

# Journal Pre-proof



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

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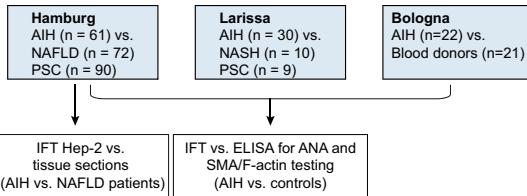
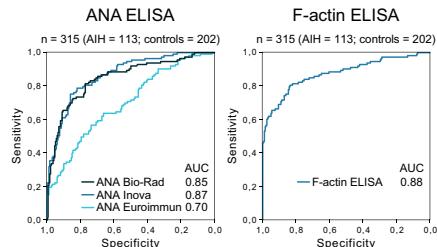
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**Diagnostic performance of ANA and F-actin ELISA for the diagnosis of AIH****The simplified criteria for the diagnosis of AIH – update of serological criteria**

Variable	Cutoff	Points
ANA or SMA/F-Actin	Positive <sup>1</sup>	1
ANA or SMA/F-Actin or LKM or SLA	Strongly positive <sup>2</sup> ≥1:40 Positive	2
IgG	>Upper normal limit >1.1 times upper normal limit	1 2
Liver histology (with evidence of hepatitis)	Compatible with AIH Typical AIH	1 2
Absence of viral hepatitis	Yes	2

≥6: probable AIH  
≥7: definite AIH

<sup>1</sup>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 cut-offs validated locally;

<sup>2</sup>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 validated locally;

Note: if ELISA-based autoantibody assessment is negative despite of a high clinical suspicion for autoimmune hepatitis, IFT should be performed.

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

3

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

53 Schakat M, Zachou K, Muratori P, Lampalzer S, Haag F, Lenzi M, Dalekos GN:  
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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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76

77

78

79 **Abstract**

80

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

102

103 **Introduction**

104

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

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

126 HEp-2 cells are widely used as substrate for ANA evaluation. In addition to a higher  
127 sensitivity, characteristic staining patterns evaluated on HEp-2 cells are useful in  
128 guiding further confirmatory testing. However, a consensus statement by the IAIHG  
129 committee for autoimmune serology advises against the use of HEp-2 cells at a  
130 screening stage [2] because of a high positivity rate in healthy individuals at low

131 cutoff titers [3]. If HEp-2 cells are used, the IAIHG suggests titers should be halved  
132 for the simplified score to be applicable [1]. However, this possible correction factor  
133 suggestion has never been validated by comparative studies [4].

134 SMA constitute a heterogeneous group of autoantibodies that primarily target F-actin,  
135 [5]. On kidney tissue sections, Bottazzo and colleagues distinguished three  
136 immunofluorescence patterns: SMA-V (vessels), SMA-VG (vessels/glomeruli), and  
137 SMA-VGT (vessels/ glomeruli/ tubuli) [6]. In contrast to the SMA-V pattern, SMA-  
138 VG/T correlates with F-actin reactivity and is more specific for AIH [6-8]. Similarly,  
139 anti-F-actin antibodies stain microfilaments (MF) on HEp-2 cells [9]. Overall,  
140 sensitivity and specificity of SMA positivity strongly depend on fluorescence patterns,  
141 which is not taken into consideration by current AIH scoring systems.

142 Since IFT is time-consuming, requires experienced technicians and lacks  
143 standardization, ELISA have emerged as a widely used alternative for routine  
144 autoantibody testing in many laboratories, especially in the United States. These  
145 tests were originally developed for use in the evaluation of rheumatic diseases and  
146 their diagnostic value in liver disease is unknown. ELISA testing can minimize  
147 interobserver variability inherent to IFT. However, it is unclear whether ELISA can  
148 replace IFT for the detection of the heterogeneous autoantibodies ANA and SMA  
149 with their range of antigenic specificities. To complicate matters even further, up to  
150 30% of ANA-positive AIH patients do not react with any known nuclear antigens [10]  
151 and might thus be missed by ELISA testing, which are based primarily on known  
152 nuclear antigens. In addition, commercially available ANA ELISA lack standardization  
153 – they differ in their antigenic profiles and assay-specific cutoff values.

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

157 autoantibody testing for the diagnosis of AIH to make these applicable in diagnosing  
158 AIH.

159

160 **Patients and methods**

161

162 **Study population**

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

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

179

180 **Autoantibody assessment by IFT**

181 IFT was performed in the respective center in which sera were collected. At the  
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  
184 epithelioma (HEp-2) cells (Mosaic Basic Profile 3, Euroimmun, Germany). The assay  
185 was performed manually according to the manufacturer's instructions at a dilution of  
186 1:40. Further dilutions up to 1:1280 were processed by the Helios automated IFA  
187 system (Aesku Diagnostics, Wendelsheim, Germany), using the same substrates  
188 and conditions. Reactivity patterns were assessed under a fluorescence microscope  
189 (Eurostar, Euroimmun, Germany). ANA and SMA reactivity were separately  
190 evaluated on all four substrates. SMA reactivity on kidney sections was assessed  
191 according to Bottazzo et al. [6]. The observers were blinded to clinical data.

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

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

202

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

204 All ELISA testing was performed at the University Medical Center Hamburg-  
205 Eppendorf. Antinuclear antibodies were assessed using enzyme immunoassays from  
206 three different manufacturers (Quanta Lite ANA ELISA, Inova Diagnostics, US; ANA  
207 Screening Test, Bio-Rad, US; ANA Screen ELISA, Euroimmun, Germany). All assays  
208 detect autoantibodies of IgG subtype and display antigenic specificities to dsDNA,

209 histones, Sm/RNP, SS-A, SS-B, Scl-70, centromere, and Jo-1. The Quanta Lite ANA  
210 ELISA is additionally coated with highly purified proliferating cell nuclear antigen  
211 (PCNA), mitochondrial M2 antigen, and ribosomal-P proteins. Besides individual  
212 antigens, immunoassays from both Inova Diagnostics and Bio-Rad include HEp-2  
213 cell nuclei extracts.

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

218

### 219 **Statistical analyses**

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

230

### 231 **Results**

232

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

234 We first investigated the diagnostic value of HEp-2 cells in comparison to tissue  
235 sections as substrates for ANA IFT in the context of AIH. To this end, sera from 61  
236 AIH patients and 72 patients with biopsy-proven NAFLD treated at the University  
237 Medical Center Hamburg-Eppendorf were evaluated for autoantibodies by IFT.  
238 Clinical characteristics of the patient groups at the time of sampling are summarized  
239 in supplemental Table 1.

240 Sensitivity and specificity of ANA IFT for HEp-2 cells and tissue sections are shown  
241 in Table 1. Among tissue sections, primate liver showed the highest diagnostic value  
242 for ANA evaluation. Sensitivity and specificity were 83.6% and 69.4% at a titer of  
243 1:40, respectively, and 68.9% and 80.6% at a titer of 1:80, respectively. Specificity  
244 increased to 91.7% at a titer of 1:160 at the cost of a lower sensitivity of 47.5%. As  
245 expected, the use of HEp-2 cells led to higher titers. Specificity was inadequate at a  
246 1:40 dilution. At a titer of 1:80, sensitivity was 91.8% at a low specificity of 36.1%. At  
247 higher titers, sensitivity and specificity were comparable to those observed on liver  
248 sections: 75.4% and 73.6%, respectively, at a titer of 1:160; 72.1% and 76.4%,  
249 respectively, at a titer of 1:320. The homogenous pattern was significantly more  
250 frequent in AIH patients (41.0%) than in NAFLD patients (6.9%,  $P < 0.001$ ).

251

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

254 We next assessed the diagnostic value of several SMA fluorescence patterns at  
255 different titers (Table 2). As expected, at a 1:40 titer, the SMA-V pattern on kidney  
256 sections, staining of smooth muscle on stomach sections as well as consideration of  
257 any SMA positivity resulted in a low specificity of 33.3% – 45.8%. In contrast, the  
258 SMA-VG pattern was more specific for the diagnosis of AIH even at low titers.  
259 Sensitivity and specificity were 72.1% and 70.8%, respectively, at a titer of 1:40, and

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.

268

### 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  $\geq 1$  RU), 79.6% and 78.2%  
283 for the ANA Inova assay (recommended cutoff  $\geq 20$  RU), and 22.1% and 95.0% for  
284 the ANA Euroimmun assay (recommended cutoff  $\geq 1$  RU), respectively (Table 3).

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

291

## 292 **ELISA- compared to IFT-based evaluation of autoantibodies**

293 We next compared ELISA- and IFT-based ANA evaluation. To account for the inter-  
294 laboratory variability inherent to IFT, ELISA assessment was compared to IFT results  
295 obtained by the respective centers according to local standards. Figure 3 and 4 show  
296 the diagnostic performance of ELISA vs. IFT for ANA and SMA/F-actin, respectively,  
297 for each center. ANA testing by ELISA and IFT performed similarly for all cohorts,  
298 except for the Euroimmun ELISA that showed a significantly lower AUC compared to  
299 IFT for the Hamburg cohort (Euroimmun ANA ELISA, AUC 0.65; ANA IFT, AUC 0.82  
300 – 0.83;  $P < 0.001$ ).

301 In addition to the patient groups shown in Figure 1, we tested sera from 26 PBC  
302 patients known to frequently present with ANA. Clinical characteristics of PBC  
303 patients are detailed in supplemental Table 4. While 17/26 (65.4%) of PBC patients  
304 tested positive for ANA by IFT on HEp-2 cells at a cut-off of 1:80, 23/26 (88.4%) and  
305 25/26 (96.2%) tested positive by the Bio-Rad and Inova ANA ELISA, respectively.  
306 Importantly, median values of the Inova ANA ELISA were significantly higher in PBC  
307 patients compared to AIH patients (49.6 RU AIH vs. 161.7 RU PBC;  $P < 0.001$ ) while  
308 there was no statistical significant difference for the Bio-Rad ELISA (1.6 RU AIH vs.  
309 2.0 RU PBC;  $P = 0.25$ ).

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  
336 IFT-based autoantibody assessment. However, assays vary considerably in their  
337 performance and cut-offs need to be validated for the diagnosis of AIH. If these  
338 aspects are taken under consideration and local cut-offs established, ELISA-based  
339 autoantibody testing as proposed in Table 4 can be used in the diagnostic work-up of  
340 liver disease patients.

341

## 342 **Discussion**

343

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

349 We first aimed to validate the use of HEp-2 cells as substrate for ANA IFT in patients  
350 with AIH. As expected, at low titers, ANA as evaluated on HEp-2 cells showed a high  
351 sensitivity at the expense of a low specificity. It is precisely the low specificity at a

352 1:40 titer that led the IAIHG to advise against use of HEp-2 cells for ANA evaluation  
353 at a screening stage [2]. However, to our knowledge, the diagnostic value of ANA IFT  
354 on HEp-2 cells has not been assessed at higher titers in the context of liver disease.

355 A previous study investigating ANA IFT in liver disease reported an increased  
356 sensitivity of ANA IFT using HEp-2 cells, but was restricted to a 1:40 dilution [15].

357 Our results suggest that HEp-2 cells are a valid alternative to tissue sections, if  
358 threshold titers are adapted. We here propose increasing cutoff titers to 1:160 and  
359 1:320 for the simplified diagnostic score to be applicable. As outlined above, a cutoff  
360 titer of 1:160 is also the recommended cutoff for ANA screening in rheumatic

361 diseases [16]. However, titers vary depending on reagents and equipment used and  
362 should be validated locally. In addition, the difference in immunofluorescence  
363 intensity between tissue sections and HEp-2 cells is not the same for all subtypes of  
364 ANA, but highly dependent on the respective ANA pattern. Nevertheless, overall,  
365 HEp-2 cells are a valid alternative to tissue sections for ANA evaluation in AIH.

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

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

387 recombinant and purified nuclear antigens to account for unrecognized autoantigens.

388 In contrast, the Euroimmun assay is only comprised of selected nuclear antigens. Its

389 antigenic specificities are therefore better defined, ensuring high specificity for the

390 diagnosis of rheumatic diseases. However, our data suggest that this comes at the

391 cost of a low diagnostic value in autoimmune hepatitis. With regard to ELISA

392 formulations, it is also worth mentioning that the Inova ANA ELISA is the only assay

393 in this study including purified ribosomal P and mitochondrial M2 antigen. In a study

394 by Calich and colleagues, autoantibodies against ribosomal P were found in 9/93

395 (9.7%) AIH patients and none of the healthy controls [22]. In contrast, the

396 incorporation of mitochondrial antigens is not expected for an ANA screening assay

397 and carries considerable potential for confusion. Indeed, if the Inova ANA ELISA

398 were to be used for the diagnostic workup of elevated liver enzymes, distinction

399 between ANA and antimitochondrial antibodies (AMA) would not be possible in a

400 reasonable fashion. Incorporation of mitochondrial antigens also likely explains the

401 significantly higher values of the Inova ANA ELISA in PBC patients compared to AIH

402 patients. Overall, while the careful choice of ELISA formulation and validation of cut-

403 offs is critical, our data suggest that in principle ELISA testing represents a potentially

404 good alternative to ANA IFT. Importantly, if ELISA-based autoantibody assessment is

405 negative despite clinical suspicion of AIH, additional IFT should be performed.

406 In the present study, we further compared IFT-based SMA evaluation to an anti-F-

407 actin ELISA. Consistent with previous results [23], we found that anti-F-actin had a

408 significantly higher diagnostic value for the diagnosis of AIH. Interestingly, while

409 hypergammaglobulinemia potentiated the predictive value of anti-F-actin for the

410 diagnosis of AIH, F-actin autoantibodies were still a strong predictor of AIH in the

411 subgroup of AIH patients with IgG within the normal range (AUC 0.79).

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

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

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

433 In conclusion, our results suggest that both IFT evaluation on HEp-2 cells as well as  
434 ELISA-based autoantibody assessment are potential alternatives to IFT on tissue  
435 sections. Our data indicate that (1) HEp-2 cells can be used for ANA assessment in  
436 AIH if scoring cutoff titers are increased, (2) The SMA-VG/T pattern and anti-MF  
437 reactivity on HEp-2 cells are highly specific even at low titers while generic SMA is

438 specific only at higher titers, (3) ANA and F-actin ELISA show at least equivalent  
439 diagnostic performance compared to IFT, but ELISA kits for ANA assessment should  
440 include HEp-2 nuclear extracts to account for unknown nuclear antigens and cutoffs  
441 need to be validated for the use in AIH. In the future, cut-off values for autoantibody  
442 testing should be determined and validated by industry on standardized AIH sera and  
443 controls and re-validated by diagnostic laboratories, as technical details may  
444 influence the exact values. Nonetheless, the objective nature of these tests will make  
445 them more attractive in the future avoiding observation errors due to the subjective  
446 assessment of staining patterns as in SMA testing on tissue sections. Based on our  
447 results, under the prerequisite of careful choice of ELISA formulation and validation  
448 of cut-offs, we propose an adaptation of the simplified diagnostic score for AIH as  
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 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, interglan- dular 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.

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**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
		≥ 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

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 <sup>1</sup>
ANA or SMA/F-Actin	Positive <sup>2</sup>	1
ANA or SMA/F-Actin or LKM or SLA	Strongly positive <sup>3</sup> $\geq 1:40$ Positive	
IgG	$>$ Upper normal limit $>1.1$ times upper normal limit	1 2
Liver histology (with evidence of hepatitis)	Compatible with AIH Typical AIH	1 2
Absence of viral hepatitis	Yes	2
		$\geq 6$ : probable AIH
		$\geq 7$ : definite AIH

<sup>1</sup>Addition of points achieved (maximum 2 points for autoantibodies);<sup>2</sup>IFT:  $\geq 1:40$  when assessed on tissue sections;  $\geq 1:80$  or 1:160 for ANA when assessed on HEp-2 cells, depending on local standards. ELISA with locally established cut-offs;<sup>3</sup>IFT:  $\geq 1:80$  when assessed on tissue sections;  $\geq 1:160$  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**

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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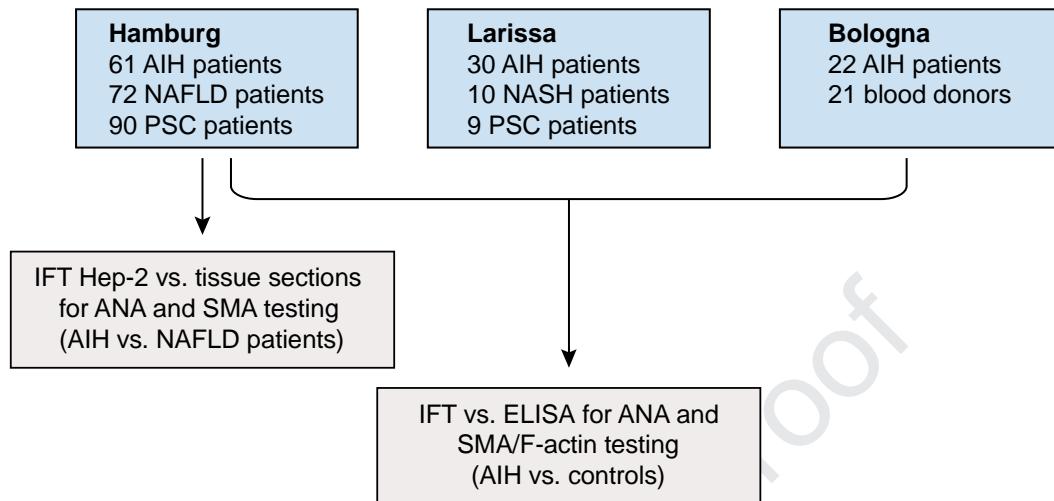
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590 **Figures**

591 **Figure 1:**

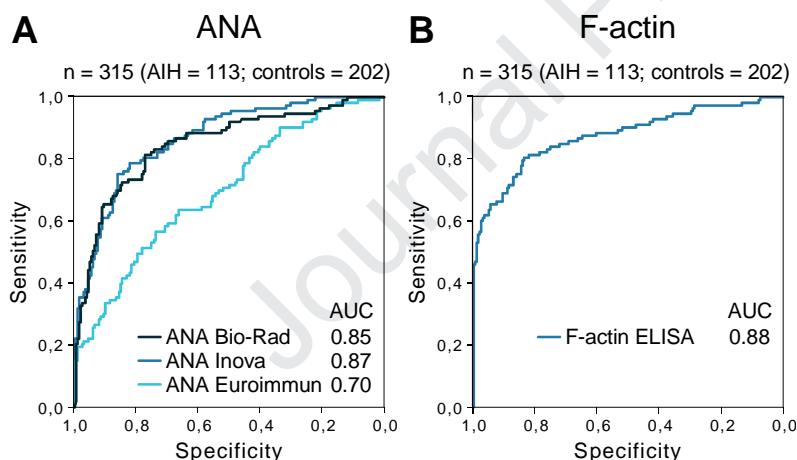
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594 **Figure 2:**

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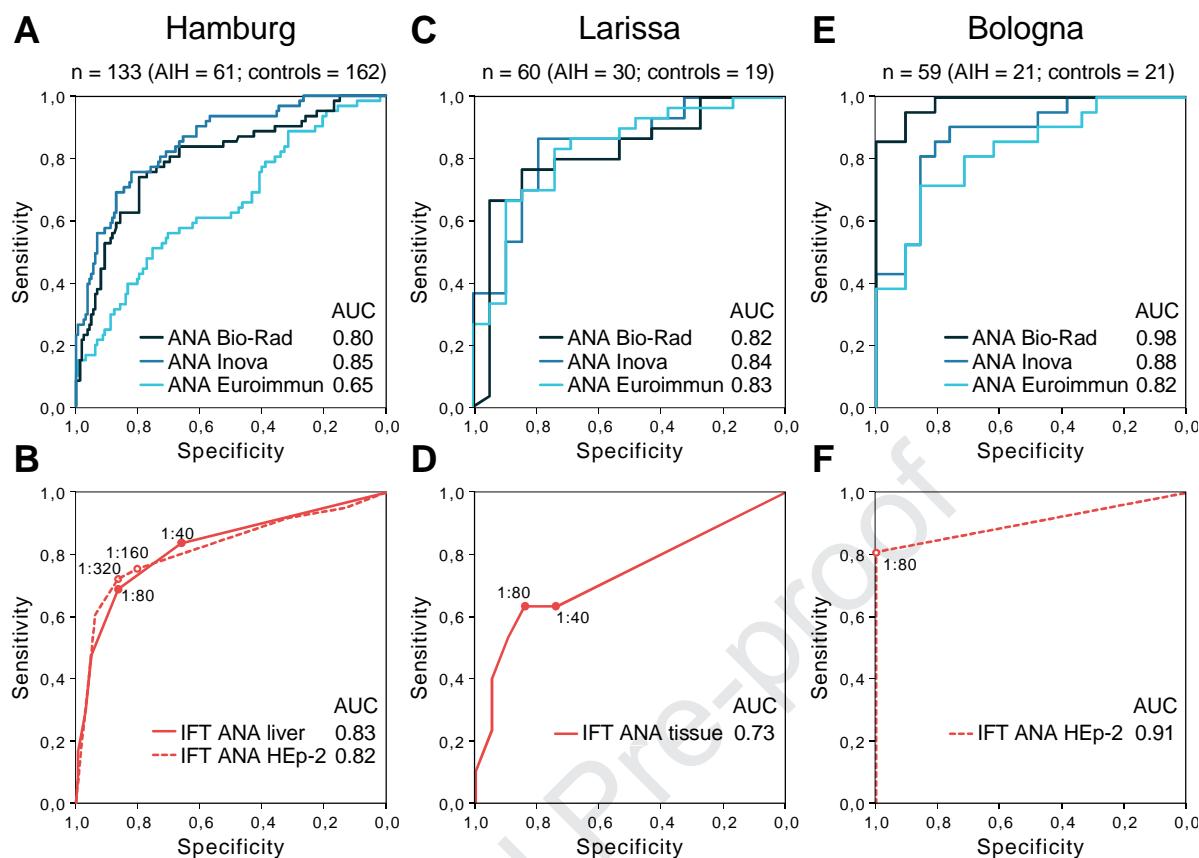
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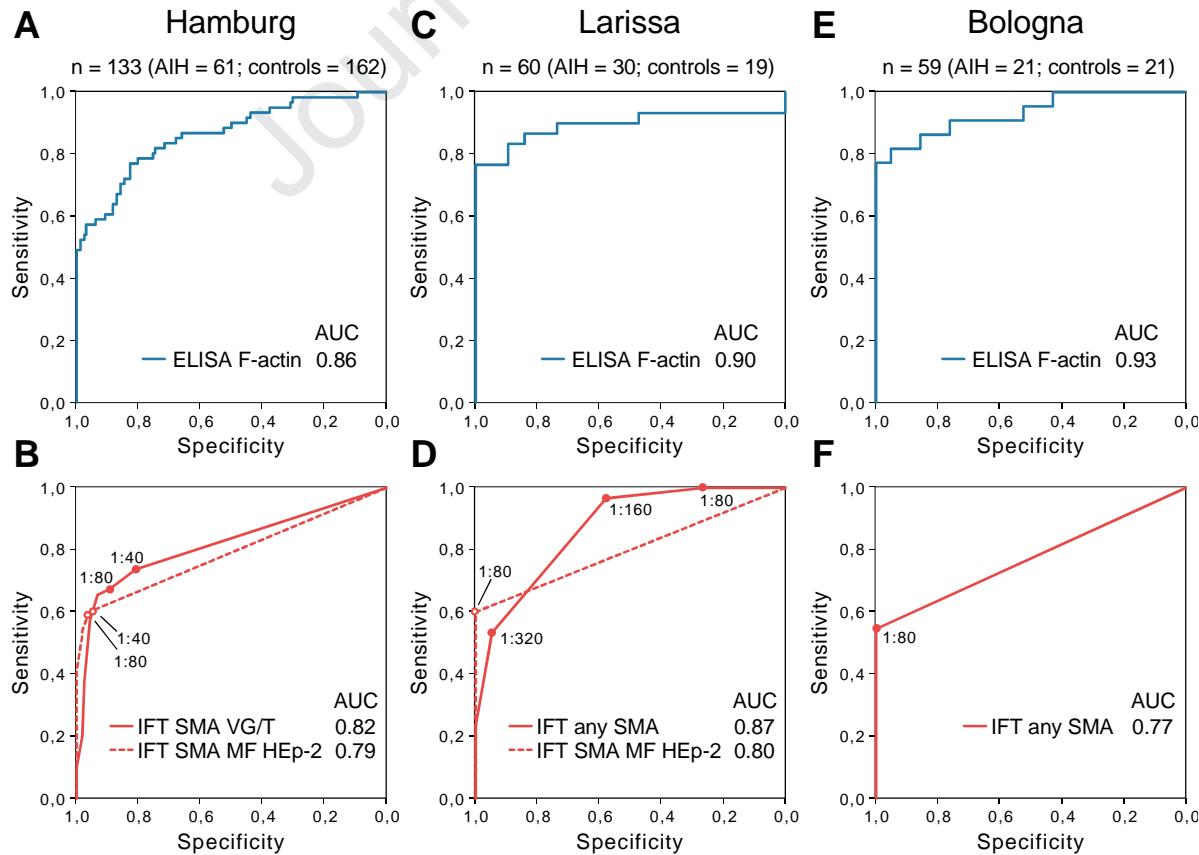
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**Figure 3:**

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**Figure 4:**

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