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1 **Update of the simplified criteria for autoimmune hepatitis: evaluation of the**
2 **methodology for immunoserological testing**

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33

34 **Conflict of Interest**

35 Weiler-Normann C reports speaker's fees from Euroimmun and Werfen (Inova) to her
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42

43 **Data availability statement**

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

46

47 **Author contributions**

48 Galaski J: substantial contribution to conception and design, data acquisition and
49 analysis, interpretation of data, drafting of the article

50 Weiler-Normann C: substantial contribution to conception and design, data
51 acquisition and interpretation of data, critical revision of the article for important
52 intellectual content

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53 Schakat M, Zachou K, Muratori P, Lampalzer S, Haag F, Lenzi M, Dalekos GN:
54 substantial contribution to data acquisition, critical revision

55 Schramm, C: critical revision of the article for important intellectual content

56 Lohse AW: substantial contribution to conception and design, interpretation of data,
57 critical revision of the article for important intellectual content

58 All authors approved submission.

59 **Lay summary**

60 Autoantibodies are a hallmark of autoimmune hepatitis and are traditionally tested for
61 by immunofluorescence assays on rodent tissue sections. Herein, we demonstrate
62 that both HEp-2 cells as substrate for ANA IFT and ELISA-based testing are
63 potentially reliable alternatives for autoantibody assessment in autoimmune hepatitis.
64 We propose the implementation of these testing methods into the simplified criteria
65 for the diagnosis of autoimmune hepatitis.

66

67 **Highlights**

- 68 • IFT on HEp-2 cells is a valid alternative to the standard ANA assessment on
69 rodent tissue sections in AIH when cutoffs titers are increased
- 70 • ANA ELISA and F-actin ELISA represent potential alternatives to IFT in the
71 diagnosis of AIH
- 72 • ANA ELISA kits should include HEp-2 nuclear extracts to account for
73 unrecognized autoantigens
- 74 • ELISA cutoffs need to be validated locally to be predictive in diagnosing AIH

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79 Abstract

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5 81 **Background & Aims:** The simplified criteria for the diagnosis of autoimmune
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7 82 hepatitis (AIH) include immunofluorescence testing (IFT) of antinuclear and smooth
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9 83 muscle autoantibodies (ANA and SMA) on rodent tissue sections. We aimed to
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11 84 establish scoring criteria for implementation of ANA IFT on HEp-2 cells and ELISA-
12
13 85 based testing. **Methods:** ANA and SMA reactivity of 61 AIH sera and 72 non-
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15 86 alcoholic fatty liver disease (NAFLD) controls were separately assessed on tissue
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17 87 sections and human epithelioma (HEp-2) cells to compare the diagnostic value at
18
19 88 increasing titers. A total of 113 AIH patients at diagnosis and 202 controls from three
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21 89 European centers were assessed by IFT as well as three different commercially
22
23 90 available ANA ELISA and one anti-F-actin ELISA. **Results:** ANA assessment by IFT
24
25 91 on liver sections had 83.6% sensitivity and 69.4% specificity for AIH at a titer of 1:40.
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27 92 On HEp-2 cells, sensitivity and specificity were 75.4% and 73.6%, respectively, at an
28
29 93 adjusted titer of 1:160. Area under the curve (AUC) values of ANA ELISA ranged
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31 94 from 0.70 – 0.87, with ELISA coated with HEp-2 extracts in addition to selected
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33 95 antigens performing significantly better. SMA assessment by IFT had the highest
34
35 96 specificity for the SMA-VG/T pattern and anti-MF reactivity on HEp-2 cells. ELISA-
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37 97 based anti-F-actin evaluation was a strong predictor of AIH (AUC 0.88) and
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39 98 performed better than SMA assessment by IFT (AUC 0.77 – 0.87). **Conclusion:** At
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41 99 adjusted cutoffs, both ANA IFT using HEp-2 cells and ELISA-based autoantibody
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43 100 evaluation for ANA and SMA are potential alternatives to tissue-based IFT for the
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45 101 diagnosis of AIH.
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58 103 Introduction

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105 Autoimmune hepatitis (AIH) is a chronic immune-mediated liver disease. Due to
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2 106 heterogeneity of the presentation, the diagnosis remains challenging. An early
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4 107 diagnosis is, however, critical for timely initiation of life-saving immunosuppressive
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7 108 therapy. To assist diagnostic evaluation, a simplified diagnostic score was
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10 109 established by the International Autoimmune Hepatitis Group (IAIHG) in 2008 for use
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12 110 in clinical practice [1]. Scoring criteria include characteristic findings on liver
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14 111 histology, the absence of viral hepatitis, an elevation of immunoglobulin G (IgG), and
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17 112 circulating autoantibodies.

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19 113 Autoantibodies associated with AIH include antinuclear antibodies (ANA), smooth
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21 114 muscle antibodies (SMA), liver kidney microsomal type 1 (LKM1) antibodies, liver
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24 115 cytosol type 1 (LC1) antibodies, and soluble liver antigen/liver pancreas (SLA/LP)
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26 116 antibodies. Screening for liver disease-associated autoantibodies is traditionally
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29 117 performed by immunofluorescence testing (IFT) on rodent tissue sections.
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32 118 Accordingly, the simplified AIH score refers to autoantibody titers as measured by
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34 119 IFT using tissue sections at a cutoff titer of 1:40. However, in several laboratories,
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36 120 there has been a shift of autoantibody assessment towards human epithelioma (HEp-
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39 121 2) cells rather than tissue sections as substrate for IFT. Furthermore, enzyme-linked
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41 122 immunosorbent assays (ELISA), for which the score does not account for, are
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44 123 frequently used in some countries. In order to make the simplified AIH score usable
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46 124 across the world, adaptation of the score to different immunoserology methods is
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49 125 urgently needed.

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51 126 HEp-2 cells are widely used as substrate for ANA evaluation. In addition to a higher
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53 127 sensitivity, characteristic staining patterns evaluated on HEp-2 cells are useful in
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56 128 guiding further confirmatory testing. However, a consensus statement by the IAIHG
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58 129 committee for autoimmune serology advises against the use of HEp-2 cells at a
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61 130 screening stage [2] because of a high positivity rate in healthy individuals at low
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131 cutoff titers [3]. If HEp-2 cells are used, the IAIHG suggests titers should be halved
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2 132 for the simplified score to be applicable [1]. However, this possible correction factor
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5 133 suggestion has never been validated by comparative studies [4].
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7 134 SMA constitute a heterogeneous group of autoantibodies that primarily target F-actin,
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10 135 [5]. On kidney tissue sections, Bottazzo and colleagues distinguished three
11
12 136 immunofluorescence patterns: SMA-V (vessels), SMA-VG (vessels/glomeruli), and
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14 137 SMA-VGT (vessels/ glomeruli/ tubuli) [6]. In contrast to the SMA-V pattern, SMA-
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17 138 VG/T correlates with F-actin reactivity and is more specific for AIH [6-8]. Similarly,
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19 139 anti-F-actin antibodies stain microfilaments (MF) on HEp-2 cells [9]. Overall,
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22 140 sensitivity and specificity of SMA positivity strongly depend on fluorescence patterns,
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24 141 which is not taken into consideration by current AIH scoring systems.
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26 142 Since IFT is time-consuming, requires experienced technicians and lacks
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29 143 standardization, ELISA have emerged as a widely used alternative for routine
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32 144 autoantibody testing in many laboratories, especially in the United States. These
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34 145 tests were originally developed for use in the evaluation of rheumatic diseases and
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36 146 their diagnostic value in liver disease is unknown. ELISA testing can minimize
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39 147 interobserver variability inherent to IFT. However, it is unclear whether ELISA can
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41 148 replace IFT for the detection of the heterogeneous autoantibodies ANA and SMA
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44 149 with their range of antigenic specificities. To complicate matters even further, up to
45
46 150 30% of ANA-positive AIH patients do not react with any known nuclear antigens [10]
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49 151 and might thus be missed by ELISA testing, which are based primarily on known
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51 152 nuclear antigens. In addition, commercially available ANA ELISA lack standardization
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53 153 – they differ in their antigenic profiles and assay-specific cutoff values.
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55
56 154 Taken together, the AIH simplified score does not account for ANA and SMA as
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58 155 evaluated by IFT on HEp-2 cells or for ELISA, even though these tests are widely
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61 156 used. We therefore set out to study the diagnostic validity of IFT and ELISA-based
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157 autoantibody testing for the diagnosis of AIH to make these applicable in diagnosing
1 158 AIH.

2 159

3 160 **Patients and methods**

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5 162 **Study population**

6 163 This multicenter study included a total of 113 patients with AIH at diagnosis and 202
7 164 controls (82 NAFLD patients, 99 primary sclerosing cholangitis (PSC) patients and 21
8 165 healthy controls) from three centers: Hamburg (Germany), Bologna (Italy), and
9 166 Larissa (Greece). A flow-chart of patient cohorts is shown in Figure 1. The large
10 167 majority of AIH patients (106/113, 93.8%) were treatment-naïve at the time of
11 168 sampling. In addition, sera from 26 patients with primary biliary cholangitis (PBC)
12 169 were tested and analyzed separately. Sera were collected between December 2006
13 170 and March 2020 and stored at -80°C until use. The study was approved by the local
14 171 ethics committee (PV4081-0005, PV 4081-0008).

15 172 The diagnosis of AIH was based on clinical, serological, and histopathological
16 173 criteria, consistent with the EASL clinical practice guidelines [11], and confirmed by
17 174 long-term follow-up in all patients. Patients with AIH and features of PSC or PBC
18 175 were excluded from the study. Diagnoses of disease controls were based on
19 176 established diagnostic criteria [12-14]. Blood donors with liver enzymes within the
20 177 normal range, negative for HBV/HCV, and negative for autoantibodies by IFT were
21 178 included as healthy controls.

22 179

23 180 **Autoantibody assessment by IFT**

24 181 IFT was performed in the respective center in which sera were collected. At the
25 182 University Medical Center Hamburg-Eppendorf sera were tested using a Biochip

183 Mosaic of primate liver, rat kidney, and rat stomach tissue sections as well as human
1
2 184 epithelioma (HEp-2) cells (Mosaic Basic Profile 3, Euroimmun, Germany). The assay
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5 185 was performed manually according to the manufacturer's instructions at a dilution of
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7 186 1:40. Further dilutions up to 1:1280 were processed by the Helios automated IFA
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10 187 system (Aesku Diagnostics, Wendelsheim, Germany), using the same substrates
11
12 188 and conditions. Reactivity patterns were assessed under a fluorescence microscope
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14 189 (Eurostar, Euroimmun, Germany). ANA and SMA reactivity were separately
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16
17 190 evaluated on all four substrates. SMA reactivity on kidney sections was assessed
18
19 191 according to Bottazzo et al. [6]. The observers were blinded to clinical data.

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22 192 Sera from the University Hospital of Bologna, Italy, were tested by IFT on both tissue
23
24 193 sections and HEp-2 cells (Euroimmun, Germany) and were automatically processed
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27 194 at a starting dilution of 1:80 up to 1:640. ANA titers were mainly reported as
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29 195 assessed on HEp-2 cells and thus these data were used for comparison with ANA
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31 196 ELISA.

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34 197 Sera from the University Hospital of Larissa, Greece, were tested by
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36 198 immunofluorescence on in-house fresh cryostat liver, kidney and stomach rat
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39 199 sections and HEp-2 cells (Inova Diagnostics). ANA titers were mainly reported as
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41 200 assessed on tissue sections and thus these data were used for comparison with ANA
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44 201 ELISA. Sera were processed manually at a starting dilution of 1:40 up to 1:640.

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48 203 **Detection of antinuclear and F-actin antibodies by ELISA**

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51 204 All ELISA testing was performed at the University Medical Center Hamburg-
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53 205 Eppendorf. Antinuclear antibodies were assessed using enzyme immunoassays from
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56 206 three different manufacturers (Quanta Lite ANA ELISA, Inova Diagnostics, US; ANA
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58 207 Screening Test, Bio-Rad, US; ANA Screen ELISA, Euroimmun, Germany). All assays
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61 208 detect autoantibodies of IgG subtype and display antigenic specificities to dsDNA,
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209 histones, Sm/RNP, SS-A, SS-B, Scl-70, centromere, and Jo-1. The Quanta Lite ANA
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2 210 ELISA is additionally coated with highly purified proliferating cell nuclear antigen
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4 211 (PCNA), mitochondrial M2 antigen, and ribosomal-P proteins. Besides individual
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7 212 antigens, immunoassays from both Inova Diagnostics and Bio-Rad include HEp-2
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9 213 cell nuclei extracts.

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12 214 Antibodies to F-actin were detected using a commercial ELISA (Quanta Lite Actin
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14 215 IgG, Inova Diagnostics, US). All enzyme immunoassays were performed in
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17 216 duplicates according to the manufacturer's recommendations. Investigators who
18
19 217 carried out immunoassays were blinded to clinical data and the results of IFT.

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23 24 219 **Statistical analyses**

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26 220 Data was expressed as median (range), or n (%) as appropriate. Statistical
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29 221 significance between groups was assessed with Fisher's exact test for categorical
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31 222 variables and the Mann-Whitney *U* test for continuous variables. Correlations were
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34 223 evaluated using Spearman correlation coefficients. The diagnostic value of variables
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36 224 in discriminating AIH from controls was assessed by receiver operating characteristic
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39 225 (ROC) analysis. Statistical significance between area under the curve (AUC) values
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41 226 was assessed by the DeLong test. All reported *P* values are based on two-sided
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44 227 tests and a *P* value < 0.05 was considered statistically significant. Statistical analyses
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46 228 were performed using GraphPad Prism (version 6), IBM SPSS (version 23), and R
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48 229 software (version 3.5.1).

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52 53 231 **Results**

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57 58 233 **Comparison of HEp-2 cells and tissue sections as substrates for ANA IFT**

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234 We first investigated the diagnostic value of HEp-2 cells in comparison to tissue
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2 235 sections as substrates for ANA IFT in the context of AIH. To this end, sera from 61
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4 236 AIH patients and 72 patients with biopsy-proven NAFLD treated at the University
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7 237 Medical Center Hamburg-Eppendorf were evaluated for autoantibodies by IFT.
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10 238 Clinical characteristics of the patient groups at the time of sampling are summarized
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12 239 in supplemental Table 1.
13
14 240 Sensitivity and specificity of ANA IFT for HEp-2 cells and tissue sections are shown
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17 241 in Table 1. Among tissue sections, primate liver showed the highest diagnostic value
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19 242 for ANA evaluation. Sensitivity and specificity were 83.6% and 69.4% at a titer of
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22 243 1:40, respectively, and 68.9% and 80.6% at a titer of 1:80, respectively. Specificity
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24 244 increased to 91.7% at a titer of 1:160 at the cost of a lower sensitivity of 47.5%. As
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26 245 expected, the use of HEp-2 cells led to higher titers. Specificity was inadequate at a
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29 246 1:40 dilution. At a titer of 1:80, sensitivity was 91.8% at a low specificity of 36.1%. At
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32 247 higher titers, sensitivity and specificity were comparable to those observed on liver
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34 248 sections: 75.4% and 73.6%, respectively, at a titer of 1:160; 72.1% and 76.4%,
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36 249 respectively, at a titer of 1:320. The homogenous pattern was significantly more
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39 250 frequent in AIH patients (41.0%) than in NAFLD patients (6.9%, $P < 0.001$).

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44 252 **Sensitivity and specificity of SMA fluorescence patterns on tissue sections and**
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46 253 **HEp-2 cells**

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48 254 We next assessed the diagnostic value of several SMA fluorescence patterns at
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51 255 different titers (Table 2). As expected, at a 1:40 titer, the SMA-V pattern on kidney
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53 256 sections, staining of smooth muscle on stomach sections as well as consideration of
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56 257 any SMA positivity resulted in a low specificity of 33.3% – 45.8%. In contrast, the
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58 258 SMA-VG pattern was more specific for the diagnosis of AIH even at low titers.
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61 259 Sensitivity and specificity were 72.1% and 70.8%, respectively, at a titer of 1:40, and
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260 65.6% and 88.9%, respectively, at a titer of 1:80. The highest specificity was seen for
261 the SMA-VGT pattern and anti-MF reactivity on HEp-2 cells. At a 1:40 dilution,
262 specificity was 93.1% – 94.4% at a sensitivity of 52.5% – 60.7%. Of note, with
263 increasing titers, staining of the SMA-VGT pattern first faded for tubuli, then
264 glomeruli, and finally vessels. In other terms, the SMA-VGT pattern changed to SMA-
265 VG and finally to SMA-V with increasing dilutions. Taken together, SMA positivity
266 was highly specific even at low titers for SMA-VG/T and anti-MF reactivity on HEp-2
267 cells, but only at higher titers for other SMA patterns.

269 **ELISA-based autoantibody testing for the diagnosis of AIH**

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

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

278 Correlation analyses between the ANA ELISA results found the strongest correlation
279 between the Bio-Rad and Inova ANA ELISA ($r_s = 0.72$; $P < 0.001$) (Supplemental
280 Figure 1). Test characteristics of the ANA ELISA kits varied greatly at cutoffs
281 recommended by the manufacturers. In fact, sensitivity and specificity were 65.5%
282 and 88.6% for the Bio-Rad assay (recommended cutoff ≥ 1 RU), 79.6% and 78.2%
283 for the ANA Inova assay (recommended cutoff ≥ 20 RU), and 22.1% and 95.0% for
284 the ANA Euroimmun assay (recommended cutoff ≥ 1 RU), respectively (Table 3).

285 Like for ANA, we assessed the diagnostic value of a F-actin ELISA. ROC analysis
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2 286 revealed anti-F-actin as a strong predictor of AIH (AUC 0.89) (Figure 2B). At a cutoff
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4 287 of 20 RU, sensitivity and specificity were 81.4% and 82.2%, respectively; at a cutoff
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7 288 of 30 RU, sensitivity and specificity were 66.4% and 92.6%, respectively (Table 3).
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9 289 Importantly, anti-F-actin was still a predictor of AIH in the subgroup of patients with
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12 290 normal range IgG (≤ 16 g/l; $n = 35/109$) (AUC 0.79).
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14 291 **ELISA- compared to IFT-based evaluation of autoantibodies**

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16
17 292 We next compared ELISA- and IFT-based ANA evaluation. To account for the inter-
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19 293 laboratory variability inherent to IFT, ELISA assessment was compared to IFT results
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22 294 obtained by the respective centers according to local standards. Figure 3 and 4 show
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24 295 the diagnostic performance of ELISA vs. IFT for ANA and SMA/F-actin, respectively,
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26 296 for each center. ANA testing by ELISA and IFT performed similarly for all cohorts,
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29 297 except for the Euroimmun ELISA that showed a significantly lower AUC compared to
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32 298 IFT for the Hamburg cohort (Euroimmun ANA ELISA, AUC 0.65; ANA IFT, AUC 0.82
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34 299 – 0.83; $P < 0.001$).
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39 301 In addition to the patient groups shown in Figure 1, we tested sera from 26 PBC
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41 302 patients known to frequently present with ANA. Clinical characteristics of PBC
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43
44 303 patients are detailed in supplemental Table 4. While 17/26 (65.4%) of PBC patients
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46 304 tested positive for ANA by IFT on HEp-2 cells at a cut-off of 1:80, 23/26 (88.4%) and
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48
49 305 25/26 (96.2%) tested positive by the Bio-Rad and Inova ANA ELISA, respectively.
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51 306 Importantly, median values of the Inova ANA ELISA were significantly higher in PBC
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53 307 patients compared to AIH patients (49.6 RU AIH vs. 161.7 RU PBC; $P < 0.001$) while
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55
56 308 there was no statistical significant difference for the Bio-Rad ELISA (1.6 RU AIH vs.
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58 309 2.0 RU PBC; $P = 0.25$).
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310 The F-actin ELISA yielded higher AUC values compared to IFT for each center,
311 reaching statistical significance for the Hamburg cohort when compared to anti-MF
312 reactivity on HEp-2 cells (F-actin ELISA, AUC 0.86; anti-MF AUC 0.79; $P = 0.003$)
313 and for the Bologna cohort when compared to any SMA reactivity (F-actin ELISA,
314 AUC 0.93; any SMA, AUC 0.77; $P = 0.002$).

315 We further assessed the performance of ELISA-based autoantibody testing in the
316 subgroup of patients with a histological diagnosis of liver cirrhosis. Overall, 24 AIH
317 patients and 15 controls (4 PSC patients, 11 NAFLD patients) with cirrhosis were
318 identified. ANA IFT assessed on tissue sections (available for $n = 35$; 20 AIH patients
319 vs. 15 controls) reached an AUC of 0.84 whereas ELISA-based ANA assessment
320 yielded higher AUC values of 0.88 – 0.93, without reaching statistical significance
321 (supplemental Figure 2A). In contrast, anti-F-actin ($n = 39$) was again a strong
322 predictor of AIH (AUC 0.91) and performed significantly better than SMA assessment
323 by IFT (SMA-VG/T; AUC 0.80; $P = 0.049$) (supplemental Figure 2B).

324 325 **Concordance between IFT- and ELISA-based ANA testing**

326 We next assessed concordance between IFT- and ELISA-based autoantibody testing
327 and were specifically interested in the proportion of AIH patients that tested positive
328 by IFT but were missed when tested by ELISA. Of 51 AIH patients from the Hamburg
329 cohort that tested positive for ANA by IFT on liver tissue sections, the ANA ELISA by
330 Inova, Bio-Rad and Euroimmun detected 40/51 (78.4%), 28/51 (54.9%), and 10/51
331 (19.6%) cases at recommended cut-offs, respectively. Conversely, of 10 AIH patients
332 that tested negative for ANA by IFT, 6 (60%) tested positive by the Inova ELISA and
333 4 (40%) by the Bio-Rad ELISA. Furthermore, the Inova and Bio-Rad assays detected
334 all but one of ANA-positive AIH cases from the Larissa and Bologna cohorts.

335 Together, the ROC analysis indicates that ELISA represent a potential alternative to
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2 336 IFT-based autoantibody assessment. However, assays vary considerably in their
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5 337 performance and cut-offs need to be validated for the diagnosis of AIH. If these
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7 338 aspects are taken under consideration and local cut-offs established, ELISA-based
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10 339 autoantibody testing as proposed in Table 4 can be used in the diagnostic work-up of
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12 340 liver disease patients.

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17 342 Discussion

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22 344 This is the first study to comprehensively evaluate IFT- and ELISA-based
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24 345 assessment of ANA and SMA/anti-F-actin in AIH. In analogy to the simplified IAIHG
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26 346 diagnostic score that largely refers to autoantibody assessment as evaluated by IFT
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29 347 on tissue sections, we propose the implementation of autoantibody testing as
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31 348 measured by IFT on HEp-2 cells and ELISA.

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34 349 We first aimed to validate the use of HEp-2 cells as substrate for ANA IFT in patients
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36 350 with AIH. As expected, at low titers, ANA as evaluated on HEp-2 cells showed a high
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39 351 sensitivity at the expense of a low specificity. It is precisely the low specificity at a
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41 352 1:40 titer that led the IAIHG to advise against use of HEp-2 cells for ANA evaluation
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44 353 at a screening stage [2]. However, to our knowledge, the diagnostic value of ANA IFT
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46 354 on HEp-2 cells has not been assessed at higher titers in the context of liver disease.

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49 355 A previous study investigating ANA IFT in liver disease reported an increased
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51 356 sensitivity of ANA IFT using HEp-2 cells, but was restricted to a 1:40 dilution [15].
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53 357 Our results suggest that HEp-2 cells are a valid alternative to tissue sections, if
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56 358 threshold titers are adapted. We here propose increasing cutoff titers to 1:160 and
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58 359 1:320 for the simplified diagnostic score to be applicable. As outlined above, a cutoff
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61 360 titer of 1:160 is also the recommended cutoff for ANA screening in rheumatic
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361 diseases [16]. However, titers vary depending on reagents and equipment used and
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2 362 should be validated locally. In addition, the difference in immunofluorescence
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5 363 intensity between tissue sections and HEp-2 cells is not the same for all subtypes of
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7 364 ANA, but highly dependent on the respective ANA pattern. Nevertheless, overall,
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10 365 HEp-2 cells are a valid alternative to tissue sections for ANA evaluation in AIH.

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12 366 We further compared the diagnostic value of different SMA patterns for the diagnosis
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14 367 of AIH. In line with a study by Muratori and colleagues [9], we found that specificity
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17 368 was highest for SMA-VGT and anti-MF reactivity at a titer of 1:40. Complementing
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19 369 this previous study, we additionally assessed SMA patterns at further dilutions.
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22 370 Interestingly, sensitivity and specificity of generic SMA at higher titers was
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24 371 comparable to the diagnostic value of SMA-VG/T and anti-MF reactivity at a 1:40
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27 372 titer. Furthermore, as previously described [6], we observed a shift from SMA-VGT
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29 373 to SMA-G and then SMA-V with increasing dilutions for individual samples. It thus
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32 374 appears likely that the SMA-VGT pattern is a reflection of high-titer SMA with
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34 375 specificity for F-actin. In contrast, the SMA-V pattern can be seen for both low-titer
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36 376 SMA with anti-F-actin reactivity or SMA targeting other cytoskeletal components.
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39 377 Taken together, our results add to the literature [6, 7, 9] that highlights the
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41 378 importance of reporting SMA patterns, in both the scientific and clinical context.

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44 379 Several studies have assessed ANA evaluation by ELISA in rheumatic diseases [17-
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46 380 21], but analogous studies in AIH are lacking. To fill this gap, we assessed the
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49 381 diagnostic value of three different ANA ELISA in AIH patients. We observed
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51 382 significant differences depending on the ELISA used, with the Bio-Rad and Inova
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53 383 assays performing best. In contrast, at the cut-off recommended by the
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56 384 manufacturer, the Euroimmun ANA ELISA had a low sensitivity of 22.1% at a 95%
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58 385 specificity. These results might be explained by differing ELISA formulations. Indeed,
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61 386 both the Inova and Bio-Rad ANA ELISA include HEp-2 nuclear extracts in addition to
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387 recombinant and purified nuclear antigens to account for unrecognized autoantigens.

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2 388 In contrast, the Euroimmun assay is only comprised of selected nuclear antigens. Its
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5 389 antigenic specificities are therefore better defined, ensuring high specificity for the
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7 390 diagnosis of rheumatic diseases. However, our data suggest that this comes at the
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10 391 cost of a low diagnostic value in autoimmune hepatitis. With regard to ELISA
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12 392 formulations, it is also worth mentioning that the Inova ANA ELISA is the only assay
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14 393 in this study including purified ribosomal P and mitochondrial M2 antigen. In a study
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17 394 by Calich and colleagues, autoantibodies against ribosomal P were found in 9/93
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19 395 (9.7%) AIH patients and none of the healthy controls [22]. In contrast, the
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22 396 incorporation of mitochondrial antigens is not expected for an ANA screening assay
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24 397 and carries considerable potential for confusion. Indeed, if the Inova ANA ELISA
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27 398 were to be used for the diagnostic workup of elevated liver enzymes, distinction
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29 399 between ANA and antimitochondrial antibodies (AMA) would not be possible in a
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32 400 reasonable fashion. Incorporation of mitochondrial antigens also likely explains the
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34 401 significantly higher values of the Inova ANA ELISA in PBC patients compared to AIH
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36 402 patients. Overall, while the careful choice of ELISA formulation and validation of cut-
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39 403 offs is critical, our data suggest that in principle ELISA testing represents a potentially
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41 404 good alternative to ANA IFT. Importantly, if ELISA-based autoantibody assessment is
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44 405 negative despite clinical suspicion of AIH, additional IFT should be performed.

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46 406 In the present study, we further compared IFT-based SMA evaluation to an anti-F-
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49 407 actin ELISA. Consistent with previous results [23], we found that anti-F-actin had a
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51 408 significantly higher diagnostic value for the diagnosis of AIH. Interestingly, while
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53 409 hypergammaglobulinemia potentiated the predictive value of anti-F-actin for the
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56 410 diagnosis of AIH, F-actin autoantibodies were still a strong predictor of AIH in the
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58 411 subgroup of AIH patients with IgG within the normal range (AUC 0.79).

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412 Several limitations to the present study warrant further discussion. First, IFT allows
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2 413 for the detection of additional autoantibodies such as AMA and provides
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4 414 characteristic staining patterns that point towards antigenic specificities of ANA. The
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7 415 benefit of this relevant information was not assessed in the present study. While ANA
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10 416 ELISA do not provide such additional information, some specific and reliable tests
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12 417 exist to further assess antigen specificity of ANA-positive sera. Indeed, most of the
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14 418 PBC sera we tested were highly positive both in the Inova ANA ELISA, which does
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17 419 however also include M2 antigen, the key target of antimitochondrial antibodies
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19 420 characteristic of PBC, as well as in the Bio-Rad ANA ELISA. Thus, for discrimination
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22 421 between AIH and PBC sera, further systematic testing by a specific M2-AMA ELISA
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24 422 and by sp100 and gp210 ELISA would be required. However, this would have been
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26 423 beyond the scope of the present study.

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29 424 Second, we included only one F-actin ELISA. However, compared to the
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31 425 heterogeneous group of ANA, F-actin is a defined antigen and the F-actin ELISA
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34 426 used in this study was investigated in two previous studies [7, 23].

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36 427 Furthermore, while control cohorts were well characterized, relevant patient groups
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39 428 such as drug-induced liver injury patients were not included in the present study.

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41 429 Finally, the gender distribution between AIH and controls was somewhat unbalanced
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44 430 reflecting the natural sex differences in these conditions. Although this potentially
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46 431 influenced the frequency of autoantibodies in patient groups, it most probably did not
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49 432 affect how the various autoantibody assays compared to one another.

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51 433 In conclusion, our results suggest that both IFT evaluation on HEp-2 cells as well as
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53 434 ELISA-based autoantibody assessment are potential alternatives to IFT on tissue
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56 435 sections. Our data indicate that (1) HEp-2 cells can be used for ANA assessment in
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58 436 AIH if scoring cutoff titers are increased, (2) The SMA-VG/T pattern and anti-MF
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61 437 reactivity on HEP-2 cells are highly specific even at low titers while generic SMA is
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438 specific only at higher titers, (3) ANA and F-actin ELISA show at least equivalent
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2 439 diagnostic performance compared to IFT, but ELISA kits for ANA assessment should
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4 440 include HEp-2 nuclear extracts to account for unknown nuclear antigens and cutoffs
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7 441 need to be validated for the use in AIH. In the future, cut-off values for autoantibody
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10 442 testing should be determined and validated by industry on standardized AIH sera and
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12 443 controls and re-validated by diagnostic laboratories, as technical details may
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14 444 influence the exact values. Nonetheless, the objective nature of these tests will make
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17 445 them more attractive in the future avoiding observation errors due to the subjective
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19 446 assessment of staining patterns as in SMA testing on tissue sections. Based on our
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22 447 results, under the prerequisite of careful choice of ELISA formulation and validation
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24 448 of cut-offs, we propose an adaptation of the simplified diagnostic score for AIH as
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27 449 summarized in Table 4 for everyday use in different laboratory settings.
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525 **Tables**526 **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

AIH n=61; NAFLD n=72; ANA, antinuclear antibodies; HEp-2 cells, human epithelioma-2 cells; IFT, immunofluorescence test; NPV, negative predictive value; PPV, positive predictive value.

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528 **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	1:40	75.4	69.4	67.7	76.9	72.2
HEp2	1:80	68.9	88.9	84.0	77.1	79.7
microfilaments	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	1:40	59.0	83.3	75.0	70.6	72.2
(bile canaliculi)	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	1:40	83.6	45.8	56.7	76.7	63.2
(tunica muscularis,	1:80	75.4	72.2	69.7	77.6	73.7
lamina muscularis	1:160	72.1	80.6	75.9	77.3	76.7
mucosa, interglandular	1:320	68.9	90.3	85.7	77.4	80.5
fibrils)	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

AIH n=61; NAFLD 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.

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

ELISA	Assay	Cutoff	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
ANA ELISA	Bio-Rad	≥ 1.0	65.5	88.6	76.3	82.1	80.3
		≥ 20	79.6	78.2	67.2	87.3	78.7
	Euroimmun	≥ 30	69.0	86.6	74.3	83.3	80.3
		≥ 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

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

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

Variable	Cutoff	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 times 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

¹Addition of points achieved (maximum 2 points for autoantibodies);²IFT: ≥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;³IFT: ≥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.

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564 **Figure legends**

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565 **Figure 1. Flow-chart of patient cohorts included in this study.**

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567 **Figure 2. Receiver-operating-characteristic (ROC) curves showing the**
568 **diagnostic value of ELISA for the diagnosis of AIH.** Diagnostic performance of (A)
569 three different ANA ELISA and (B) a F-actin ELISA to discriminate between AIH and
570 controls (distribution of diagnoses as shown in Figure 1). Area under the curve (AUC)
571 values are indicated.

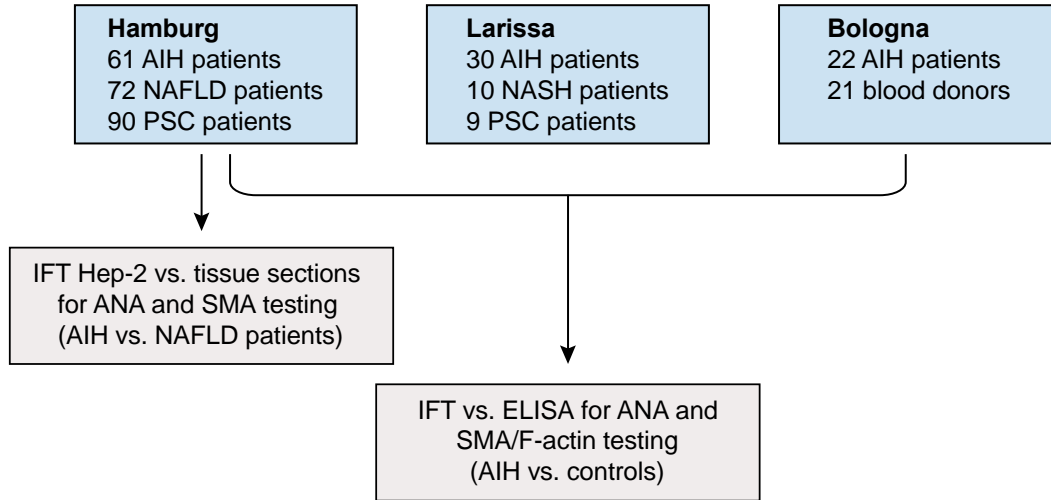
572
573 **Figure 3. Receiver-operating-characteristic (ROC) curves showing the**
574 **diagnostic performance of three different ANA ELISA in comparison with ANA**
575 **immunofluorescence for the diagnosis of AIH.** Diagnostic performance is
576 separately shown for cohorts from (A–B) Hamburg, (C–D) Larissa, and (E–F)
577 Bologna. The distribution of diagnoses is shown in Figure 1. Area under the curve
578 (AUC) values are indicated.

579
580 **Figure 4. Receiver-operating-characteristic (ROC) curves showing the**
581 **diagnostic performance of a F-actin ELISA in comparison with SMA**
582 **immunofluorescence for the diagnosis of AIH.** Diagnostic performance is
583 separately shown for cohorts from (A–B) Hamburg, (C–D) Larissa, and (E–F)
584 Bologna. The distribution of diagnoses is shown in Figure 1. Area under the curve
585 (AUC) values are indicated.

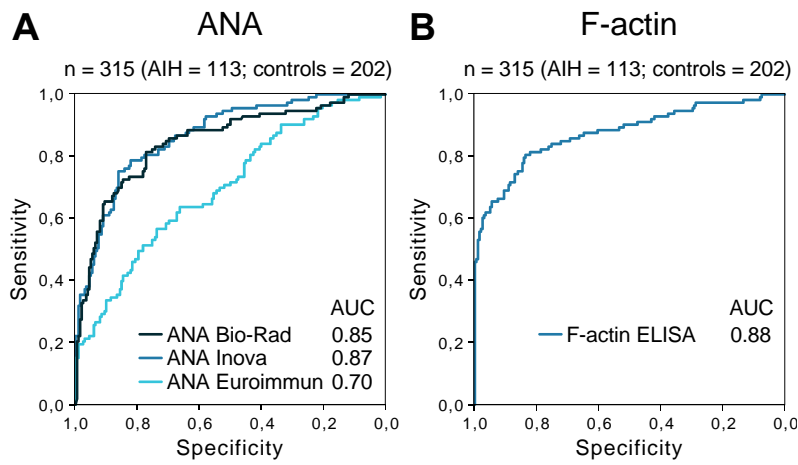
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590 **Figures**

591 **Figure 1:**



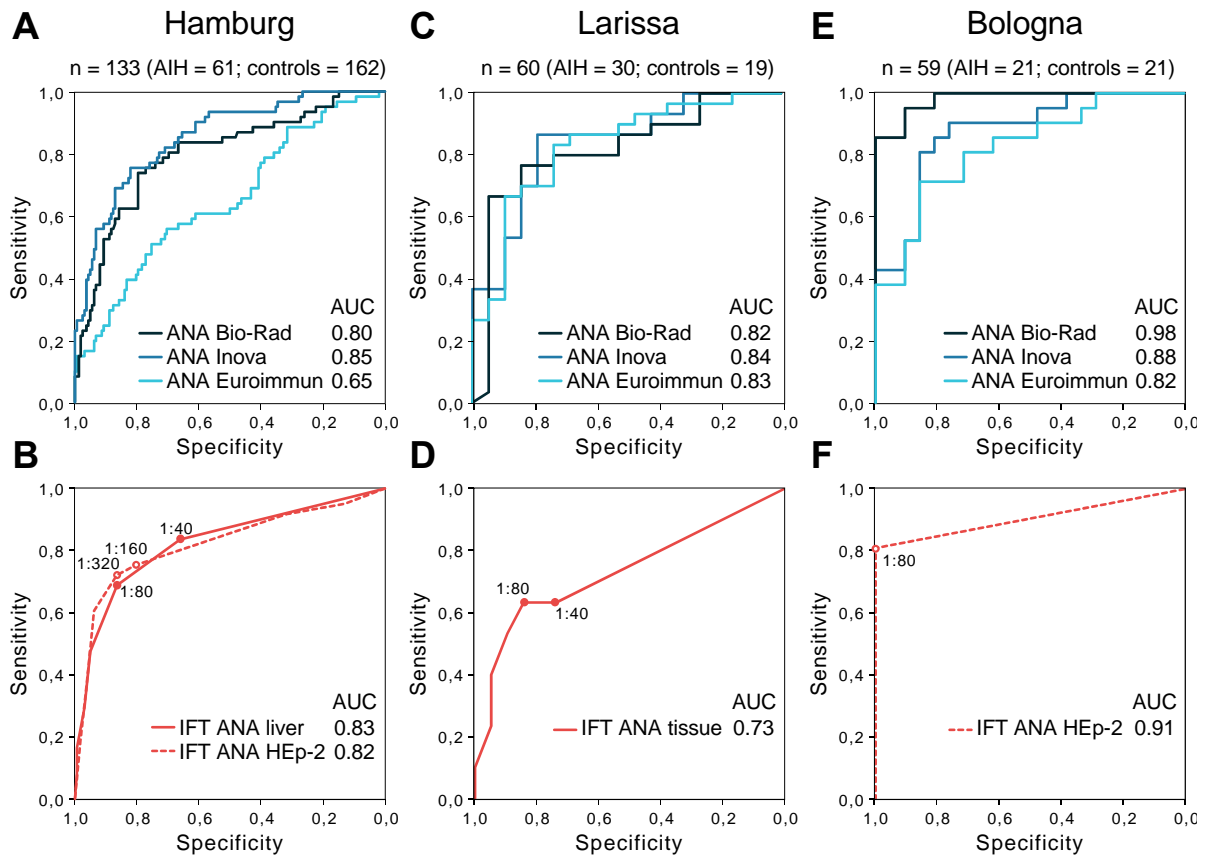
593 **Figure 2:**



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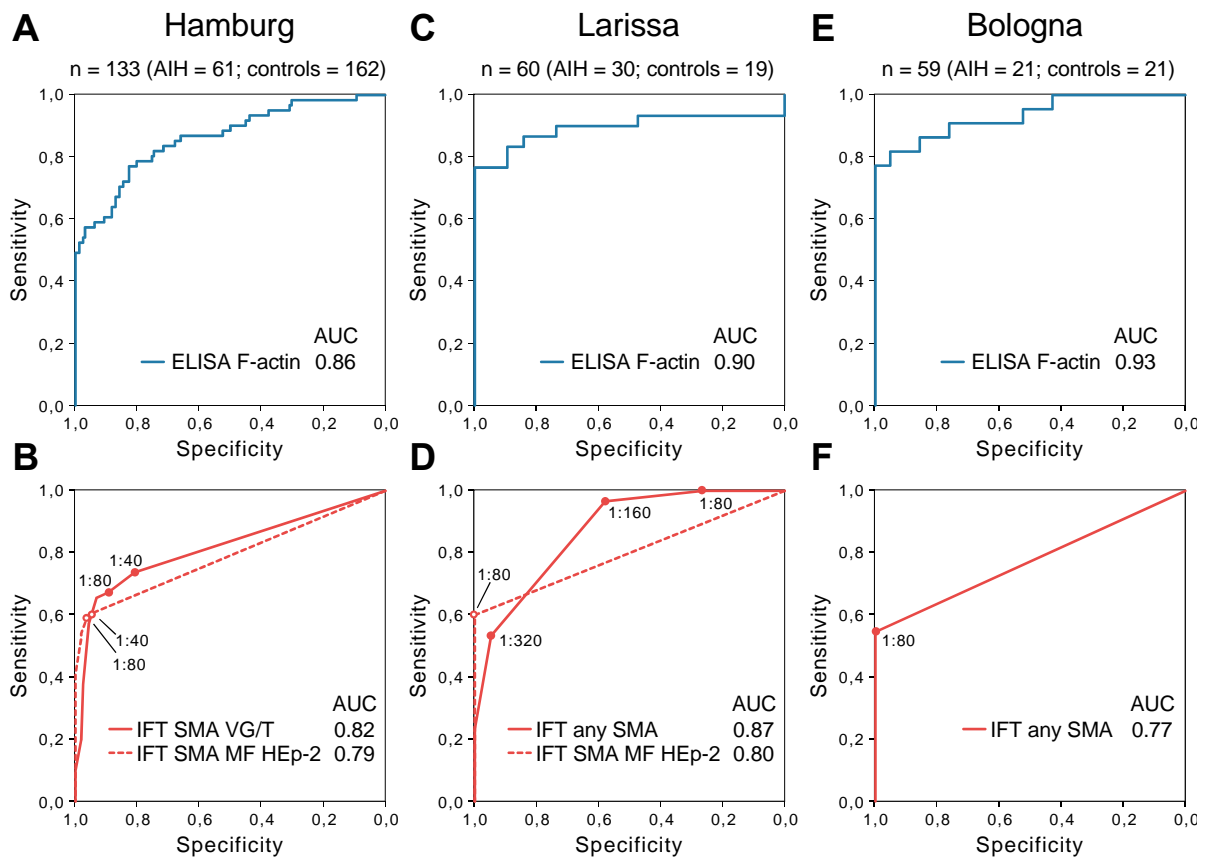
608 **Figure 3:**



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611 **Figure 4:**



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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

AIH n=61; NAFLD n=72; ANA, antinuclear antibodies; HEp-2 cells, human epithelioma-2 cells; IFT, immunofluorescence test; NPV, negative predictive value; PPV, positive predictive value.

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

AIH n=61; NAFLD 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.

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

ELISA	Assay	Cutoff	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
ANA ELISA	Bio-Rad	≥ 1.0	65.5	88.6	76.3	82.1	80.3
		≥ 20	79.6	78.2	67.2	87.3	78.7
	Euroimmun	≥ 30	69.0	86.6	74.3	83.3	80.3
		≥ 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

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

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

Variable	Cutoff	Points ¹
ANA or SMA/F-Actin	Positive ²	1
ANA or SMA/F-Actin or LKM or SLA	Strongly positive ³ ≥1:40 Positive	2
IgG	>Upper normal limit	1
	>1.1 times 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

¹Addition of points achieved (maximum 2 points for autoantibodies);²IFT: ≥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;³IFT: ≥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.

Figure 1

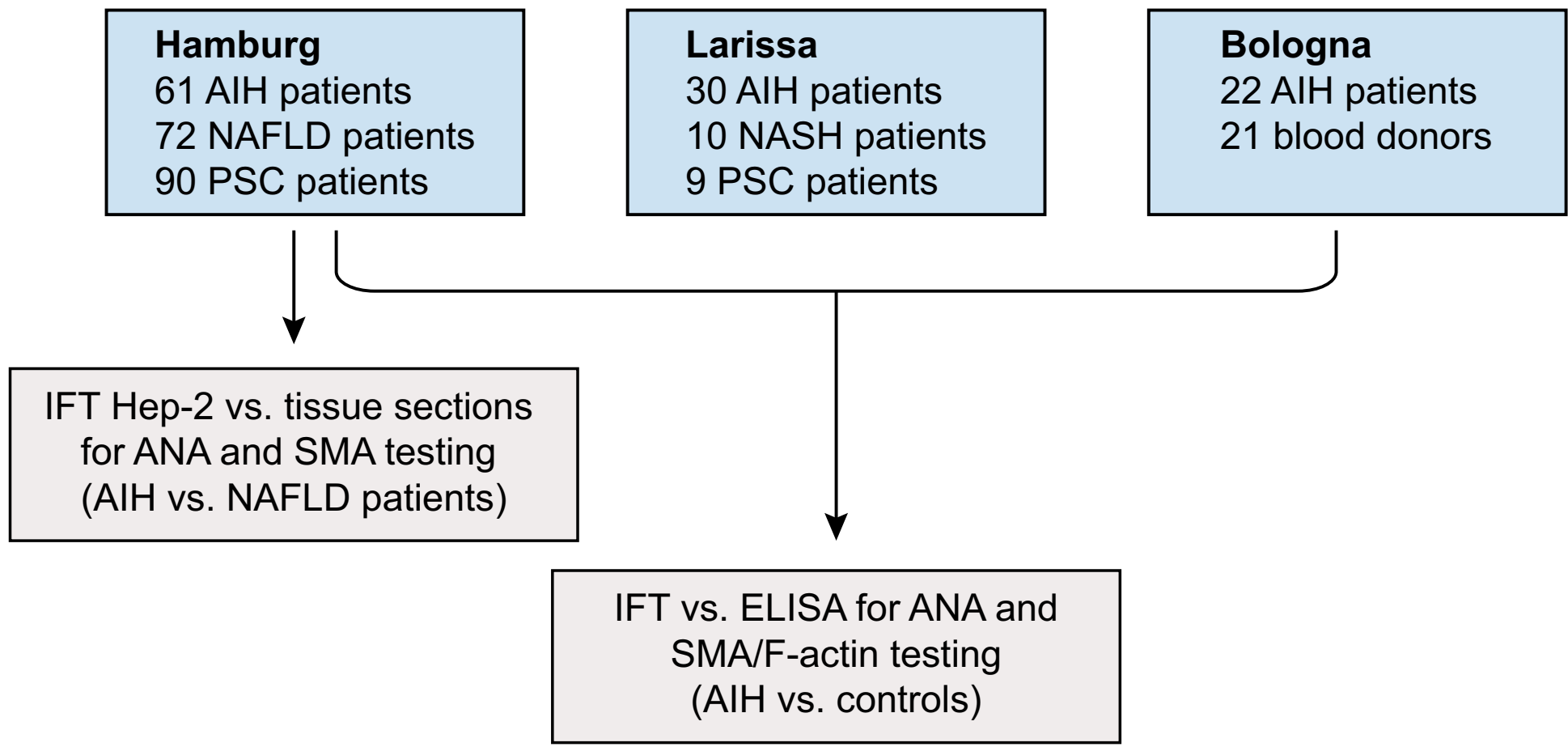


Figure 2

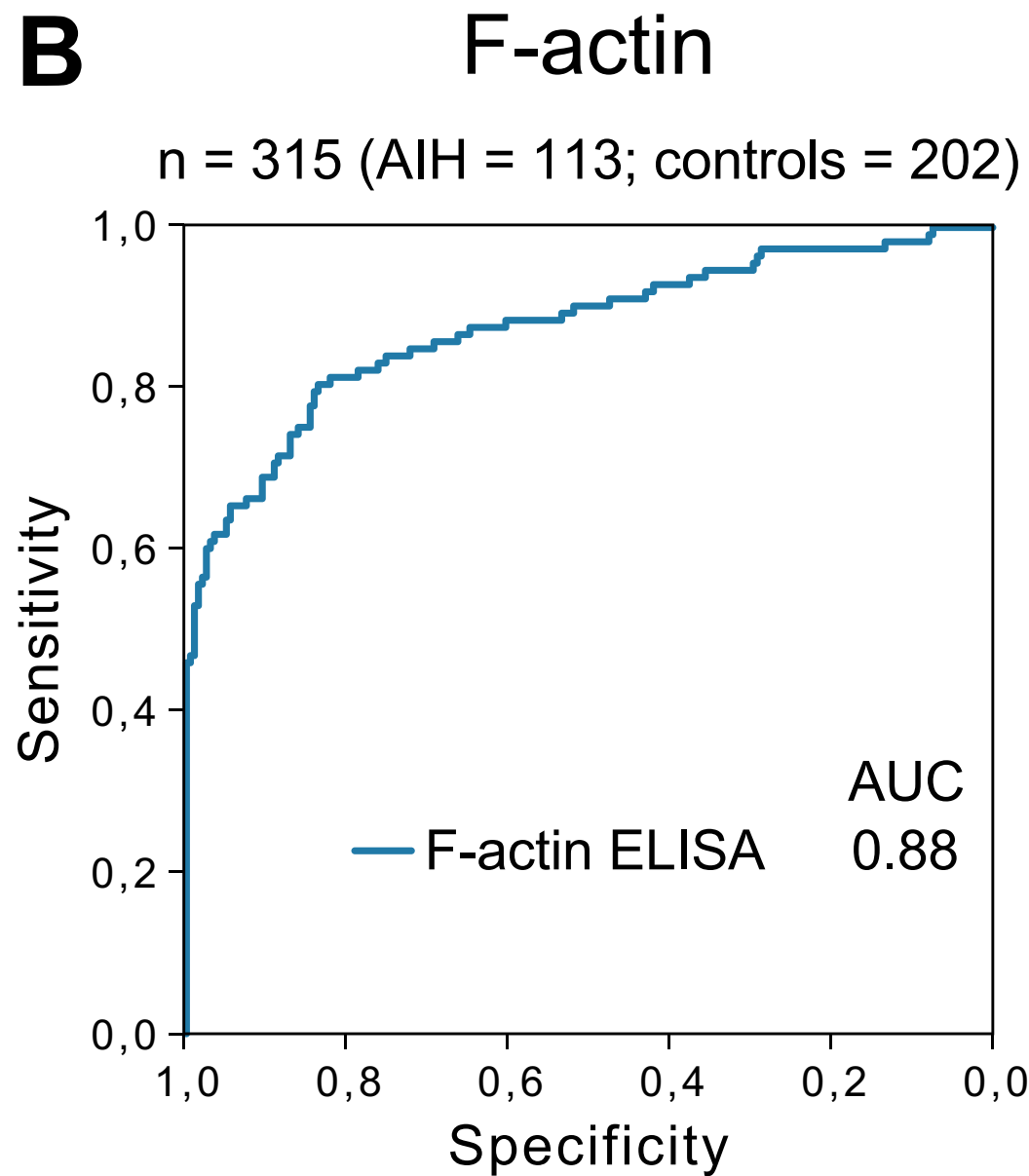
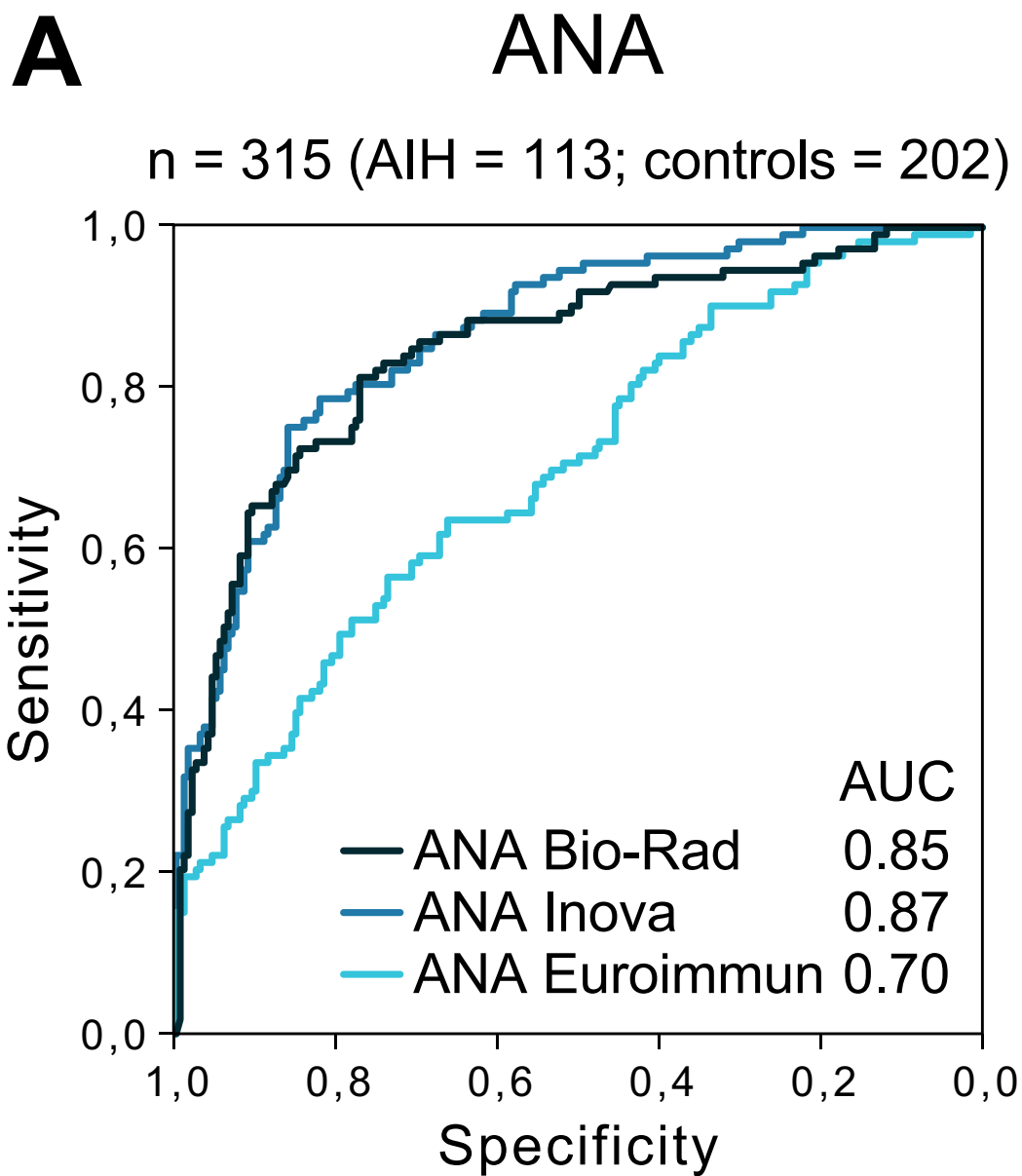


Figure 3

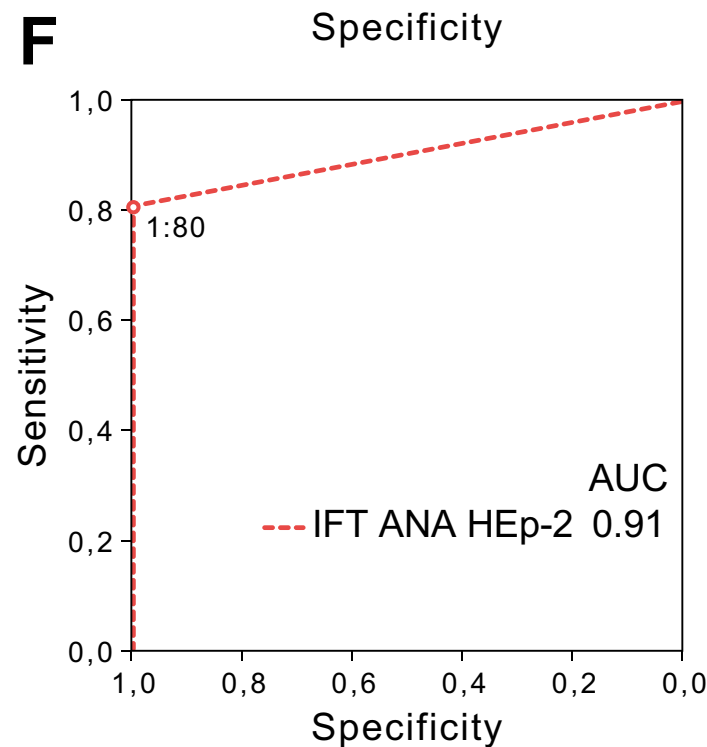
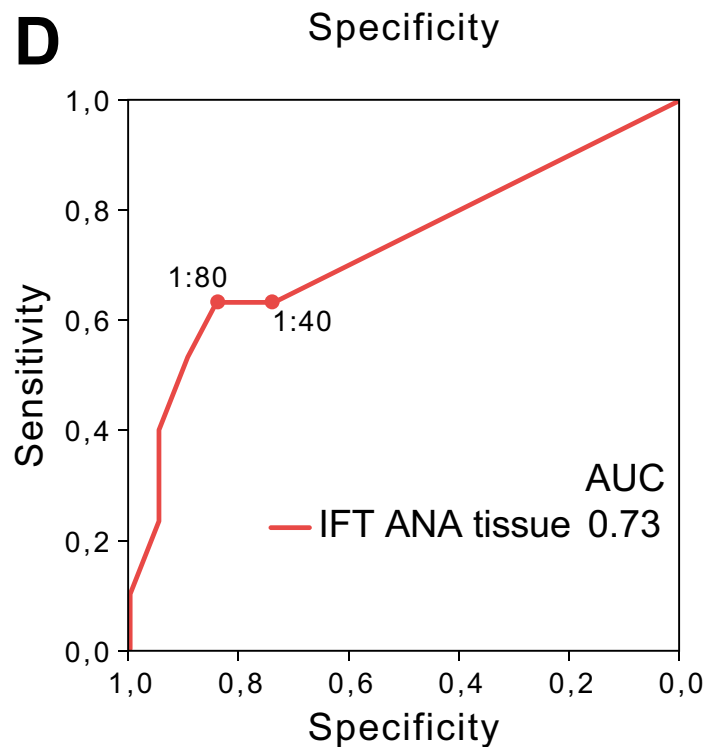
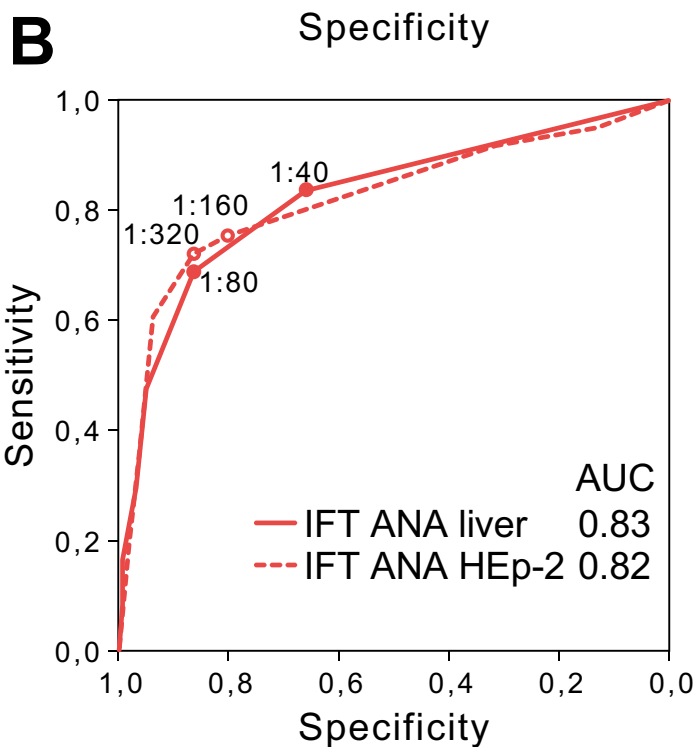
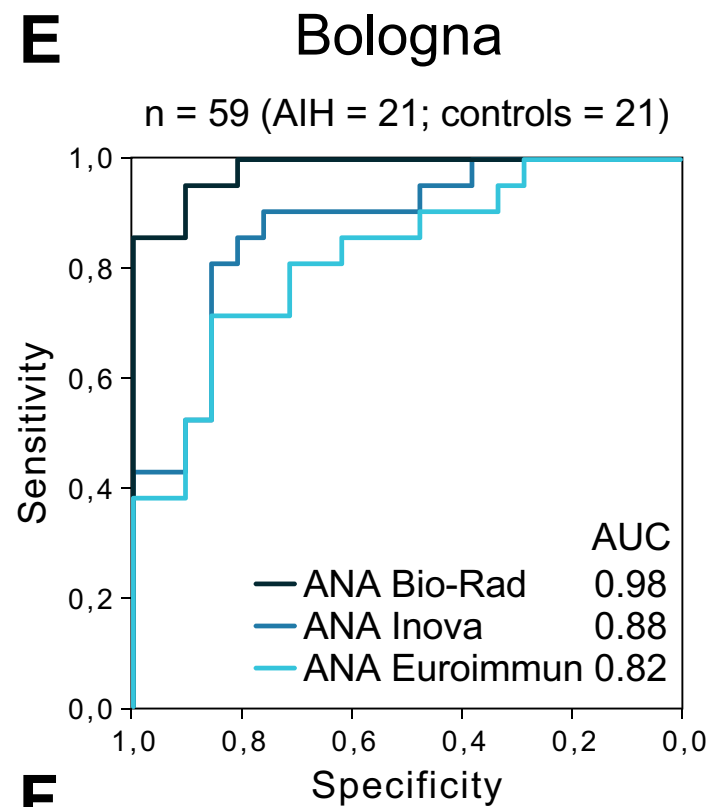
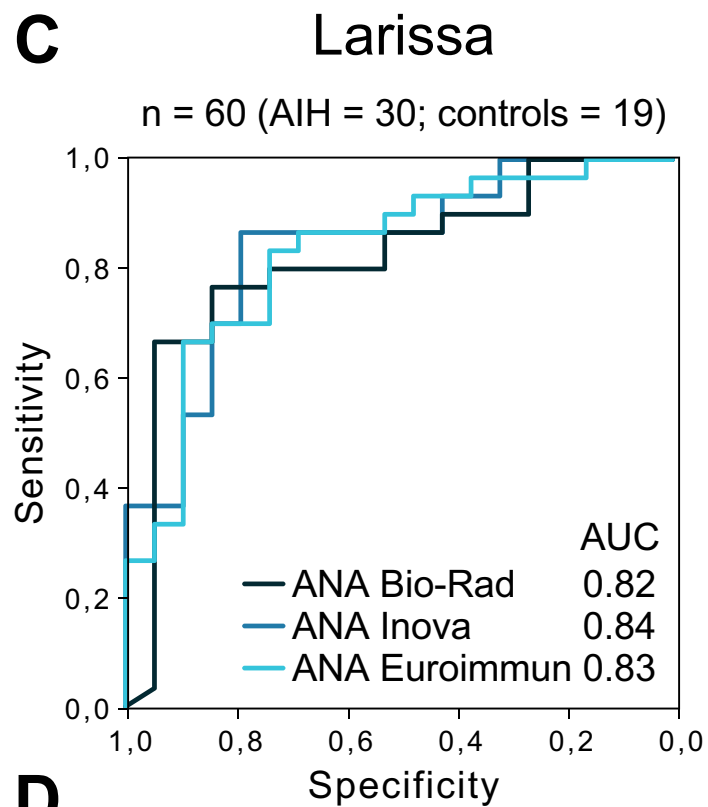
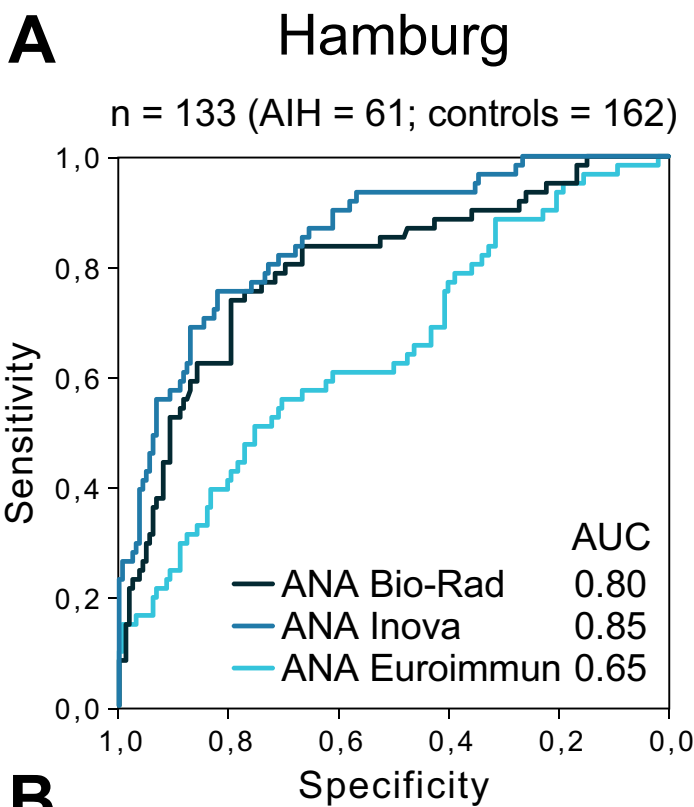
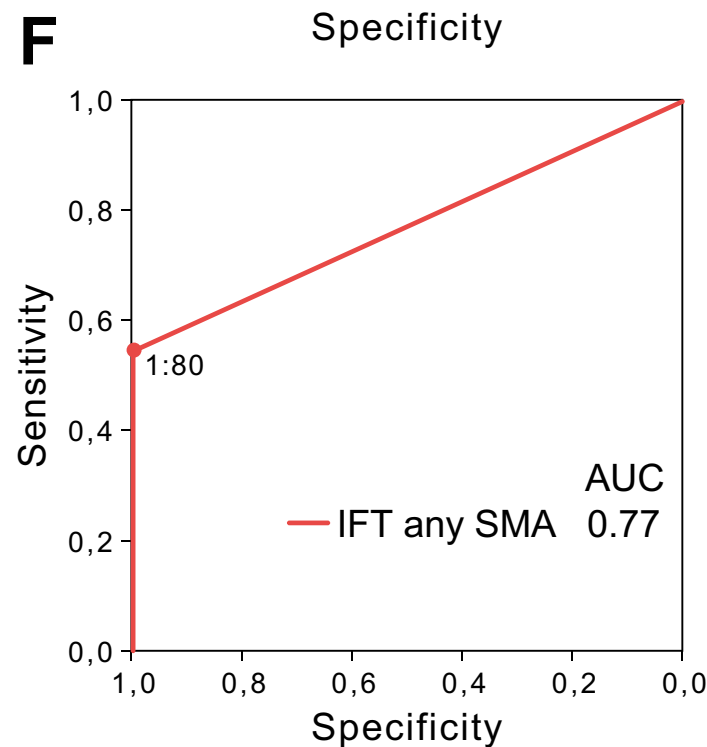
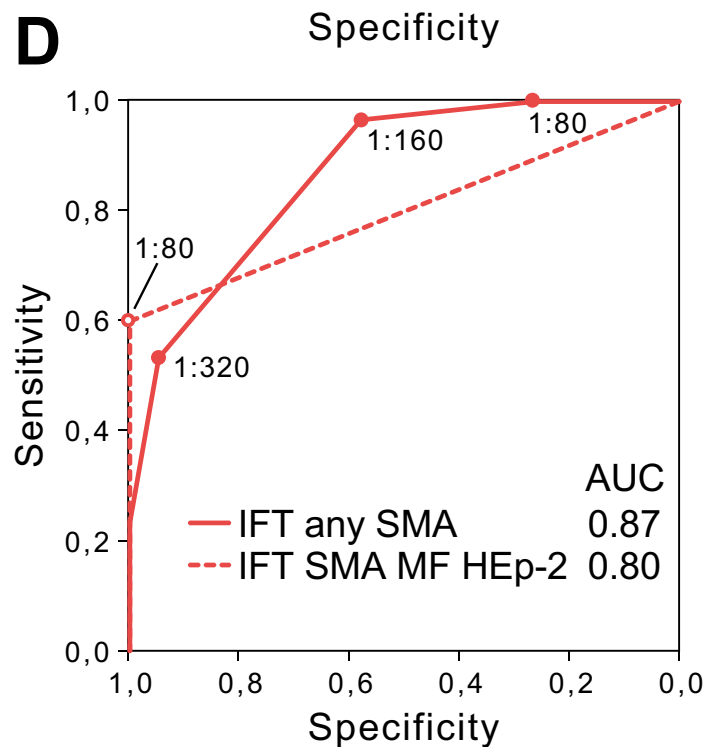
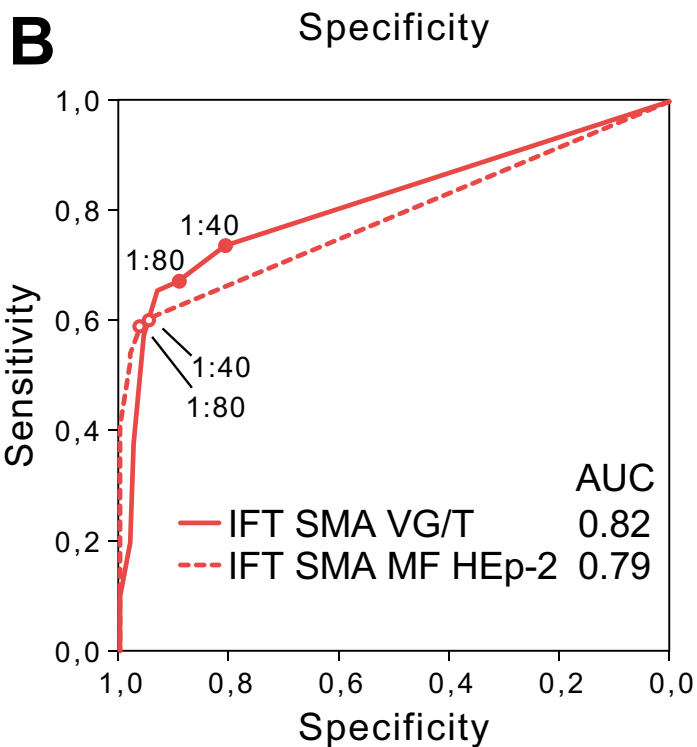
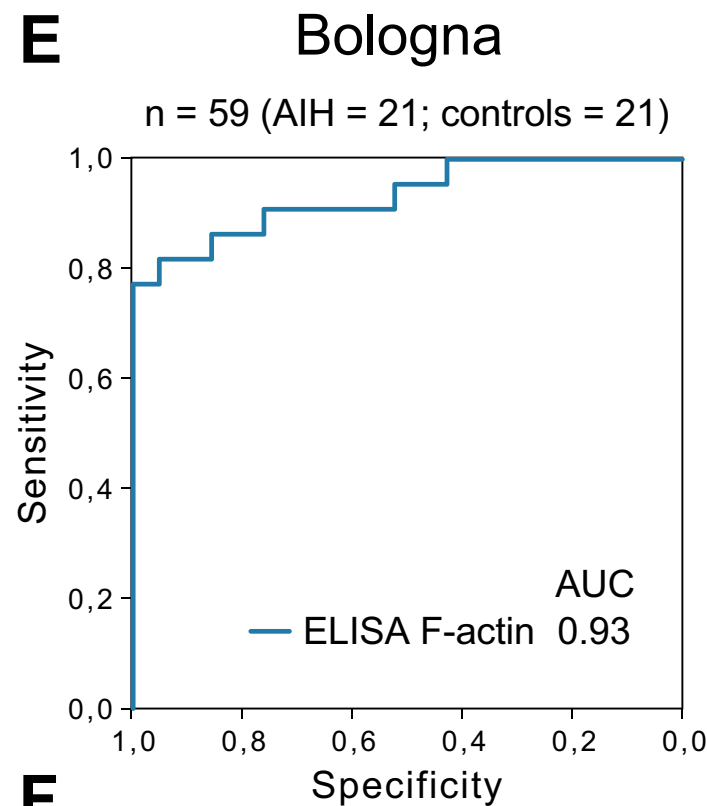
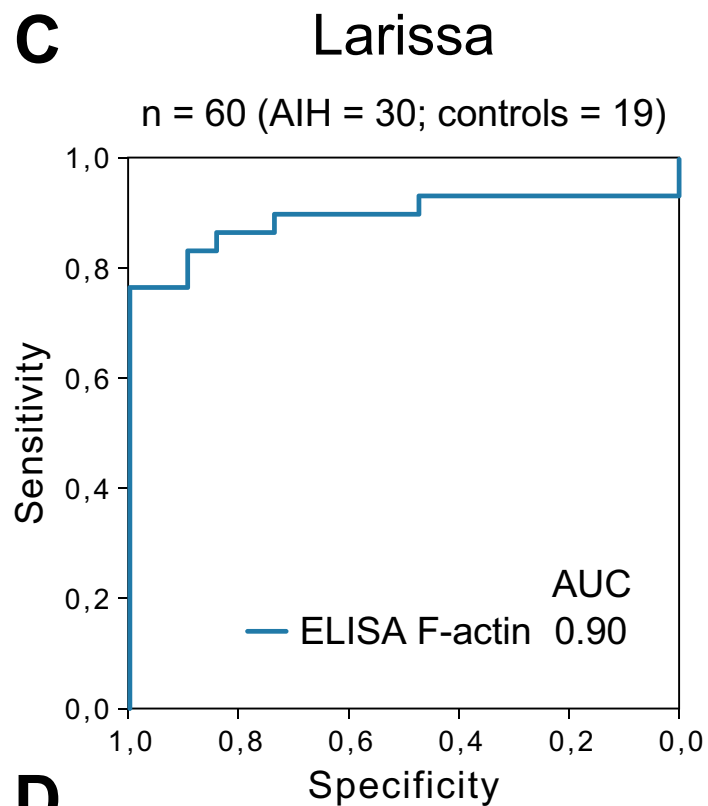
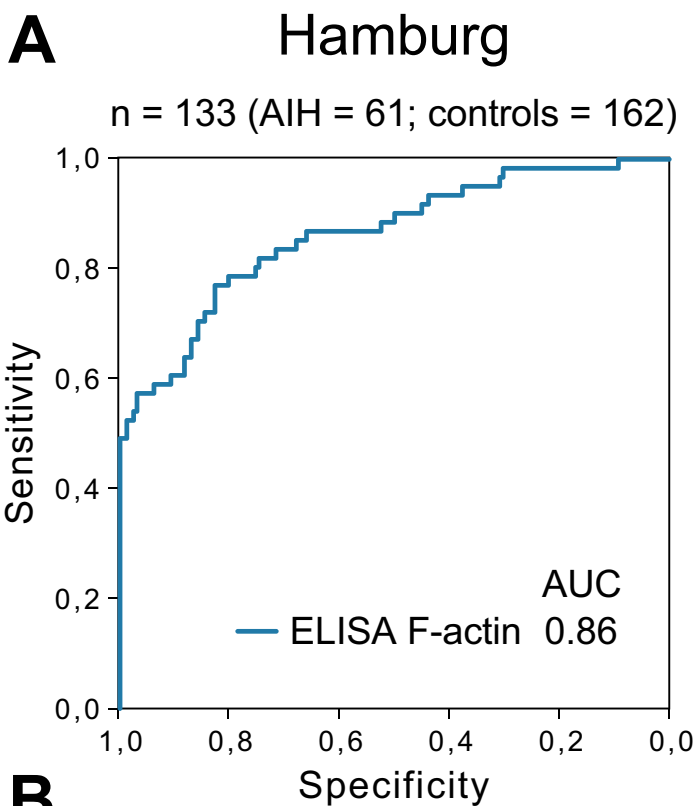
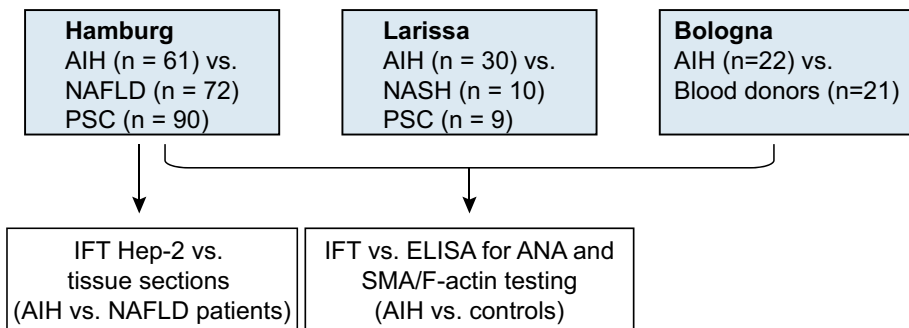
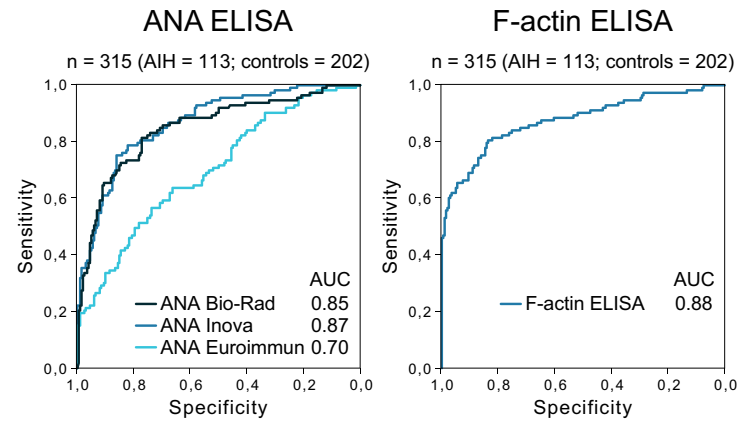


Figure 4





Diagnostic performance of ANA and F-actin ELISA for the diagnosis of AIH



The simplified criteria for the diagnosis of AIH – update of serological criteria

Variable	Cutoff	Points
ANA or SMA/F-Actin	Positive ¹	1
ANA or SMA/F-Actin or LKM or SLA	Strongly positive ² ≥1:40 Positive	2
IgG	>Upper normal limit	1
	>1.1 times 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

¹IFT: ≥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 cut-offs validated locally;
²IFT: ≥1:80 when assessed on tissue sections; ≥ 1:160 or 1:320 for ANA when assessed on HEp-2 cells. ELISA with cut-offs validated locally;
 Note: if ELISA-based autoantibody assessment is negative despite of a high clinical suspicion for autoimmune hepatitis, IFT should be performed.

Highlights

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