilution of 1:40 up to 1:640.

Detection of antinuclear and F-actin antibodies by ELISA

All ELISA testing was performed at the University Medical Center Hamburg-Eppendorf. Antinuclear antibodies were assessed using enzyme immunoassays from 3 different manufacturers (Quanta Lite ANA ELISA, Inova Diagnostics, US; ANA Screening Test, Bio-Rad, US; ANA Screen ELISA, Euroimmun, Germany). All assays detect autoantibodies of IgG subtype and display antigenic specificities to dsDNA, histones, Sm/RNP, SS-A, SS-B, Scl-70, centromere, and Jo-1. The Quanta Lite ANA ELISA is additionally

Table 1. Sensitivity and specificity of ANA IFT for different tissue sections.

Substrate	Titer	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
HEp-2 cells	1:40	95.1	8.3	46.8	66.7	48.1
	1:80	91.8	36.1	54.9	83.9	61.7
	1:160	75.4	73.6	70.8	77.9	74.4
	1:320	72.1	76.4	72.1	76.4	74.4
	1:640	60.7	87.5	80.4	72.4	75.2
Primate liver	1:40	83.6	69.4	69.9	83.3	75.9
	1:80	68.9	80.6	75.0	75.3	75.2
	1:160	47.5	91.7	82.9	67.4	71.4
	1:320	47.5	91.7	82.9	67.4	71.4
	1:640	29.5	94.4	81.8	61.3	64.7
Rat kidney	1:40	75.4	73.6	70.8	77.9	74.4
	1:80	65.6	81.9	75.5	73.8	74.4
	1:160	52.5	87.5	78.1	68.5	71.4
	1:320	47.5	91.7	82.9	67.4	71.4
	1:640	34.4	93.1	80.8	62.6	66.2
Rat stomach	1:40	78.7	70.8	69.6	79.7	74.4
	1:80	67.2	81.9	75.9	74.7	75.2
	1:160	52.5	88.9	80.0	68.8	72.2
	1:320	44.3	91.7	81.8	66.0	69.9
	1:640	36.1	93.1	81.5	63.2	66.9
Any tissue positivity (primate liver, rat kidney, rat stomach)	1:40	85.3	65.3	67.5	83.9	74.4
	1:80	73.8	77.8	73.8	77.8	75.9
	1:160	52.5	87.5	78.1	68.5	71.4
	1:320	50.8	91.7	83.8	68.8	72.9
	1:640	37.7	93.1	82.1	63.8	67.7

Autoimmune hepatitis: n = 61; non-alcoholic fatty liver disease: n = 72.
ANA, antinuclear antibodies; HEp-2 cells, human epithelioma-2 cells; IFT, immunofluorescence test; NPV, negative predictive value; PPV, positive predictive value.

coated with highly purified proliferating cell nuclear antigen (PCNA), mitochondrial M2 antigen, and ribosomal P proteins. Besides individual antigens, immunoassays from both Inova Diagnostics and Bio-Rad include HEp-2 cell nuclei extracts.

Antibodies to F-actin were detected using a commercial ELISA (Quanta Lite Actin IgG, Inova Diagnostics, US). All enzyme immunoassays were performed in duplicates according to the manufacturer's recommendations. Investigators who carried out immunoassays were blinded to clinical data and the results of IFT.

Statistical analyses

Data was expressed as median (range), or n (%) as appropriate. Statistical significance between groups was assessed with Fisher's exact test for categorical variables and the Mann-Whitney *U* test for continuous variables. Correlations were evaluated using Spearman correlation coefficients. The diagnostic value of variables in discriminating AIH from controls was assessed by receiver-operating characteristic (ROC) analysis. Statistical significance between area under the curve (AUC) values was assessed by the DeLong test. All reported *p* values are based on 2-sided tests and a *p* value <0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism (version 6), IBM SPSS (version 23), and R software (version 3.5.1).

Results

Comparison of HEp-2 cells and tissue sections as substrates for ANA IFT

We first investigated the diagnostic value of HEp-2 cells in comparison to tissue sections as substrates for ANA IFT in the context of AIH. To this end, sera from 61 patients with AIH and 72 patients with biopsy-proven NAFLD treated at the University Medical Center Hamburg-Eppendorf were evaluated for autoantibodies by IFT. Clinical characteristics of the patient groups at the time of sampling are summarized in Table S1.

Sensitivity and specificity of ANA IFT for HEp-2 cells and tissue sections are shown in Table 1. Among tissue sections, primate liver showed the highest diagnostic value for ANA evaluation. Sensitivity and specificity were 83.6% and 69.4% at a titer of 1:40, respectively, and 68.9% and 80.6% at a titer of 1:80, respectively. Specificity increased to 91.7% at a titer of 1:160 at the cost of a lower sensitivity of 47.5%. As expected, the use of HEp-2 cells led to higher titers. Specificity was inadequate at a 1:40 dilution. At a titer of 1:80, sensitivity was 91.8% at a low specificity of 36.1%. At higher titers, sensitivity and specificity were comparable to those observed on liver sections: 75.4% and 73.6%, respectively, at a titer of 1:160; 72.1% and 76.4%, respectively, at a titer of 1:320. The homogenous pattern was significantly more frequent in patients with AIH (41.0%) than in patients with NAFLD (6.9%, p <0.001).

Sensitivity and specificity of SMA fluorescence patterns on tissue sections and HEp-2 cells

We next assessed the diagnostic value of several SMA fluorescence patterns at different titers (Table 2). As expected, at a 1:40 titer, the SMA-V pattern on kidney sections, staining of smooth muscle on stomach sections as well as consideration of any SMA positivity resulted in a low specificity of 33.3%–45.8%. In contrast, the SMA-VG pattern was more specific for the diagnosis of AIH even at low titers. Sensitivity and specificity were 72.1% and 70.8%, respectively, at a titer of 1:40, and 65.6% and 88.9%, respectively, at a titer of 1:80. The highest specificity was seen for the SMA-VGT pattern and anti-MF reactivity on HEp-2 cells. At a 1:40 dilution, specificity was 93.1%–94.4% at a sensitivity of 52.5%–60.7%. Of note, with increasing titers, staining of the SMA-VGT pattern first faded for tubuli, then glomeruli, and finally vessels. In other terms, the SMA-VGT pattern changed to

Table 2. Sensitivity and specificity of SMA IFT for different patterns.

Substrate	Titer	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
HEp-2 (microfilaments)	1:40	60.7	94.4	90.2	73.9	79.9
	1:80	59.0	98.6	97.3	74.0	80.5
	1:160	54.1	98.6	97.1	71.7	78.2
	1:320	52.5	98.6	97.0	71.0	77.4
	1:640	41.0	100	100	66.7	72.9
Kidney SMA-V (vessels)	1:40	78.7	45.8	55.2	71.7	60.9
	1:80	73.8	72.2	69.2	76.5	72.9
	1:160	68.9	80.6	75.0	75.3	75.2
	1:320	62.3	88.9	82.6	73.6	76.7
	1:640	49.2	98.6	96.8	69.6	75.9
Kidney SMA-VG (vessels, glomeruli)	1:40	72.1	70.8	67.7	75.0	71.4
	1:80	65.6	88.9	83.3	75.3	78.2
	1:160	63.9	94.4	90.7	75.6	80.5
	1:320	55.7	97.2	94.4	72.2	78.2
	1:640	36.1	100	100	64.9	70.7
Kidney SMA-VGT (vessels, glomeruli tubuli)	1:40	52.5	93.1	86.5	69.8	74.4
	1:80	49.2	93.1	85.7	68.4	72.9
	1:160	44.3	95.8	90.0	67.0	72.2
	1:320	31.2	97.2	90.5	62.5	66.9
	1:640	23.0	100	100	60.5	64.7
Kidney SMA-VG or HEp2 (microfilaments)	1:40	75.4	69.4	67.7	76.9	72.2
	1:80	68.9	88.9	84.0	77.1	79.7
	1:160	65.6	94.4	90.9	76.4	81.2
	1:320	62.3	97.2	95.0	75.3	81.2
	1:640	44.3	100	100	67.9	74.4
Liver (bile canaliculi)	1:40	59.0	83.3	75.0	70.6	72.2
	1:80	49.2	95.8	90.9	69.0	74.4
	1:160	42.6	98.6	96.3	67.0	72.9
	1:320	39.3	98.6	96.0	65.7	71.4
	1:640	26.2	100	100	61.5	66.2
Stomach (tunica muscularis, lamina muscularis mucosa, interglandular fibrils)	1:40	83.6	45.8	56.7	76.7	63.2
	1:80	75.4	72.2	69.7	77.6	73.7
	1:160	72.1	80.6	75.9	77.3	76.7
	1:320	68.9	90.3	85.7	77.4	80.5
	1:640	54.1	97.2	94.3	71.4	77.4
Any SMA positivity	1:40	86.9	37.5	54.1	77.1	60.2
	1:80	80.3	69.4	69.0	80.7	74.4
	1:160	72.1	79.2	74.6	77.0	75.9
	1:320	72.1	88.9	84.6	79.0	81.2
	1:640	60.7	97.2	94.9	74.5	80.5

Autoimmune hepatitis: n = 61; non-alcoholic fatty liver disease: n = 72.
HEp-2 cells, human epithelioma-2 cells; IFT, immunofluorescence test; NPV, negative predictive value; PPV, positive predictive value; SMA, smooth muscle antibodies; VGT, vessel, glomeruli, tubuli.

SMA-VG and finally to SMA-V with increasing dilutions. Taken together, SMA positivity was highly specific even at low titers for SMA-VG/T and anti-MF reactivity on HEp-2 cells, but only at higher titers for other SMA patterns.

ELISA-based autoantibody testing for the diagnosis of AIH

We next assessed the diagnostic value of ELISA-based autoantibody evaluation to discriminate between AIH and controls. Sera from 3 European centers were reassessed by 3 different ANA ELISA and 1 F-actin ELISA. Clinical characteristics of the patient groups at the time of sampling are summarized in Tables S1–3.

ANA testing by the Bio-Rad and Inova ANA ELISA had a similar diagnostic accuracy (AUC 0.85 and 0.87, respectively; $p = 0.32$) and performed significantly better compared to the ANA Euroimmun ELISA (AUC 0.70; $p < 0.001$) (Fig. 2A).

Correlation analyses between the ANA ELISA results found the strongest correlation between the Bio-Rad and Inova ANA ELISA ($r_s = 0.72$; $p < 0.001$) (Fig. S1). Test characteristics of the ANA ELISA kits varied greatly at cut-offs recommended by the manufacturers. In fact, sensitivity and specificity were 65.5% and 88.6% for the Bio-Rad assay (recommended cut-off ≥1 RU), 79.6% and 78.2% for the ANA Inova assay (recommended cut-off ≥20 RU), and 22.1% and 95.0% for the ANA Euroimmun assay (recommended cut-off ≥1 RU), respectively (Table 3).

Like for ANA, we assessed the diagnostic value of a F-actin ELISA. ROC analysis revealed anti-F-actin as a strong predictor of AIH (AUC 0.89) (Fig. 2B). At a cut-off of 20 RU, sensitivity and specificity were 81.4% and 82.2%, respectively; at a cut-off of 30 RU, sensitivity and specificity were 66.4% and 92.6%, respectively (Table 3). Importantly, anti-F-actin was still a predictor of AIH in the subgroup of patients with normal range IgG (≤ 16 g/L; n = 35/109) (AUC 0.79).

ELISA- compared to IFT-based evaluation of autoantibodies

We next compared ELISA- and IFT-based ANA evaluation. To account for the inter-laboratory variability inherent to IFT, ELISA assessment was compared to IFT results obtained by the respective centers according to local standards. Fig. 3 and 4 show the diagnostic performance of ELISA *vs.* IFT for ANA and SMA/F-actin, respectively, for each center. ANA testing by ELISA and IFT

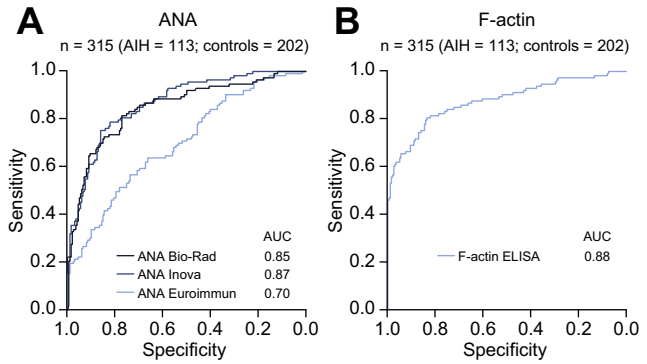

Fig. 2. Receiver-operating-characteristic curves showing the diagnostic value of ELISA for the diagnosis of AIH. Diagnostic performance of (A) 3 different ANA ELISA and (B) a F-actin ELISA to discriminate between AIH and controls (distribution of diagnoses as shown in Fig. 1). AUC values are indicated. AIH, autoimmune hepatitis; ANA, antinuclear antibodies; AUC, area under the curve.

performed similarly for all cohorts, except for the Euroimmun ELISA that showed a significantly lower AUC compared to IFT for the Hamburg cohort (Euroimmun ANA ELISA, AUC 0.65; ANA IFT, AUC 0.82–0.83; p <0.001).

In addition to the patient groups shown in Fig. 1, we tested sera from 26 patients with PBC known to frequently present with ANA. Clinical characteristics of the patients with PBC are detailed in Table S4. While 17/26 (65.4%) patients with PBC tested positive for ANA by IFT on HEp-2 cells at a cut-off of 1:80, 23/26 (88.4%) and 25/26 (96.2%) tested positive by the Bio-Rad and Inova ANA ELISA, respectively. Importantly, median values of the Inova ANA ELISA were significantly higher in patients with PBC compared to patients with AIH (49.6 relative units [RU] AIH *vs.* 161.7 RU PBC; p <0.001) while there was no statistical significant difference for the Bio-Rad ELISA (1.6 RU AIH *vs.* 2.0 RU PBC; p = 0.25).

The F-actin ELISA yielded higher AUC values compared to IFT for each center, reaching statistical significance for the Hamburg cohort when compared to anti-MF reactivity on HEp-2 cells (F-actin ELISA, AUC 0.86; anti-MF AUC 0.79; p = 0.003) and for the Bologna cohort when compared to any SMA reactivity (F-actin ELISA, AUC 0.93; any SMA, AUC 0.77; p = 0.002).

We further assessed the performance of ELISA-based autoantibody testing in the subgroup of patients with a histological diagnosis of cirrhosis. Overall, 24 patients with AIH and 15 controls (4 patients with PSC, 11 patients with NAFLD) with cirrhosis were identified. ANA IFT assessed on tissue sections (available for n = 35; 20 patients with AIH *vs.* 15 controls) reached an AUC of 0.84 whereas ELISA-based ANA assessment yielded higher AUC values of 0.88–0.93, without reaching statistical significance (Fig. S2A). In contrast, anti-F-actin (n = 39) was again a strong predictor of AIH (AUC 0.91) and performed significantly better than SMA assessment by IFT (SMA-VG/T; AUC 0.80; p = 0.049) (Fig. S2B).

Concordance between IFT- and ELISA-based ANA testing

We next assessed concordance between IFT- and ELISA-based autoantibody testing and were specifically interested in the proportion of patients with AIH that tested positive by IFT but were missed when tested by ELISA. Of 51 patients with AIH from the Hamburg cohort that tested positive for ANA by IFT on liver tissue sections, the ANA ELISA by Inova, Bio-Rad and Euroimmun detected 40/51 (78.4%), 28/51 (54.9%), and 10/51 (19.6%) cases at recommended cut-offs, respectively. Conversely, of 10 patients with AIH that tested negative for ANA by IFT, 6 (60%) tested positive by the Inova ELISA and 4 (40%) by the Bio-Rad ELISA. Furthermore, the Inova and Bio-Rad assays detected all but 1 of the ANA-positive AIH cases from the Larissa and Bologna cohorts.

Together, the ROC analysis indicates that ELISA-based tests represent a potential alternative to IFT-based autoantibody assessment. However, assays vary considerably in their performance and cut-offs need to be validated for the diagnosis of AIH. If these aspects are taken into consideration and local cut-offs established, ELISA-based autoantibody testing as proposed in Table 4 can be used in the diagnostic work-up of patients with liver disease.

Discussion

This is the first study to comprehensively evaluate IFT- and ELISA-based assessment of ANA and SMA/anti-F-actin in AIH. In analogy to the simplified IAIHG diagnostic score that largely refers to autoantibody assessment as evaluated by IFT on tissue sections, we propose the implementation of autoantibody testing as measured by IFT on HEp-2 cells and ELISA.

We first aimed to validate the use of HEp-2 cells as substrate for ANA IFT in patients with AIH. As expected, at low titers, ANA as evaluated on HEp-2 cells showed a high sensitivity at the expense of a low specificity. It is precisely the low specificity at a 1:40 titer that led the IAIHG to advise against the use of HEp-2 cells for ANA evaluation at a screening stage.[2] However, to our knowledge, the diagnostic value of ANA IFT on HEp-2 cells has not been assessed at higher titers in the context of liver disease. A previous study investigating ANA IFT in liver disease reported an increased sensitivity of ANA IFT using HEp-2 cells, but was restricted to a 1:40 dilution.[15] Our results suggest that HEp-2 cells are a valid alternative to tissue sections, if threshold titers are adapted. Herein, we propose increasing cut-off titers to 1:160 and 1:320 for the simplified diagnostic score to be applicable. As outlined above, a cut-off titer of 1:160 is also the recommended cut-off for ANA screening in rheumatic diseases.[16] However, titers vary depending on reagents and equipment used and should be validated locally. In addition, the difference in immunofluorescence intensity between tissue sections and HEp-2 cells is not the same for all subtypes of ANA, but highly dependent on the respective ANA pattern. Nevertheless, overall, HEp-2 cells are a valid alternative to tissue sections for ANA evaluation in AIH.

We further compared the diagnostic value of different SMA patterns for the diagnosis of AIH. In line with a study by Muratori and colleagues,[9] we found that specificity was highest for SMA-VGT and anti-MF reactivity at a titer of 1:40. Complementing this previous study, we additionally assessed SMA patterns at further dilutions. Interestingly, sensitivity and specificity of generic SMA at higher titers was comparable to the diagnostic value of SMA-VG/T and anti-MF reactivity at a 1:40 titer. Furthermore, as previously described,[6] we observed a shift from SMA-VGT to SMA-G and then SMA-V with increasing dilutions for individual samples. It thus appears likely that the SMA-VGT pattern is a reflection of high-titer SMA with specificity for F-actin. In contrast, the SMA-V pattern can be seen for both low-titer SMA with anti-F-actin reactivity or SMA targeting other cytoskeletal components. Taken together, our results add to the literature[6,7,9] that highlights the importance of reporting SMA patterns, in both the scientific and clinical context.

Table 3. Diagnostic value of ANA and F-Actin ELISA at cut-offs recommended by manufacturers.

ELISA	Assay	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
ANA ELISA	Bio-Rad	≥1.0	65.5	88.6	76.3	82.1	80.3
	Inova	≥20	79.6	78.2	67.2	87.3	78.7
		≥30	69.0	86.6	74.3	83.3	80.3
	Euroimmun	≥1.0	22.1	95.0	71.4	68.6	68.9
F-Actin ELISA	Inova	≥20	81.4	82.2	71.9	88.8	81.9
		≥30	66.4	92.6	83.3	83.1	83.2

Autoimmune disease: n = 113; controls; n = 202; distribution of diagnoses as shown in Fig. 1.
ANA, antinuclear antibodies; NPV, negative predictive value; PPV, positive predictive value.

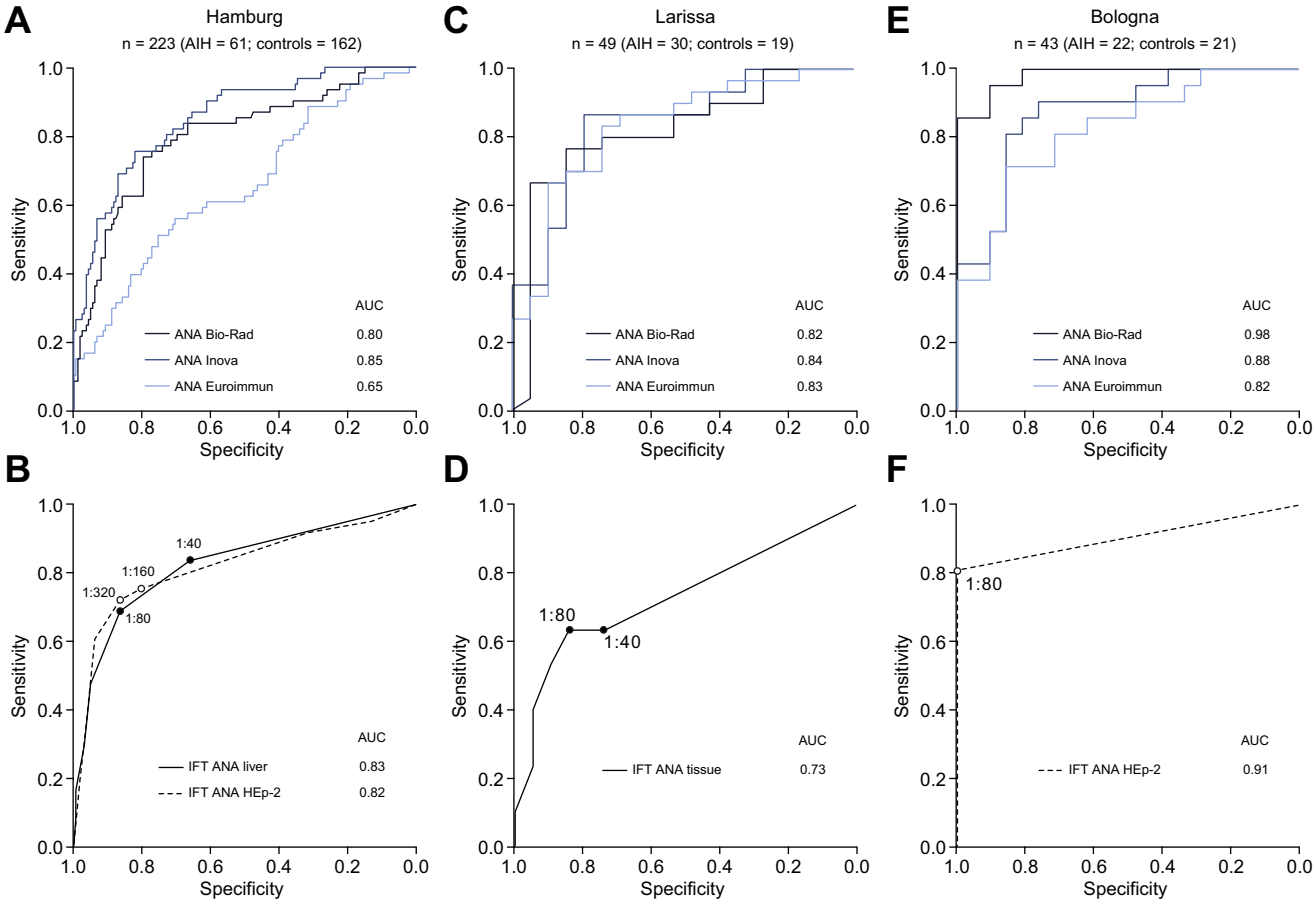

Fig. 3. Receiver-operating-characteristic curves showing the diagnostic performance of 3 different ANA ELISA in comparison with ANA immunofluorescence for the diagnosis of AIH. Diagnostic performance is separately shown for cohorts from (A–B) Hamburg, (C–D) Larissa, and (E–F) Bologna. The distribution of diagnoses is shown in Fig. 1. AUC values are indicated. AIH, autoimmune hepatitis; ANA, antinuclear antibodies; AUC, area under the curve.

Several studies have assessed ANA evaluation by ELISA in rheumatic diseases,[17–21] but analogous studies in AIH are lacking. To fill this gap, we assessed the diagnostic value of 3 different ANA ELISA in patients with AIH. We observed significant differences depending on the ELISA used, with the Bio-Rad and Inova assays performing best. In contrast, at the cut-off recommended by the manufacturer, the Euroimmun ANA ELISA had a low sensitivity of 22.1% at a 95% specificity. These results might be explained by differing ELISA formulations. Indeed, both the Inova and Bio-Rad ANA ELISA include HEp-2 nuclear extracts in addition to recombinant and purified nuclear antigens to account for unrecognized autoantigens. In contrast, the Euroimmun assay is only comprised of selected nuclear antigens. Its antigenic specificities are therefore better defined, ensuring high specificity for the diagnosis of rheumatic diseases. However, our data suggest that this comes at the cost of a low diagnostic value in autoimmune hepatitis. With regard to ELISA formulations, it is also worth mentioning that the Inova ANA ELISA is the only assay in this study including purified ribosomal P and mitochondrial M2 antigen. In a study by Calich and colleagues, autoantibodies against ribosomal P were found in 9/93 (9.7%) patients with AIH and none of the healthy controls.[22] In contrast, the incorporation of mitochondrial antigens is not expected for an ANA screening assay and carries considerable potential for confusion. Indeed, if the Inova ANA ELISA were to be used for the diagnostic work-up of elevated liver enzymes, distinction between ANA and

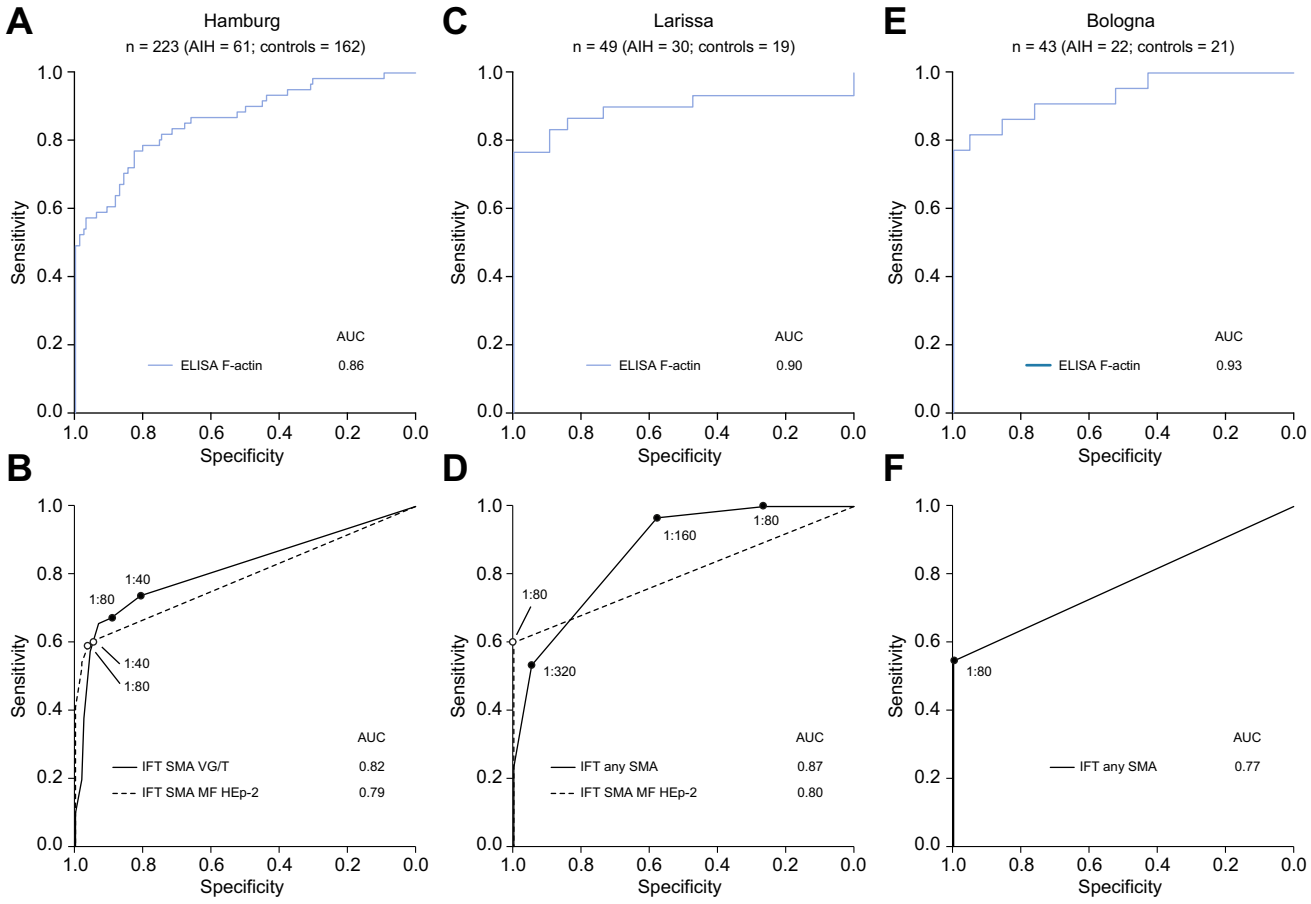

Fig. 4. Receiver-operating-characteristic curves showing the diagnostic performance of a F-actin ELISA in comparison with SMA immunofluorescence for the diagnosis of AIH. Diagnostic performance is separately shown for cohorts from (A–B) Hamburg, (C–D) Larissa, and (E–F) Bologna. The distribution of diagnoses is shown in Fig. 1. AUC values are indicated. AIH, autoimmune hepatitis; AUC, area under the curve; SMA, smooth muscle antibody.

Table 4. Simplified criteria for autoimmune hepatitis – Update of serological criteria.

Variable	Cut-off	Points[1]
ANA or SMA/F-Actin	Positive[2]	1
ANA or SMA/F-Actin	Strongly positive[3]	
or LKM	≥1:40	2
or SLA	Positive	
IgG	>Upper normal limit	1
	>1.1× upper normal limit	2
Liver histology (with evidence of hepatitis)	Compatible with AIH	1
	Typical AIH	2
Absence of viral hepatitis	Yes	2
		≥6: probable AIH
		≥7: definite AIH

[1]Addition of points achieved (maximum 2 points for autoantibodies).
[2]IFT: ≥1:40 when assessed on tissue sections; ≥1:80 or 1:160 for ANA when assessed on HEp-2 cells, depending on local standards. ELISA with locally established cut-offs.
[3]IFT: ≥1:80 when assessed on tissue sections; ≥1:160 or 1:320 for ANA when assessed on HEp-2 cells. ELISA with cut-offs established locally; Note: if ELISA-based autoantibody assessment is negative despite high clinical suspicion of autoimmune hepatitis, IFT should be performed in addition.

antimitochondrial antibodies (AMA) would not be possible in a reasonable fashion. Incorporation of mitochondrial antigens also likely explains the significantly higher values of the Inova ANA ELISA in patients with PBC compared to patients with AIH. Overall, while the careful choice of ELISA formulation and validation of cut-offs is critical, our data suggest that, in principle, ELISA testing represents a potentially good alternative to ANA IFT. Importantly, if ELISA-based autoantibody assessment is negative despite clinical suspicion of AIH, additional IFT should be performed.

In the present study, we further compared IFT-based SMA evaluation to an anti-F-actin ELISA. Consistent with previous results,[23] we found that anti-F-actin had a significantly higher diagnostic value for the diagnosis of AIH. Interestingly, while hypergammaglobulinemia potentiated the predictive value of anti-F-actin for the diagnosis of AIH, F-actin autoantibodies were still a strong predictor of AIH in the subgroup of patients with AIH and IgG within the normal range (AUC 0.79).

Several limitations to the present study warrant further discussion. First, IFT allows for the detection of additional autoantibodies such as AMA and provides characteristic staining patterns that point towards antigenic specificities of ANA. The benefit of this relevant information was not assessed in the present study. While ANA ELISA do not provide such additional information, some specific and reliable tests exist to further assess antigen specificity of ANA-positive sera. Indeed, most of the PBC sera we tested were highly positive both in the Inova ANA ELISA, which does however include the M2 antigen, the key target of antimitochondrial antibodies characteristic of PBC, as

well as in the Bio-Rad ANA ELISA. Thus, for discrimination between AIH and PBC sera, further systematic testing by a specific M2-AMA ELISA and by sp100 and gp210 ELISA would be required. However, this would have been beyond the scope of the present study.

Second, we included only 1 F-actin ELISA. However, compared to the heterogeneous group of ANA, F-actin is a defined antigen and the F-actin ELISA used in this study was investigated in 2 previous studies.[7,23]

Furthermore, while control cohorts were well characterized, relevant patient groups, such as patients with drug-induced liver injury, were not included in the present study. Finally, the gender distribution between AIH and controls was somewhat unbalanced reflecting the natural sex differences in these conditions. Although this potentially influenced the frequency of autoantibodies in patient groups, it most probably did not affect how the various autoantibody assays compared to one another.

In conclusion, our results suggest that both IFT evaluation on HEp-2 cells as well as ELISA-based autoantibody assessment are potential alternatives to IFT on tissue sections. Our data indicate that i) HEp-2 cells can be used for ANA assessment in AIH if scoring cut-off titers are increased; ii) The SMA-VG/T pattern and anti-MF reactivity on HEP-2 cells are highly specific even at low titers, while generic SMA is specific only at higher titers; iii) ANA and F-actin ELISA show at least equivalent diagnostic performance compared to IFT, but ELISA kits for ANA assessment should include HEp-2 nuclear extracts to account for unknown nuclear antigens and cut-offs need to be validated for the use in AIH. In the future, cut-off values for autoantibody testing should be determined and validated by industry on standardized AIH sera and controls and re-validated by diagnostic laboratories, as technical details may influence the exact values. Nonetheless, the objective nature of these tests will make them more attractive in the future, avoiding observation errors due to the subjective assessment of staining patterns as in SMA testing on tissue sections. Based on our results, under the prerequisite of careful selection of ELISA formulation and validation of cut-offs, we propose an adaptation of the simplified diagnostic score for AIH as summarized in Table 4 for everyday use in different laboratory settings.

Abbreviations

AMA, antimitochondrial antibodies; ANA, antinuclear antibodies; AUC, area under the curve; HEp-2, human epithelioma-2; IAIHG, International Autoimmune Hepatitis Group; IFT, immunofluorescence testing; MF, microfilament; PBC, primary biliary cholangitis; PPV, positive predictive value; PSC, primary sclerosing cholangitis; ROC, receiver-operating characteristic; RU, relative units; SMA, smooth muscle antibodies.

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Conflict of interest

Weiler-Normann C reports speaker's fees from Euroimmun and Werfen (Inova) to her institution. All other authors declare no conflict of interest with respect to this study.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Galaski J: substantial contribution to conception and design, data acquisition and analysis, interpretation of data, drafting of the article. Weiler-Normann C: substantial contribution to conception and design, data acquisition and interpretation of data, critical revision of the article for important intellectual content. Schakat M, Zachou K, Muratori P, Lampalzer S, Haag F, Lenzi M, Dalekos GN: substantial contribution to data acquisition, critical revision. Schramm, C: critical revision of the article for important intellectual content. Lohse AW: substantial contribution to conception and design, interpretation of data, critical revision of the article for important intellectual content. All authors approved submission.

Data availability statement

The dataset generated during this study is available from the corresponding author upon reasonable request.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2020.07.032.

References

[1] Hennes EM, Zeniya M, Czaja AJ, Pares A, Dalekos GN, Krawitt EL, et al. Simplified criteria for the diagnosis of autoimmune hepatitis. Hepatology 2008;48:169–176.
[2] Vergani D, Alvarez F, Bianchi FB, Cancado EL, Mackay IR, Manns MP, et al. Liver autoimmune serology: a consensus statement from the committee for autoimmune serology of the International Autoimmune Hepatitis Group. J Hepatol 2004;41:677–683.
[3] Tan EM, Feltkamp TE, Smolen JS, Butcher B, Dawkins R, Fritzler MJ, et al. Range of antinuclear antibodies in "healthy" individuals. Arthritis Rheum 1997;40:1601–1611.
[4] Tozzoli R, Villalta D, Bizzaro N. Challenges in the standardization of autoantibody testing: a comprehensive review. Clin Rev Allergy Immunol 2017;53:68–77.
[5] Dighiero G, Lymberi P, Monot C, Abuaf N. Sera with high levels of anti-smooth muscle and anti-mitochondrial antibodies frequently bind to cytoskeleton proteins. Clin Exp Immunol 1990;82:52–56.
[6] Bottazzo GF, Florin-Christensen A, Fairfax A, Swana G, Doniach D, Groeschel-Stewart U. Classification of smooth muscle autoantibodies detected by immunofluorescence. J Clin Pathol 1976;29:403–410.
[7] Granito A, Muratori L, Muratori P, Pappas G, Guidi M, Cassani F, et al. Antibodies to filamentous actin (F-actin) in type 1 autoimmune hepatitis. J Clin Pathol 2006;59:280–284.
[8] Liaskos C, Bogdanos DP, Davies ET, Dalekos GN. Diagnostic relevance of anti-filamentous actin antibodies in autoimmune hepatitis. J Clin Pathol 2007;60:107–108.
[9] Muratori P, Muratori L, Agostinelli D, Pappas G, Veronesi L, Granito A, et al. Smooth muscle antibodies and type 1 autoimmune hepatitis. Autoimmunity 2002;35:497–500.
[10] Czaja AJ, Nishioka M, Morshed SA, Hachiya T. Patterns of nuclear immunofluorescence and reactivities to recombinant nuclear antigens in autoimmune hepatitis. Gastroenterology 1994;107:200–207.
[11] EASL Clinical Practice Guidelines: autoimmune hepatitis. J Hepatol 2015;63:971–1004.
[12] EASL Clinical Practice Guidelines: management of cholestatic liver diseases. J Hepatol 2009;51:237–267.
[13] European Association for the Study of the Liver. EASL Clinical Practice Guidelines: the diagnosis and management of patients with primary biliary cholangitis. J Hepatol 2017;67:145–172.
[14] EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. J Hepatol 2016;64:1388–1402.
[15] Cassani F, Bianchi FB, Lenzi M, Volta U, Pisi E. Immunomorphological characterisation of antinuclear antibodies in chronic liver disease. J Clin Pathol 1985;38:801–805.
[16] Agmon-Levin N, Damoiseaux J, Kallenberg C, Sack U, Witte T, Herold M, et al. International recommendations for the assessment of

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OF HEPATOLOGY

autoantibodies to cellular antigens referred to as anti-nuclear antibodies. Ann Rheum Dis 2014;73:17–23.

[17] Copple SS, Sawitzke AD, Wilson AM, Tebo AE, Hill HR. Enzyme-linked immunosorbent assay screening then indirect immunofluorescence confirmation of antinuclear antibodies: a statistical analysis. Am J Clin Pathol 2011;135:678–684.

[18] Reisner BS, DiBlasi J, Goel N. Comparison of an enzyme immunoassay to an indirect fluorescent immunoassay for the detection of antinuclear antibodies. Am J Clin Pathol 1999;111:503–506.

[19] Bernardini S, Infantino M, Bellincampi L, Nuccetelli M, Afeltra A, Lori R, et al. Screening of antinuclear antibodies: comparison between enzyme immunoassay based on nuclear homogenates, purified or recombinant antigens and immunofluorescence assay. Clin Chem Lab Med 2004;42:1155–1160.

[20] Gniewek RA, Sandbulte C, Fox PC. Comparison of antinuclear antibody testing methods by ROC analysis with reference to disease diagnosis. Clin Chem 1997;43:1987–1989.

[21] Fenger M, Wiik A, Hoier-Madsen M, Lykkegaard JJ, Rozenfeld T, Hansen MS, et al. Detection of antinuclear antibodies by solid-phase immunoassays and immunofluorescence analysis. Clin Chem 2004;50:2141–2147.

[22] Calich AL, Viana VS, Cancado E, Tustumi F, Terrabuio DR, Leon EP, et al. Anti-ribosomal P protein: a novel antibody in autoimmune hepatitis. Liver Int 2013;33:909–913.

[23] Frenzel C, Herkel J, Luth S, Galle PR, Schramm C, Lohse AW. Evaluation of F-actin ELISA for the diagnosis of autoimmune hepatitis. Am J Gastroenterol 2006;101:2731–2736.