Quantification of polyreactive immunoglobulin G facilitates the diagnosis of autoimmune hepatitis


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Quantification of polyreactive immunoglobulin G facilitates the diagnosis of autoimmune hepatitis

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Abbreviations: HU, Hounsfield unit; AIH, autoimmune hepatitis; ALF, acute liver failure; ALT, alanine aminotransferase; ANAs, antinuclear antibodies; asAIH, acute-severe AIH; ASGPR, asialoglycoprotein receptor; AUROC, area under the receiver operating characteristic; BSA, bovine serum albumin; DILI, drug-induced liver injury; HCs, healthy controls; HGO, hypergammaglobulinemia; HILI, herb-induced liver injury; HIP1R, huntingtin-interacting protein 1-related protein; HSA, human serum albumin; IF, immunofluorescence; INR, international normalized ratio; ITSN1, intersectin 1; anti-LC1, antiliver cytosol antibodies type 1; anti-LKM, anti-liver kidney microsomal antibodies; LP, liver pancreas antigen; MAZ, Myc-associated zinc finger protein; nAU, normalized arbitrary unit; non-AIH-LD, non-AIH liver diseases; pIgG, polyreactive IgG; SLA, soluble liver antigen; anti-SMA, anti-smooth muscle antibodies; SR, Spearman’s rank-correlation coefficient; UBC, ubiquitin.

Richard Taubert and Bastian Engel first authors.

Information for the “UK-AIH Consortium” can be found in the Acknowledgment section at the end of the article.

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INTRODUCTION

Autoimmune hepatitis (AIH) is an immune-mediated liver disease with a chronic progressive disease course leading to cirrhosis and its sequelae if it is insufficiently treated.[1,2] Although rare, there has been an increasing incidence of AIH in genetically susceptible persons across several countries in recent decades.[3-5]

It can be challenging to diagnose AIH; because clinical manifestation is variable, pathognomonic features are lacking and other, more likely causes of hepatitis, such as viral infections, have to be excluded. Typical findings in AIH are elevated transaminases, a polyclonal hypergammaglobulinemia (HGG) with a predominant IgG elevation, and elevated titers of autoantibodies. A liver biopsy is finally required to diagnose AIH; because clinical manifestation is variable, pathognomonic features are lacking and other, more likely causes of hepatitis, such as viral infections, have to be excluded. Typical findings in AIH are elevated transaminases, a polyclonal hypergammaglobulinemia (HGG) with a predominant IgG elevation, and elevated titers of autoantibodies. A liver biopsy is finally required to diagnose AIH.
AIH. Typical histological features are predominant lymphoplasmacytic infiltrates with interface hepatitis, rosette formation, emperipolesis, and Kupffer cell hyaline globules.\[8-8\]

Testing for autoantibodies is the most relevant non-invasive diagnostic tool to support an autoimmune origin in patients with nonviral hepatitis. The traditional gold standard of autoantibody testing in AIH is still the titration of patient serum using immunofluorescence (IF) on three rodent tissue sections, adding a secondary IF antibody followed by microscopy, and evaluation of autoantibody staining pattern. This approach is time-consuming and requires highly trained staff. Therefore, liver autoantibodies are frequently detected by ELISAs in the USA as well as other countries. Antinuclear antibodies (ANAs) and anti-smooth muscle antibodies (anti-SMAs), the hallmarks of the most frequent AIH type 1 (AIH-1; 90% of all AIH patients), are also commonly found in other chronic liver diseases. More disease-specific anti-liver kidney microsomal antibodies 1 (anti-LKM1) and liver cytosol antibodies type 1 (anti-LC1) define the less-frequent AIH type 2 (AIH- 2; 10% of all AIH patients).\[9-11\] The best disease specificity for AIH-1 is provided by autoantibodies directed against soluble liver antigen/liver pancreas antigen (anti-SLA/LP).\[10,11\]

A recent meta-analysis found only a moderate sensitivity and specificity for ANA (65% and 75%), a moderate sensitivity and good specificity for anti-SMA (59% and 93%), and a low sensitivity with an excellent specificity for anti-SLA/LP (19% and 99%).\[9\]

The aim of this study was to identify autoantibodies with a higher sensitivity and specificity than the conventional autoantibodies used for the diagnosis of AIH and generate a clinically useful autoantibody test. After screening for autoantibodies with a protein macroarray in AIH patients, reactivity against candidate autoantigens should be tested with solid-phase ELISA in various liver diseases in direct comparison to the current standard diagnostic autoantibodies, ANA, anti-SMA, anti-LKM, and anti-SLA/LP. Similar approaches with protein microarrays identified promising autoantibodies in AIH (e.g., anti-CD124 antibodies).\[12,13\] In our study, the aforementioned approach led to the discovery of polyreactivity of IgG (plgG) to multiple human and nonhuman proteins with independent diagnostic significance.

**PATIENTS AND METHODS**

**Patients**

Three adult (age ≥18 years) patient cohorts were analyzed for this study. (1) The first cohort was a retrospective, single-center cohort of patients with AIH (before immunosuppressive therapy, n = 83; under ongoing immunosuppressive therapy, n = 42), other non-AIH-LDs (n = 160), rheumatological (n = 71, of which 19 had systemic lupus erythematosus) and neurological (n = 35) autoimmune diseases, and healthy controls (HCs; n = 112) from Hannover Medical School (Hannover, Germany). Samples were contributed from existing local biorepositories. (2) A multicenter adult validation cohort (n = 640) was recruited from existing biomaterial repositories from nine centers from eight European countries. (3) A prospectively collected single-center validation cohort (n = 235) of adult patients with AIH (n = 44) and other non-AIH-LD (n = 191), who had a liver biopsy during the diagnostic workup of their liver disease at Hannover Medical School, was recruited. Plasma samples from healthy blood donors (n = 190) were collected at the Institute of Transfusion Medicine and Transplant Engineering at Hannover Medical School. Patient data are summarized in Table 1 and Table S1.

Criteria for untreated AIH were: biopsy-proven diagnosis with the currently available scoring systems from the International Autoimmune Hepatitis Group: the original revised diagnostic score ≥10\[7\] and/or the simplified diagnostic system ≥6\[14\]; no evidence of overlapping features of primary biliary cholangitis or primary sclerosing cholangitis; and no previous immunosuppressive therapy.\[10,11\] AIH patients under ongoing therapy were analyzed separately. Criteria for acute-severe AIH (asAIH) and AIH with acute liver failure (ALF) were in accordance with current guidelines (asAIH: acute presentation of AIH without preexisting liver disease, international normalized ratio [INR] >1.5 and <2.0, and no signs of HE; AIH with ALF: acute presentation of AIH without preexisting liver disease, INR ≥2.0, and clinically overt HE).\[10,11\]

Patients’ sera/EDTA plasma samples were cryoconserved at ≤−20°C. EDTA plasma samples from the prospective adult cohort were collected within 24 hours around a liver biopsy in a prospective biorepository. Serum samples from external centers were cryoconserved according to local protocols and sent frozen to Hannover Medical School.

**Ethics**

Written informed consent was obtained from all patients from the prospective cohort (approval no.: 5582, with last update 2018) and from patients with extrahepatic diseases in the retrospective cohort (approval no.: 1322-2012). Use of retained samples from our clinical laboratories from patients with liver diseases within the retrospective cohort was approved by the local ethical committee (approval no.: 2817-2015).

Use of material and data from external patients in the multicenter cohort was approved by the respective local ethical committees.
The study conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in an a priori approval by the institution’s human research committee. All experiments were performed in accordance with relevant guidelines and regulations.

Statistical analysis

Statistical analysis was performed using SPSS (version 15.0; SPSS, Inc., Chicago, IL), GraphPad Prism (version 5; GraphPad Software Inc., La Jolla, CA), and MedCalc software (version 19.4.1; MedCalc Software Ltd, Ostend, Belgium). The Mann-Whitney U test and Student t test were used to compare quantitative data between two groups, and the Kruskal-Wallis test was used for more than two groups. Fisher’s exact test was used to prepare contingency tables with two groups. Correlation analyses were calculated with Spearman’s rank correlation. Area under the receiver operating characteristic (AUROC) analyses and Youden’s index were used to guide identification of cut-off values. Dependent AUROC curves were compared by Delong’s test.

Accuracy of the diagnostic test was calculated as:
(true positive + true negative)/total number. Sensitivities and specificities were compared with McNemar’s test. Overall accuracies were compared by the comparison of the 95% CI. p values <0.05 (two-tailed) were considered significant in all analyses.

Further methods are outlined in the Supporting Information.

RESULTS
plgG in AIH

First, 10 adult plasma samples (9× untreated AIH, 1× acute HAV as non-AIH control) from a prospective biorepository were screened for IgG autoantibodies on a protein array (Figure S1A-C). Thereby, four autoantigens were recognized by IgGs in more than 2 of 9 AIH patients and not by the HAV patient: 5× intersectin 1 (ITSN1); 5× ubiquitin (UBC); 3× huntingtin-interacting protein 1-related protein (HIP1R); and 3× Myc-associated zinc finger protein (MAZ). Anti-MAZ autoantibodies were already known to be associated with cardiovascular disease manifestations. Antibodies against ITSN1, UBC, and HIP1R were not found in other patients with mostly rheumatological autoimmune diseases in our previous studies. Next, ELISA assays with full-length UBC and recombinant fragments from ITSN1 and HIP1R, which largely corresponded to the fragments spotted on the protein array, were established to screen larger patient numbers.

During establishment of the respective ELISA, substantial binding of IgG from AIH patients to all 10 applied...
protein and nonprotein blocking reagents, which were used to prevent unspecific binding to ELISA plates, was recognized, when ELISA plates were not coated with target antigens (Figure 1A). The lowest binding was found for human serum albumin (HSA) and the highest for bovine serum albumin (BSA). Binding to blocking reagents could be decreased only partially by further dilution steps of serum from AIH patients (Figure S2A). Whereas binding of anti-LKM autoantibodies was hardly affected by increasing concentrations of NaCl or Tween 20, indicative of strong antibody affinity, the binding to BSA was attenuated by these measures (Figure S2B).

**FIGURE 1** Presence of plgG in untreated AIH. (A) Reactivity of IgG from untreated autoimmune hepatitis (AIH Dx) and healthy controls (HC) to blocking reagents. (B) IgG reactivity in liver disease patients (AIH and non-AIH liver disease (non-AIH-LD)) and HCs (mean with SD; *p < 0.05; **p < 0.01; Kruskal-Wallis’ test). (C) Coating of HIP1R to ELISA plates increased reactivity (HIP1R/BSA binding) of AIH patients’ serum samples compared to uncoated plates blocked with bovine serum albumin (BSA). Patients were ordered according to increasing reactivity to BSA. (D) IgG reactivity against HIP1R and ITSN1 in human serum albumin (HSA)-coated ELISA plates of AIH patients’ serum samples compared to uncoated plates blocked with HSA. (E) BSA reactivity with and without HIP1R preincubation. Abbreviations: FCS, fetal calf serum; OD, optical density.
Liquid-liquid preincubation of patients’ sera with BSA and solid-phase extraction of patients’ IgG on BSA-coated plates had only a minimal effect on BSA reactivity, arguing against a highly specific antibody-antigen interaction (Figure S2C). In addition, binding of IgG from AIH patients to blocking reagents was not sensitive to repetitive freeze-and-thaw cycles as well as prolonged storage of serum at room temperature (Figure S2D).

To exclude complexes of multiple IgGs as a cause for polyreactivity against blocking reagents, sera from AIH patients and HCs were ultracentrifuged at 100,000 g for 1 hour. Thereby, binding to BSA was preserved in centrifuged sera without the aggregated IgG (supernatant after ultracentrifugation) of AIH patients (Figure S2E).

Binding of IgG to blocking reagents, such as BSA and HSA, was mostly found in patients with untreated AIH and much less in patients with non-AIH-LD or in HCs (Figure 1B). IgG of patients with untreated AIH and HCs exhibited significantly different glycosylation patterns (Figure S3). To exclude a bias in favor of polyreactivity induced by these different IgG glycosylation patterns, N-glycans from purified IgG were cleaved enzymatically by incubation with PNGase F. Whereas deglycosylation reduced overall optical density in the HIP1R/BSA ELISA, the relative difference between AIH and HC remained unchanged by deglycosylation (Figure S4). In addition, IgG from AIH and HC samples were titrated to the same target concentration of 13 g/L to exclude a bias by the HGG in AIH in these analyses (Figure S4). Purification of IgG by protein A columns should have resulted in a removal of other molecules noncovalently attached to IgG. So, the polyreactivity that persisted after IgG purification cannot be explained by putatively different IgG molecule complexes in AIH and HC.

Coating of ELISA plates with HIP1R as an exemplary target autoantigen increased absorbance compared to the mere binding of the blocking reagent, as shown for HSA and BSA, in AIH patients (Figure 1C-E). This binding of HIP1R to blocking reagent could be decreased by specific preincubation of sera with HIP1R (Figure 1E). In contrast, addition of ITSN1, which was also recognized by AIH patients’ IgG on the protein array, did not result in an increased IgG binding above the background of BSA or HSA (Figure 1D). In addition, we could prove hepatic expression of HIP1R in various liver diseases (Figure S5).

In summary, patients with untreated AIH exhibited plgG with binding capacities to all blocking reagents as well as to autoantigens identified in the protein array screen.

Next, the diagnostic utility of plgG for the diagnosis of AIH was explored with HIP1R, ITSN1, and UBC, the autoantigens that were recognized by the highest number of AIH sera on the protein array, in a larger test set of patients with untreated AIH and non-AIH-LD from a retrospective cohort of liver disease patients. Therefore, further ELISA test parameters were optimized to increase the ratio of AIH patients to HCs. High inter- and intra-assay reproducibility assured a comparability of measurements of larger patient cohorts (Figure S6). These ELISAs exhibited a higher sensitivity to detect plgG than the protein array used for autoantibody screening (Figure S1D).

In an AUROC analysis to distinguish untreated AIH from non-AIH-LD, IgG reactive to HIP1R/BSA exhibited a significantly higher AUROC (AUROC = 0.847) compared to ITSN1/BSA (AUROC = 0.715) and UBC/BSA (AUROC = 0.715; Figure 2A). Thus, HIP1R-coated ELISA blocked with BSA was used to explore the diagnostic fidelity of plgG to diagnose AIH in comparison to conventional autoantibodies (at least ANA, anti-SMA, anti-LKM, and anti-SLA/LP) in larger cohorts.

**HIP1R/BSA reactive IgG in adults**

Presence of plgG binding to HIP1R/BSA was initially assessed in cryoconserved serum from a retrospective single-center cohort (Table 1 and Table S1) with available status of conventional autoantibodies. Patients with untreated AIH (n = 83) exhibited the highest serum concentration of HIP1R/BSA binding IgG compared to all disease groups analyzed and HCs (Figure 2B). During the first months of immunosuppressive therapy (time under therapy: median [range], 11 [2–85] months), serum concentrations of HIP1R/BSA binding IgG declined to background levels of non-AIH-LD (Figure S7A), which itself exhibited heterogeneous concentrations of HIP1R/BSA binding IgG (Figure S9A). Patients with rheumatic autoimmune diseases without hepatic disease manifestations (n = 71, including 19 patients with systemic lupus erythematosus [SLE]) exhibited slightly elevated HIP1R/BSA binding IgG concentrations compared to HCs (n = 112; Figure 2B). In contrast, neurological autoimmune diseases did not exhibit HIP1R/BSA binding IgGs in blood (n = 35) or in cerebrospinal fluid (n = 16; Figure 2B).

To explore and validate the diagnostic fidelity of HIP1R/BSA reactive plgG, they were quantified in all three patient cohorts. An increase of HIP1R/BSA reactivity of IgG was observed with the duration of cryoconservation of serum and plasma samples (Figure S8). To prevent false high quantification of plgG, all measurements were normalized to samples with non-AIH-LD and treated AIH with a similar storage duration (Figure S8). In the following analyses, only these normalized arbitrary units (nAU) were used to describe the polyreactivity of IgG.

**HIP1R/BSA reactive IgG in adults: retrospective training cohort**

With an AUROC analysis, a cut-off level of 1.27 nAU of HIP1R/BSA binding IgG in serum could be identified for the distinction between untreated AIH and non-AIH-LD...
in the retrospective training cohort (Figure 2C). Cut-off values for titers of conventional autoantibodies were determined in the same way and were in line with current guidelines. Test criteria of all autoantibody tests to distinguish untreated AIH from non-AIH-LD are summarized in Table 2. In summary, presence of IgG binding HIP1R/BSA was more sensitive than anti-SMA, anti-LKM, and anti-SLA/LP and more specific than ANA and anti-SMA. Anti-HIP1R/BSA exhibited the highest overall accuracy to distinguish untreated AIH and non-AIH-LD.

The overall low sensitivity of anti-LKM is attributable to the low frequency of AIH-2 in our adult cohort. Of the 4 anti-LKM-positive patients with untreated AIH, 2 were also positive for plgG (50%). However, polyspecific binding to HIP1R/BSA was weakly positively correlated with the amount of total IgG (Spearman’s rank-correlation coefficient [SR] = 0.306; \( p = 0.005; n = 82 \)), but not with alanine aminotransferase (ALT; SR = 0.087; \( p = 0.435; n = 83 \))

Levels of significance in all panels: n.s., \( p \geq 0.05 \); *** \( p < 0.001 \)

FIGURE 2  HIP1R/BSA reactive IgG in the retrospective training cohort. (A) Diagnostic fidelity of HIP1R/BSA (solid line), ITSN1/BSA (dotted line), and UBC/BSA (dashed line) reactivity to diagnose untreated AIH (AIH Dx; \( n = 76 \); Delong’s test). (B) HIP1R/BSA reactive IgG (median and interquartile range) in patients with AIH Dx (\( n = 83 \)), AIH under therapy (AIH Tx; \( n = 42 \)), non-AIH-LD (\( n = 160 \)), rheumatological diseases (RD; \( n = 71 \)), neurological diseases (ND; serum, \( n = 35 \); cerebrospinal fluid [CSF], \( n = 16 \)), and HCs (\( n = 112 \)). (C) Diagnostic fidelity of HIP1R/BSA, normalized to storage duration of serum, to distinguish AIH Dx from non-AIH-LD. Gray horizontal line represents the cutoff (1.27 nAU). The table outlines the rate of HIP1R/BSA reactive IgG above this cutoff. (D-F) Presence of IgG binding HIP1R/BSA in AIH Dx according to total IgG, to the presence (sero-pos.) or absence (sero-neg.) of diagnostic conventional autoantibodies (ANA and/or anti-SMA, anti-SLA/LP, and anti-LKM1) and treatment response: incomplete remission (IR) or biochemical remission (BR; Fisher’s exact test). Levels of significance in all panels: n.s., \( p \geq 0.05 \); *** \( p < 0.001 \)
### TABLE 2 Diagnostic Accuracies of Autoantibodies to Distinguish Untreated AIH From Non-AIH-LD

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Cutoff</th>
<th>Sensitivity</th>
<th>p vs. HIP1R</th>
<th>Specificity</th>
<th>p vs. HIP1R</th>
<th>Accuracy</th>
<th>CI</th>
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</thead>
<tbody>
<tr>
<td><strong>Retrospective training cohort</strong></td>
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<tr>
<td>Anti-HIP1R/BSA</td>
<td>243 &gt;1.27 nAU</td>
<td>0.819</td>
<td>0.781</td>
<td>0.870</td>
<td>0.823-0.916</td>
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<td></td>
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<tr>
<td>ANA</td>
<td>243 &gt;1:40</td>
<td>0.819</td>
<td>1.000</td>
<td>0.669</td>
<td>0.720</td>
<td>0.659-0.776</td>
<td></td>
</tr>
<tr>
<td>Anti-SMA</td>
<td>242 &gt;1:40</td>
<td>0.427 &lt;0.001</td>
<td>0.581 &lt;0.001</td>
<td>0.529 0.464-0.593</td>
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<tr>
<td>Anti-LKM</td>
<td>220 &gt;1:40</td>
<td>0.037 &lt;0.001</td>
<td>1.000 NA</td>
<td>0.646 0.578-0.709</td>
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<tr>
<td>Anti-SLA/LP</td>
<td>187 &gt;40%</td>
<td>0.037 &lt;0.001</td>
<td>1.000 NA</td>
<td>0.578 0.503-0.649</td>
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<td><strong>European multicenter validation cohort</strong></td>
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<tr>
<td>Anti-HIP1R/BSA</td>
<td>572 &gt;1.27 nAU</td>
<td>0.584</td>
<td>0.733</td>
<td>0.673</td>
<td>0.624-0.721</td>
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<tr>
<td>ANA</td>
<td>569 &quot;</td>
<td>0.719</td>
<td>0.004</td>
<td>0.571 &lt;0.001</td>
<td>0.631 0.590-0.671</td>
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<tr>
<td>SMA</td>
<td>569 &quot;</td>
<td>0.671</td>
<td>0.067</td>
<td>0.737 0.925</td>
<td>0.710 0.671-0.747</td>
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<tr>
<td>Anti-LKM</td>
<td>406 &quot;</td>
<td>0.027 &lt;0.001</td>
<td>0.995 &lt;0.0001</td>
<td>0.473 0.424-0.523</td>
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<tr>
<td><strong>Prospective validation cohort</strong></td>
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<tr>
<td>Anti-HIP1R/BSA</td>
<td>235 &gt;1.27 nAU</td>
<td>0.705</td>
<td>0.723</td>
<td>0.719 0.656-0.775</td>
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<tr>
<td>ANA</td>
<td>234 &gt;1:40</td>
<td>0.841</td>
<td>0.210</td>
<td>0.579 0.001</td>
<td>0.628 0.563-0.690</td>
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<tr>
<td>Anti-SMA</td>
<td>235 &gt;1:40</td>
<td>0.750 0.791</td>
<td>0.539 &lt;0.001</td>
<td>0.579 0.513-0.643</td>
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<tr>
<td>Anti-LKM</td>
<td>233 &gt;1:40</td>
<td>0.068 &lt;0.001</td>
<td>0.984 &lt;0.0001</td>
<td>0.811 0.755-0.859</td>
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<tr>
<td>Anti-SLA/LP</td>
<td>136 &gt;40%</td>
<td>0.045 &lt;0.001</td>
<td>1.000 NA</td>
<td>0.691 0.606-0.768</td>
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</tbody>
</table>

Red color indicates inferiority and green color superiority compared to anti-HIP1R/BSA.
Abbreviation: NA, not applicable.
*Adjusted for duration of cryoconservation.; ** According to center standard.
Multicenter validation of HIP1R/BSA reactive IgG in adults

To validate the results, an adult multicenter validation cohort (n = 640) was recruited from nine existing biorepositories from eight European countries. ELISA measurements were performed centrally in Hannover, Germany after shipment of cryoconserved aliquots, whereas results of conventional autoantibody testing were retrieved from the centers' own testing using the same technique of IF on three rodent tissue sections. Concentrations of plgG varied between the participating centers that did not apply harmonized protocols for serum preparation, sample storage, aliquotation, and shipment (Table S3). To compensate for center differences and storage duration, plgG concentrations were normalized to the center background of non-AIH-LD and treated AIH samples with comparable durations of cryoconservation (Figure S8). HIP1R/BSA reactive plgGs were again significantly higher in untreated AIH (n = 231) compared to AIH under therapy (n = 68) and non-AIH-LD (n = 341; Figure 3A and Figures S8B and S9). The same cut-off level from the training cohort (1.27 nAU) was applied in this validation cohort.

In summary, HIP1R/BSA binding IgG were less sensitive to diagnose untreated AIH compared to ANA, but more specific than ANA, and with a comparable overall accuracy to ANA and anti-SMA (Table 2). Anti-SLA/LP was not included into the validation cohort, because it was not homogeneously tested at all collaborating centers. Of the anti-LKM- or anti-LC1-positive patients with untreated AIH, 5 were positive for HIP1R/BSA reactive IgG (56%).

As in the training cohort, concentrations of plgG were weakly positively correlated with disease severity (IgG, SR = 0.200; p = 0.003; n = 224) and ALT (SR = 0.189; p = 0.004) levels in this multicenter cohort. Again, sensitivity of HIP1R/BSA binding IgG was independent from total IgG levels (Figure 3B) and presence of diagnostic titers of conventional autoantibodies (Figure 3C; Table S2). There was no influence of sex (p = 0.119), age (SR = 0.1; p = 0.130) or presence of cirrhosis or asAIH on concentrations of plgG in this cohort (Figure S7C).

Prospective single-center validation of HIP1R/BSA reactive IgG in adults

To compensate for differences in sample processing in various laboratories in the two previous cohorts, results were validated further with EDTA plasma samples that were prospectively collected using standard operating procedures for sample processing and storage of patients with liver diseases (2010-2019) and blood donors as HCs (2020). Measurement of HIP1R/BSA binding IgG in plasma exhibited a similar high reproducibility as in serum (Figure S6). Furthermore, the same cut-off value (1.27 nAU) as in the training cohorts was applied.

Again, HIP1R/BSA binding IgG was significantly elevated in untreated AIH (n = 44) compared to non-AIH-LD (n = 191) and HCs (n = 190; Figure 4A and Figures S8C and S9). In summary, HIP1R/BSA binding IgG was more sensitive than anti-LKM and anti-SLA/LP, more specific than ANA and anti-SMA and had the highest overall accuracy compared to the most prevalent conventional autoantibodies, similar to the training cohort. Of the 3 anti-LKM-positive patients with untreated AIH, 1 had HIP1R/BSA binding IgG (33%). Again, HIP1R/BSA binding of IgG was positively correlated with total IgG (SR = 0.544; p < 0.001), whereas there was a negative correlation with ALT.
Overall diagnostic fidelity of plgG to distinguish AIH from non-AIH-LD

In summary, of all three different cohorts, HIP1R/BSA reactive plgG was significantly elevated in untreated AIH compared to non-AIH-LD (Figure 5A). Whereas sensitivity of plgG to diagnose untreated AIH was similar to anti-SMA and lower than ANA, plgG had a significantly higher specificity than ANA and anti-SMA and exhibited a significantly higher overall accuracy to distinguish untreated AIH from non-AIH-LD compared to all other conventional autoantibody tests when the same cut-off value (1.27 nAU) was applied (Figure 5B). Based on these data, we calculated a post hoc power of 100% to discriminate untreated AIH (cumulative sample number = 358) from non-AIH-LD (cumulative sample number = 692) by BSA/HIP1R reactive IgG with an alpha error of 0.05.

DISCUSSION

This study used a protein macroarray to screen for autoantibodies in human AIH. Three previous studies applied protein microarrays with smaller numbers of spotted proteins. However, all four protein array studies reported a broad and nonoverlapping panel of autoantigens recognized by IgG (e.g., >80 autoantigens; Figure S1) from AIH patients, supporting our concept of polyreactivity of AIH IgG. Whereas three studies used solid-phase ELISAs to validate their results, the present study assessed the interference by polyreactivity of patients’ IgG systematically.

Interference of immunoassays by patients’ IgG is a well-known phenomenon. Additionally, polyreactivity of patients’ IgG to a multitude of protein and nonprotein antigens can also extend to other test reagents, such as blocking reagents, and cause false-positive results. Such plgGs are commonly found in diseases with high inflammation and even more in diseases with polyclonal HGG, both characteristic of AIH. Concentration of plgG was also positively correlated with high inflammation and even more in diseases with polyclonal HGG, both characteristic of AIH. Concentration of plgG was also positively correlated with AIH severity (IgG, ALT) in this study. plgGs, including those with autoreactivity, usually have a lower affinity and are created during class switching from IgM to IgG and de novo during somatic hypermutation during the transition from mature naïve to IgG memory B cells. plgG and HGG can arise when antigen-specific helper T cells stimulate B cells that present antigens, sometimes even irrespective of their own B-cell receptor specificity. Most recent T- and B-cell receptor repertoire analyses in AIH patients showing an AIH-specific T-cell, but not a specific B-cell, receptor repertoire are in line with this pathophysiological concept, suggesting that plgG formation results from an overshooting immune response. Interestingly, even...
the disease-specific T-cell receptor repertoire in AIH is broader than in other chronic hepatitides, such as chronic hepatitis C, underlining the polyreactivity of immune response in AIH.[23] In line with these human data, experimental murine AIH is also characterized by a polyclonality of autoantibodies and IgG.[24,25]

plG could partially be reduced by a higher dilution of patient serum and the addition of adjuvants reducing low-affinity IgG binding (e.g., NaCl or Tween 20), as described recently. However, the broad binding capacities of the polyclonal HGG in AIH cannot be removed by these assay modifications.

Whereas other studies reported plG in up to 32%, patients with untreated AIH exhibited plG in up to 82% of samples.[17] Although all blocking reagents bound IgG of the majority of AIH patients, absorbance could be increased further by coating with some, but not all, tested autoantigens. The reason why HIP1R seems to be more immunogenic than ITSN1 or UBC, all of which are ubiquitously expressed, remains elusive. HIP1R is an intracellular adaptor protein involved in membrane traffic, attachment of spindle microtubules to chromosomes, and regulation of cell death through interaction with B-cell lymphoma 2 family members.[26–28] Probably, HIP1R is one of many autoantigens that could be used to trace plG. To our knowledge, only one previous study identified anti-HIP1R autoantibodies in 6.8% of colon cancer patients, but not in HCs.[29] Thus, HIP1R reactivity seems to be no common specificity of natural antibodies in HCs. Likewise, only 3 of 302 (<1%) HCs exhibited HIP1R/BSA reactive IgG in this study.

Autoantibodies against asialoglycoprotein receptor (ASGPR), or other liver-specific autoantigens, could not be identified on the protein macroarray. ASGPR is a transmembrane receptor, and anti-ASGPRs are targeted against glycosylated conformational epitopes[30] that are most likely not represented in the Escherichia coli–expressed proteins spotted on the macroarray used by us.

Additionally, no autoantibodies that have been previously identified with custom-made protein microarrays (e.g., anti-CD124) were identified with our approach with a commercial protein array from a fetal brain complementary DNA library.[12,13,18] In contrast to many previous studies that only described the significance of an autoantibody test, we aimed to compare the HIP1R/BSA ELISA against the gold standard of conventional autoantibody detection, which is indirect IF on three rodent tissue sections.[10,11] We then tested the diagnostic fidelity of HIP1R/BSA binding IgG, as a putative surrogate marker for plG, in order to discriminate between AIH and non-AIH-LD in three independent cohorts from 10 international centers; the overall finding was a significant 15-26% higher specificity of plG than ANA and anti-SMA, respectively, whereas the sensitivity of plG to diagnose AIH was slightly lower than ANA and similar to anti-SMA. However, plG was much more sensitive, but less specific, compared to the rare but highly AIH-specific autoantibodies like anti-LKM and anti-SLA/LP. In summary, plG had the highest overall accuracy to diagnose untreated AIH compared to all other conventional autoantibodies. Another important finding was that plG was present in up to 88% of so-called seronegative AIH, in which diagnostic titers of conventional autoantibodies are missing.

Given that reactivity against HIP1R/BSA potentially arose from plG, this reactivity declined under immunosuppressive therapy, most likely as an initial sign of attenuation of the overshooting immune response. This
decline limits the diagnostic value of plgG when immunosuppressive therapy has already been initiated.

Diagnosis of plgG was not homogenous in the three independent cohorts. One reason for this could be the different composition of non-AIH-LD. The retrospective training cohort contained many patients with viral hepatitis (57%) with low reactivity against HIP1R/BSA. The retrospective, multicenter validation cohort contained many patients with autoimmune liver diseases that are prone to have high autoantibody titers (65%). The prospective validation cohort contained many patients with cryptogenic liver diseases (31%), reflecting a realistic clinical scenario. We decided to include even cryptogenic liver diseases, because diagnostic fidelity of plgG was similar whether or not cryptogenic liver diseases were excluded.

Both retrospective cohorts have the potential bias of nonstandardized sample processing (different sample tubes for blood collection, time until serum centrifugation, storage temperature, etc.). Even an increase of HIP1R/BSA reactivity with the mere duration of cryoconservation could be identified. Although this increase did not influence the relative difference between AIH and non-AIH-LD, this effect was a bias that needed to be adjusted during this study with samples from biorepositories. In this sense, different background levels of optical densities in the HIP1R/BSA ELISA were observed in the retrospective multicenter cohort. Differences in optical density background in the HIP1R/BSA ELISA between two neighboring centers (Hamburg and Hannover), both located in northern Germany, support the hypothesis that different sample processing accounts for the variances, because environmental factors are quite similar within 1-2 hours' driving distance.

plgG in AIH was associated with cirrhosis at initial AIH manifestation in our study. This would probably be related to HGG, which is often found in patients with cirrhosis. Beyond that, we found no stringent association of plgG with clinical presentation, age, sex, or treatment response to subsequent immunosuppressive therapy.

HIP1R/BSA reactive IgG exhibited no homogeneously low concentrations in non-AIH-LD. Subgroups with relatively high reactivity were alcohol-associated liver disease and drug-induced liver injury (DILI). Unfortunately, these groups were represented by only a few samples, preventing reasonable subgroup analyses. However, this would be of special interest, because the distinction between AIH, DILI, and herbal-induced liver injury (HILI) or autoimmune-like DILI/HILI can be challenging. Likewise, the discrimination between asAIH and nonautoimmune severe hepatitis with or without ALF can be difficult and clinically meaningful given that asAIH may benefit from rapid steroid therapy. Unfortunately, we did not have a long-term follow-up of all patients to assure the clinical diagnosis of AIH or DILI by the clinical disease course (e.g., successful steroid withdrawal in DILI and relapse of AIH). Recruitment of an international, multicenter cohort of DILI/HILI and ALF for the assessment of anti-HIP1R/BSA reactive IgG is currently ongoing.

The high concentration of plgG in untreated AIH binding to all tested blocking reagents and autoantigens is a major source for false-positive test results in ELISAs detecting patients' IgG (e.g., autoantibodies and antivirus antibodies). Thus, such test results should be interpreted cautiously in untreated AIH until polyreactivity (e.g., with an uncoated ELISA plate) has been excluded. The potential relevance of this finding in routine clinical practice is underlined by a recent multicenter study comparing ANA and anti-SMA autoantibodies detected with IF and different commercial ELISAs. Thereby, 40-60% of AIH patients who tested negative for ANA in IF were tested positive in ELISA-based ANA tests.

This study was focused on the establishment of a clinical autoantibody test and not on the etiology of plgG in general. In the course of experiments, regarding assay interference, we found hints for a different glycosylation pattern of IgG in AIH and HCs that have to be further explored in future studies. Given that our results are in line with the recent literature on B-cell receptor repertoires in AIH, we would raise the hypothesis that the overshooting autoimmune response stimulates the secretion of nonaffinity matured polyclonal IgG in the course of untreated AIH. This overshooting IgG stimulation might also lead to the differences in the IgG glycosylation pattern compared to HCs that, however, did not influence the diagnostic fidelity of plgG. In this context, plgG rather seems to be a phenomenon of an unleashed immune response, given that plgGs decline early after initiation of therapy, whereas AIH is still not fully controlled in many patients.

Whereas we focused on the polyreactivity of IgG, a recent study found a diagnostic relevance of autoreactive IgM, which is also known to be polyreactive and secreted before the class switch to IgG. Given that these autoreactive IgMs were highly associated with ANA and anti-SMA, an assessment of the diagnostic capacities of auto- and/or polyreactive IgM for the identification of AIH is warranted. Yet, the finding of polyreactivity against SLE and rheumatic-disease-associated autoantigens in the small number of AIH patients in that study, strongly supports our findings.

In summary, plgGs are a common finding in untreated AIH irrespective of HGG. Reactivity in a HIP1R/BSA ELISA, as a surrogate marker for plgG concentration, is independent from the presence of conventional autoantibodies. plgGs have the highest overall accuracy for the distinction between untreated AIH and non-AIH-LD compared to the most common conventional autoantibodies and could therefore be valuable for the diagnostic workup of liver diseases. Quantification of plgG will not obviate the need for a liver biopsy when AIH is suggested, but, most of all, the higher specificity could help to improve the preselection of liver disease...
patients for a liver biopsy, postponing or canceling liver biopsy in those patients with a low pretest probability of AIH.

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CONFLICT OF INTEREST

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**DATA AVAILABILITY STATEMENT**

The data that support the plots within this article and other findings of this study are available from the corresponding author upon reasonable request.

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