Clinical & Experimental Gastroenterology & Hepatology

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# *Serological Differential Diagnosis of Autoimmune Liver Diseases by Line Blot Immunoassay for Parallel Detection of Nine Different Autoantibodies*

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Article info

Received 30 January 2019
Revised 14 February 2019
Published 21 February 2019

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***Abstract***

*We aimed to evaluate a multiparametric test system for diagnosing autoimmune liver diseases in a Bulgarian cohort of patients. Methods: We investigated serum samples of 67 consecutive patients: twenty with autoimmune liver diseases (autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC), fourty-seven with liver cirrhosis or viral hepatitis, and 20 healthy persons for the presence of antibodies against AMA-M2, M2-3E(BPO), Sp100, PML, gp210, LKM-1, LC-1, SLA/LP and Ro-52 by line blot technique, and AMA, anti-ASMA, ANA, and LKM antibodies by Indirect Immunofluorescence technique (IIF). Results: Twelve out of thirteen AIH patients showed antibodies against at least one of the tested antibodies (antibodies against SLA/LP, LKM-1, and anti-Ro52 (line blot), whereas 80% of them were positive for ANA and/or ASMA (IIF). We detected six out of six positive samples in the PBC group: for AMA-M2, anti-M2-3E (BPO), anti-Sp100 and anti-gp210. The PSC patient, as well as viral hepatitis group, stayed seronegative. We found a moderate correlation (r=0.67) and 100% coincidence between the results for AMA-M2 and LKM-1 antibodies testing by line blot and IIF. Conclusion: The investigated line immunoblot represents a useful diagnostic tool for AIH and PBC contributing positively to gold standard methods such as IIF.*

*Keywords: Autoimmune hepatitis; Primary biliary cirrhosis; Primary sclerosing cholangitis; Autoantibodies; Liver immune blot; AMA-M; LKM-1; SLA/LP; SP-100; gp-210*

## Introduction

Autoimmune liver diseases, although their low prevalence, lead to liver cirrhosis progression and death from liver failure. They represent the triad of autoimmune hepatitis (AIH), primary biliary cholangitis (cirrhosis) (PBC), and primary sclerosing cholangitis (PSC), along with overlapping syndromes [1]. Liver autoantibodies have a validated role in early diagnosis of these patients, particularly in asymptomatic ones, before the development of clinical symptoms [1]. Moreover, early identification of the disease and subsequent early therapeutic intervention by immunosuppression for AIH and by ursodeoxycholic acid (UDCA) for PBC can control disease progression [2].

The International Autoimmune Hepatitis group have established the following codified criteria for the diagnosis of AIH: compatible liver histopathology (interphase hepatitis), elevated serum IgG and serum transaminases, the presence of liver autoantibodies, and negative serology for viral hepatitis [3]. By autoantibody profile and by age of onset, AIH can be divided into type 1 and type 2. However, both types could not be distinguished by their clinical presentation alone [1,4]. Asymptomatic patients at the time of diagnosis may have a good prognosis without administration of immunosuppressive therapy [1].

PBC is a progressive liver disease characterized by destruction of small intrahepatic bile ducts leading to cholestasis and eventually cirrhosis. Early diagnosis is also crucial as the disease can be controlled by UDCA [1], as mentioned above. The diagnostic criteria include definite liver histopathology of granulomas around the bile ducts, the presence of liver autoantibodies to mitochondria, as well as PBC-specific anti-nuclear autoantibodies (ANA), along with elevated serum alkaline phosphatase [5].

PSC is the third autoimmune live disorder, although it exerts many differences in comparison to other two diseases. It is considered as a cholestatic liver disease characterized histopathologically by inflammation and fibrosis of both intrahepatic and extrahepatic bile ducts, leading to stricturing and cirrhosis [6]. However, there are no diagnostic autoantibodies, nor effective treatment besides liver transplantation [1].

The overlapping syndromes of the autoimmune liver disease are poorly characterized. The prevalence of overlap with AIH has been reported in 2-19% of patients with PBC and 7-14% of patients with PSC [7].

Liver autoantibodies can be classified as non-organ specific autoantibodies, such as ANA, smooth muscle antibodies (SMA), antimitochondrial antibodies (AMA), and antibodies to liver kidney microsome type-1 (LKM-1), and liver-specific autoantibodies such as antibodies to soluble liver antigen (SLA), gp210, M2-3E(BPO), Sp100, etc. [3,8]. For routine practice in immunology laboratories, liver autoantibodies are initially screened by indirect immunofluorescence technique (IIF), followed by enzyme-linked immunosorbent assay (ELISA) or line blot when required [1].

Since the key role of liver autoantibodies in the diagnosis of autoimmune liver disease, grounded also in the diagnostic criteria, we were interested in evaluating of a robust multiparametric test system-line immunoassay for parallel detection of nine different liver autoantibodies in a Bulgarian cohort of patients.

## Materials and Methods

### Subject of the study

The study included eighty-seven subjects. Sixty-seven from them were consecutive patients with liver diseases, as follows: twenty with autoimmune liver diseases and forty-seven with other liver diseases.

Thirteen patients were with AIH at mean age 42 ± 12 years, twenty-nine with liver cirrhosis (from them fifteen with autoimmune and fourteen with viral aetiology) at mean age 52 ± 8 years, eighteen with viral hepatitis (HBV or HCV) at mean age 50 ± 16 years, six with PBC at mean age 48 ± 6 years, one 56-year-old patient with PSC. From the patients with autoimmune liver diseases, sixteen (80%) were women.

Patients were recruited at the Clinic of gastroenterology at University Hospital St. Ivan Rilski, Sofia, and the diagnosis was based on the set of anamnestic, clinical, laboratory and instrumental studies following the respective guidelines and consensuses. The exclusion criteria for patients were the following but not limited to a proved infectious disease (different from HBV or HCV), other systemic severe or psychiatric illness.

The control group of healthy persons comprises of ten men and ten women at mean age 34 ± 11 years. Gastrointestinal diseases, systemic severe or psychiatric illnesses were excluded for these subjects.

All patients and healthy controls were informed about the purpose of the study, and a written informed consent was obtained from all participants. The study was approved and performed according to the Ethical Committee of the Medical University of Sofia, and the local hospital`s ethical considerations.

### Serum samples collection

Serum samples (3 ml) from each subject were collected during a routinely performed blood withdrawal using serum separator tubes and tested for autoantibodies within 2 days.

### Indirect immunofluorescence technique

AMA, ASMA, ANA, and LKM antibodies were assessed in the serum samples by IIF (Autoantibodies-RL/RK/RS IIF, rat liver/kidney/stomach, Biosystems, Spain).

### Immunoblot testing

AMA-M2 (E2 subunit of pyruvate dehydrogenase complex), M2-3E (BPO-branched-chain oxoacid-, pyruvate- and oxoglutarate dehydrogenases), Sp100 (spot-pattern 100 kDa protein), PML (promyelocytic leukaemia protein), gp210 (glycoprotein 210), LKM-1 (liver-kidney microsomes), LC-1 (liver cytosolic antigen type1), SLA/LP (soluble liver antigen/liver pancreas antigen) and Ro-52 were assessed in the serum samples by line immunoblot technique (EUROLINE Profile Autoimmune Liver Diseases, Euroimmun AG, Germany). Purified antigens AMA-M2, M2-3E (BPO), Sp100, PML, gp210, LKM-1, LC-1, SLA/LP, Ro52) have been coated as parallel lines on the test strips.

During the first incubation, the antibodies in the serum samples bond to the test immunoblot band. Then, in the second incubation, the added second antibody conjugated with an enzyme reacts with IgG, IgA, IgM from the patient's test sample already associated with the particular antigens on the test strips. After each incubation, the free material was removed by a wash cycle. Subsequently, a substrate was added and a color reaction was developed. The assay strips were scanned with IVD-registered EUROLineScan software (Euroimmun AG, Germany) on an approved scanner for eventual digital reporting of the results. The results were given as the relative value of intensity.

All immunological testing was performed at the Laboratory of Clinical Immunology, University Hospital "St. Ivan Rilski", Sofia, according to the manufacturer`s instructions.

### Statistical methods

We analyze the raw data with the software package for statistical analysis (SPSS®, IBM 2009), v. 19. The results were accepted for significant if $p<0.05$.

### Results

Twelve out of thirteen AIH patients showed antibodies against at least one of the tested antibodies (Table 1).

**Table 1:** Prevalence of liver autoantibodies, assessed by immunoblot or IIF, in different study groups. Results are presented as number (%).

| Classification | | AIH N=13 | Liver cirrhosis N=29 | | HBV/HCV N=18 | PBC N=6 | PSC N=1 | Healthy persons N=20 |
|---|---|---|---|---|---|---|---|---|
| | | | Autoimmune N=15 | Viral N=14 | | | | |
| Immunoblot | AMA-M2 | 0 | 1 (6,7%) | 0 | 0 | 5 (83,4%) | 0 | 0 |
| | M2-3E (BPO) | 0 | 0 | 0 | 0 | 2 (33,3%) | 0 | 0 |
| | Sp100 | 0 | 0 | 0 | 0 | 1 (16,6%) | 0 | 0 |
| | PML | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | gp210 | 0 | 0 | 0 | 0 | 1 (16,6%) | 0 | 0 |
| | LKM-1 | 2 (15,4%) | 0 | 0 | 0 | 0 | 0 | 0 |
| | LC-1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | SLA/LP | 3 (23,1%) | 0 | 0 | 0 | 0 | 0 | 0 |
| | Ro52 | 2 (15,4%) | 0 | 0 | 0 | 0 | 0 | 0 |
| IIF | AMA | 0 | 1 (6,7%) | 0 | 0 | 5 (83,4%) | 0 | 0 |
| | ASMA | 10 (76,9%) | 3 (20%) | 0 | 0 | 0 | 0 | 1 (5%) |
| | ANA | 11 (84,6%) | 2 (13,3%) | 0 | 0 | 1 (16,6%) | 0 | 2 (10%) |
| | LKM-1 | 2 (15,4%) | 0 | 0 | 0 | 0 | 0 | 0 |

The most prevalent autoantibodies, assessed by the immune blot, in AIH group were the following: antibodies against SLA/LP (three patients) and LKM-1 (two patients), as well as anti-Ro52 (two patients). About eighty percent of the AIH patients were positive for ANA and/or ASMA (Figure 1a), and two-for anti-LKM-1 (Figure 1b), assessed by IIF.

We detected six out of six positive samples in the PBC group: five for AMA-M2 (two of them also positive for anti-M2-3E (BPO) and one distinct patient for antibodies against Sp100 and gp210, assessed on line immunoblot. This patient exerted multiple nuclear dots staining on IIF (Figure 2a). The PSC patient, as well as viral hepatitis group, stayed seronegative, assessed by Immunoblot or IIF.

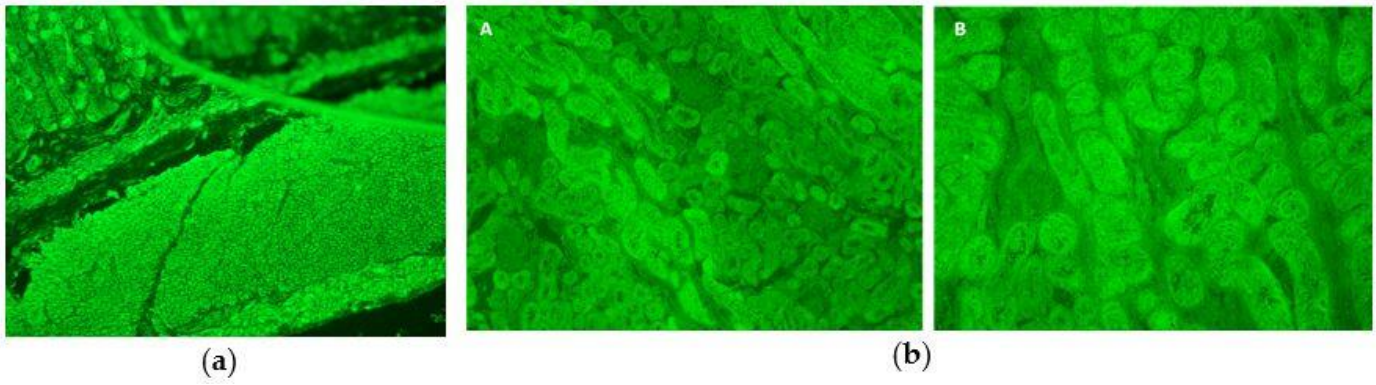


**Figure 1:** Indirect immunofluorescence staining: (a) anti-smooth muscle antibodies pattern on rat stomach tissue (IIF, 10X); (b) anti-LKM pattern on rat kidney tissue (IIF): 10X (A) and 40X (B).

According to liver cirrhosis group, we found one patient positive for AMA-M2 and four patients positive for ANA and/or ASMA (all with autoimmune cirrhosis) (Figure 2b), in contrast to patients with viral liver cirrhosis, which did not exert presence of any liver antibodies.

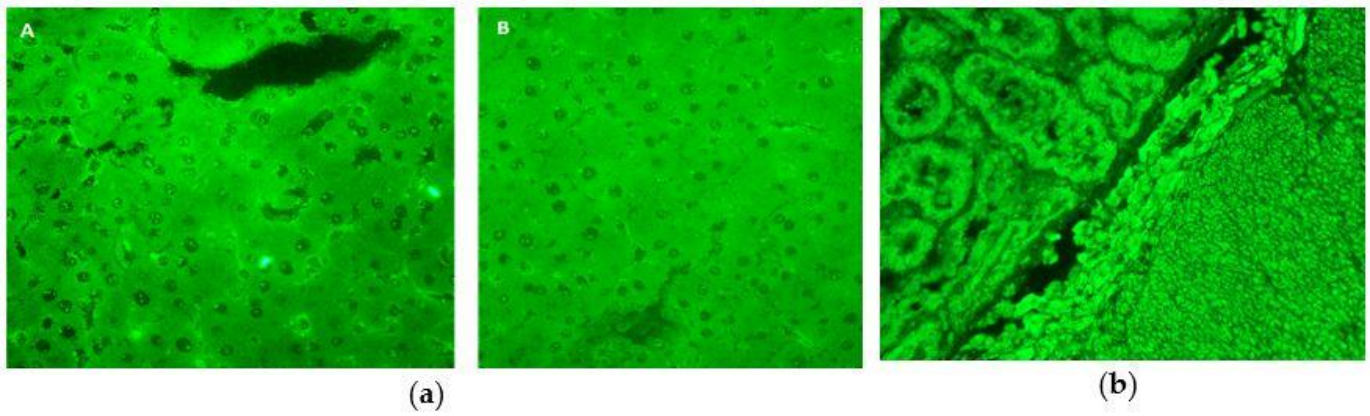


**Figure 2:** Indirect immunofluorescence staining: (a) multiple nuclear dots pattern on rat liver (IIF, 40X) on different liver areas (A and B); (b) anti-mitochondrial and anti-smooth muscle antibodies patterns on rat kidney and stomach (IIF, 40X).

No one of the tested subjects in our study was positive for antibodies against PML or LC-1 (Table 1). We detected two of the healthy persons positive for ANA, and one-positive also for ASMA. Regarding AMA-M2 and LKM antibodies, which presented in both IIF and Immunoblot testing, we found a moderate correlation ($r=0.67$; $p<0.05$) and 100% coincidence between their results (Figure 3a). The overlapping among some of the antibodies is present in Figure 3b.

**Citation:** *Velikova T, Ivanova-Todorova E, Kancheva L, et al. Serological Differential Diagnosis of Autoimmune Liver Diseases by Line Blot Immunoassay for Parallel Detection of Nine Different Autoantibodies. Clin Exp Gastroenterol Hepatol 2019; 1: 104. doi: 10.31531/edwiser.jcegh.1000104*

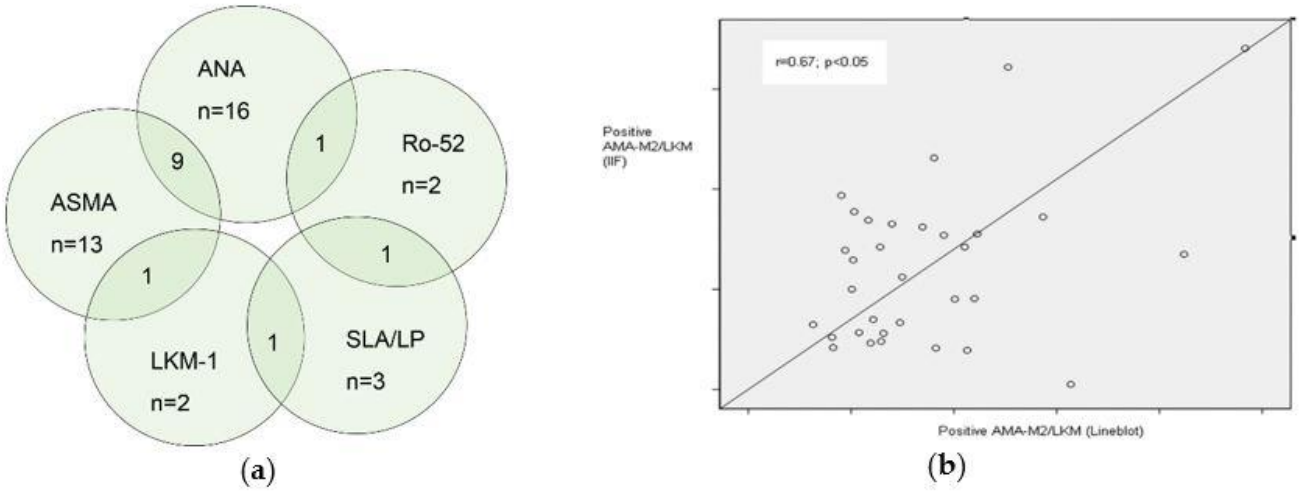


**Figure 3:** (a) Overlapping among some of the tested liver antibodies; (b) Correlation between AMA-M2 and LKM antibodies results, assessed by IIF and Immunoblot testing, among studied subjects.

## Discussion

Autoimmune diagnostics, particularly for liver diseases, in a routine clinical immunology laboratory practice is challenging. On one hand, new autoantibodies are constantly being discovered, and on the other hand-the immunoassays innovate technically every year. The challenges rice when autoantibody testing is combined in the multiparametric immunoassays [9]. Appropriate positioning of each individual antigen in the multiparametric immunoassays requires integrated knowledge of the disease criteria (grounded in the certain guidelines and consensuses), which defines also the clinical performance of the whole test system. However, the usefulness and the up keeping of the multiparametric immunoassays in the laboratory depends on the demands of the clinicians [9]. Here, we tested patients with autoimmune liver disease for the presence of different autoantibodies, by means of the gold standard IIF, as well as by line blot immunoassay for simultaneous detection of nine distinct autoantibodies.

## Autoantibodies in AIH

The prototype autoantibody for type 1 AIH-ASMA, is with specificity for F-actin microfilaments, which can be found in up to 60% of patients [1]. In our study, 76,9% of AIH patients were positive for ASMA, assessed by IIF. However, the diagnostic sensitivity of ASMA detection is 80%, thus a negative result does not exclude AIH [10]. In routine diagnostic practice, ASMA is recognized by staining of the gastric muscular external and smooth muscle fibers that extend into the lamina propria [1]. Additionally, another characteristic pattern is the staining of contractile fibrils around renal tubules and the mesangial cells of renal glomeruli together with staining of renal blood vessels [1]. It is considered a serological marker for histological and biochemical disease activity [11], but according to some authors, this correlation is limited [10]. We have to mention that low ASMA titers may also be found in viral infections (i.e., infectious mononucleosis, HCV), and rheumatic diseases, PBC, or neoplastic disease [10]. However, we did not include patients with the infectious or rheumatic disease, whereas HCV and PBC patients were negative for ASMA.

ASMA are often associated with ANA. ANA staining in AIH, assessed by IIF, gives specked or homogeneous nuclear pattern along with autoantibodies to nucleoli [1]. We documented 84,6% AIH patients positive for ANA, and 9/13 AIH patients positive for both ASMA and ANA. These nine patients were classified as having type 1 AIH.

Autoantibody to SLA/LP is an additional diagnostic marker for type 1 AIH, more recent but less frequent [12]. The target antigen is UGA suppressor tRNA-associated protein which is a serine tRNA protein complex [1] and it is present in 15-20% of AIH patients [13]. However, about 30% of anti-SLA positive AIH patients possess simultaneously ASMA or ANA [14]. This is advantageous for type 1 AIH patients who are

negative for ASMA or ANA. We detected 23,1% of AIH patients positive for SLA/LP, and none of these three patients were positive for ANA or ASMA. There are data that positive SLA antibodies indicate a more severe course and faster progression of the autoimmune disease: they correlate with a poor short- and long-term outcome, such as the 3.1-fold increased risk of hepatic related death and a 2-fold increase in the risk of relapse after drug withdrawal [1]. However, the remission rates were comparable between anti-SLA seropositive and seronegative AIH patients. Moreover, anti-SLA/LP antibodies together with anti-Ro52 also predict a poor prognosis [15]. We have also observed one AIH patient with the simultaneous presence of antibodies against SLA/LP and Ro52, but without difference in the disease behavior. In conclusion, anti-SLA/LP positive results require strict follow-up.

Regarding anti-LKM-1 antibodies, they are considered as markers for type 2 AIH [1]. Furthermore, they can be used for distinguishing between type 2 from type 1 AIH [16]. Target antigens have been identified as Cytochrome P4502D6 (CYP2D6) [1]. On IIF testing, anti-LKM autoantibodies give characteristic staining of the proximal renal tubules and hepatocytes [1]. LKM-1 antibodies exert a very high diagnostic sensitivity (70-93%), especially in young patients. However, LKM-1 antibodies could be found in patients with HCV (6-10%) [16]. Two of our AIH patients (15%) were found positive for anti-LKM-1 antibodies (IIF and line blot). Interestingly, LKM-2 and LKM-3 antibodies have also been described. Despite the former are currently only of historical interest, the latter were present in patients with a severe form of drug-induced hepatitis, patients with hepatitis D (delta), and in up to 10% of patients with AIH type 2. However, the different forms of LKM give similar immunofluorescence pattern [3]. We should emphasize that both methods of LKM-antibodies detection showed 100% coincidence of the results and a moderate correlation in our study.

Anti-LC-1 antibodies which are also largely specific for type 2 AIH, could be detected in up to 50% of young patients. The target antigen was recognized as formiminotransferase cyclodeaminase, a 62 kDa cytosolic protein [1]. By IIF, the staining pattern involves the hepatocytes but sparing the cells around the central vein. Their appearance partially overlaps with the anti-LKM-1 autoantibody [14]. Although about 30-50% of the AIH patients are positive for anti-LKM-1 and LC-1 antibodies together, each of these antibodies may present alone (10% of cases) [14]. In contrast to anti-LKM-1, anti-LC-1 antibodies parallel better disease activity, and also predict an unfavorable clinical course and a more rapid disease progression [1]. However, in our study, no one of the study subjects exerted positive result for LC-1 antibodies.

**Autoantibodies in PBC**

AMA act as diagnostic and predictive markers for PBC. They can be detected in 90-98% of cases and are primarily directed against the M2-antigen (AMA-M2) [1,17]. The target antigen was cloned and identified as the inner lipoyl domain of the E2 subunit of pyruvate dehydrogenase complex [18]. The IIF pattern is characterized by staining of distal renal tubules, gastric parietal cells, and liver hepatocytes. However, in doubtful instances, the presence of AMA should be confirmed by line blots with the target M2 antigen [1]. Eighty-three percent of our PBC patients were found positive for AMA-M2 by line blot and AMA on IIF, and one of the patients with liver cirrhosis with autoimmune etiology.

We observed excellent coincidence and correlation between both methods of detection. However, some authors raise a caution regarding the specificity of AMA-M2 antibodies due to their presence in SLE (17-23%), Sjögren's syndrome (22%), scleroderma (8-18%), and rheumatoid arthritis (10%) patients. Thus, not all positive results for AMA are associated with PBC, although it is thought that these patients have a greater risk of developing PBC in addition to their existing autoimmune disease [17]. Two of our PBC patients were positive for anti-M2-3E (BPO) which is a recombinant M2 fusion protein. The documented sensitivity/specificity rates for these antibodies are 79.4%/93.2% [19].

The PBC-specific ANA are observed mainly in AMA-negative PBC. They include antibodies to the nuclear pore complex targeting gp210 and nucleoporin p62 and antibodies to multiple nuclear dots targeting Sp100 and PML [20]. One of our PBC patients was found positive for anti-gp210 and anti-Sp100 antibodies, and no one showed positive anti-PML antibodies. Anti-gp210 antibodies showed specificity of nearly 100% for PBC, however, the sensitivity is relatively low - 21-47% of cases, depending on test [21]. Rarely, they can be found in patients with AIH, rheumatoid arthritis, polymyositis/dermatomyositis, or Sjögren's syndrome, mainly in overlapping cases. Antibodies against the gp210 were shown to be associated with extrahepatic manifestations, such as arthritis, and unfavorable outcome of PBC [21]. These antibodies give punctate nuclear rim staining on IIF testing and account for about 25% of PBC-specific ANA. Antibodies to Sp100,

visualized as multiple nuclear dots staining on IIF, have 95% specificity for PBC but again low sensitivity of 25% [21]. Rarely, they can be detected also in patients with AIH, SLE (10%), progressive systemic sclerosis (5%), rheumatoid arthritis (3%), Sjögren's syndrome (2%) [21].

PML is a transformation and cell growth suppressing protein expressed in promyelocytic leukemia cells, co-localized with Sp100 in nuclear dots [1]. Anti-PML antibodies are found in about 19% of PBC patients, often along with anti-sp100 antibodies. Regardless their low sensitivity, they are highly specific for PBC, especially AMA-negative PBC [22]. Here, we can also mention that autoantibodies to centromeres could be present in PBC patients despite they are not specific for the disease [1].

However, PBC-specific ANA should be detected on Hep2 cells substrat, as it is considered the gold standard for detection. According to ICAP nomenclature, antibodies against Sp-100 and PML are described as discrete multiple nuclear dots (AC-6), whereas against gp-210-punctate nuclear envelopestaining (AC-12) [23].

### Autoantibodies in PSC

In PSC there are no disease-specific autoantibodies. In correspondence with this, we include one patient with PSC in our study, but he stayed seronegative on IIF and line blot. However, pANCA and xANCA were described in up to 90% of cases, thus, ANCA testing could be helpful in diagnosing when PSC is suspected [16].

### Conclusion

Autoantibodies are validated as useful disease markers that facilitate early diagnosis of autoimmune liver diseases, such as AIH and PBC, allowing timely therapeutic intervention to prevent progression to liver cirrhosis and associated complication. In our study, the simultaneous testing of liver-specific autoantibodies by line immunoblot contributed to the diagnosis of 95% of our patients, thus, it was established as a beneficial diagnostic tool for patients with AIH and PBC. Furthermore, assessment of these antibodies assisted in discriminating autoimmune liver diseases from other liver diseases, ie., viral hepatitis. We could conclude that the line immunoblot with liver-specific autoantibodies represents a diagnostic tool for autoimmune liver diseases and it contributes positively to gold standard methods for autoantibody detection, such as IIF.

### Acknowledgement

None.

### Author Contribution

E. Ivanova-Todorova and D. Kyurkchiev conceived and designed the work; L. Kancheva and L. Mateva recruited the patients; Ts. Velikova, K. Tumangelova-Yuzeir and E. Krasimirova performed the immunological testing; Ts. Velikova and E. Ivanova-Todorova analyzed and interpreted the data; Ts. Velikova wrote the paper; D. Kyurkchiev substantively revised the manuscript.

### Conflict of Interest

None declared.

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*This manuscript was peer-reviewed*
*Mode of Review: Single-blinded*
*Academic Editor: Dr. Radislav Nakov and Dr. Salem Bouomrani*

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