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Autotaxin: A Potential biomarker for primary biliary cholangitis

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ABSTRACT

Background: In some patients especially those AMA negative, the diagnosis may be a challenge requiring liver biopsy. This study determined whether autotaxin, a secreted lysophospholipase D encoded by the exonucleotide pyrophosphatase phosphodiesterase 2 gene, can be used as a serum biomarker for primary biliary cholangitis.

Methods: Plasma samples were collected from 103 patients with PBC and 74 healthy controls. autotaxin levels were determined by Enzyme-linked immunosorbent assay, and its predictive value for diagnosing primary biliary cholangitis was analysed. The relationship between autotaxin and the clinical data was also evaluated.

Results: Autotaxin levels in patients with primary biliary cholangitis were significantly higher than those in healthy control (median: 60.7 ng/ml vs. 32.6 ng/ml, $P < 0.001$). The cut-off value of autotaxin in patients with primary biliary cholangitis was 38.5 ng/ml, and the positivity rate was 33.9 %, calculated twice. The sensitivity, specificity, positive predictive value, and negative predictive value were 54.3 %, 93.1 %, 84.4 %, and 74.8 %, respectively, and the area under the curve was 0.73. Autotaxin level positively correlated with immunoglobulin M level ($r = -0.22$, $P < 0.05$) and Ludwig's classification ($r = 0.76$, $P < 0.01$) in patients with primary biliary cholangitis. The positivity rate of autotaxin (50.0 %) was higher than that of anti-sp100 (16.7 %) and anti-gp210 (11.1 %) antibodies in anti-mitochondrial antibody -negative patients with primary biliary cholangitis.

Conclusions: Autotaxin may be an effective noninvasive biomarker used in diagnosis, prognosis of primary biliary cholangitis, particularly in anti-mitochondrial antibody -negative patients.

1. Introduction

Primary biliary cholangitis (PBC) is a chronic and progressive autoimmune liver disease characterised by cholestasis. As the disease progresses PBC eventually leads to liver fibrosis and the main treatment option is ursodeoxycholic acid (UDCA). Early diagnosis helps prolong survival, however accurate diagnosis of PBC is difficult due to a combination of early asymptomatic, absence of specific antibodies. The early stage of PBC typically presents with anti-mitochondrial antibody (AMA) positivity; however, clinical symptoms and laboratory tests show normal results. As the disease progresses, patients may experience fatigue and itching, and the decompensated stage manifests as liver cirrhosis [1]. Early diagnosis is important to control the continued progression of the disease. AMA is

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considered as the primary antibody used in PBC diagnosis. However, approximately 10 % of patients with PBC are negative for AMA, making it difficult to diagnose the disease.

Autotaxin (ATX), also known as exonucleotide pyrophosphatase phosphodiesterase 2 (ENPP2), is a secreted lysophospholipase D [2] that catalyses lysophosphatidylcholine (LPC) and hydrolyses it into lysophosphatidic acid (LPA). LPA is a pleiotropic growth factor similar to lysophospholipase, which can trigger various biological reactions, including cell migration, angiogenesis, neurogenesis, smooth muscle contraction, platelet aggregation, wound healing [3], and reduction of inflammation [4]. The ATX-LPA signal axis plays important role in the autophagy and migration of liver cells as well as liver fibrosis [5,6].

Early diagnosis is essential for PBC, particularly in AMA-negative cases. Researchers have attempted to identify specific serological markers for the early diagnosis and prognosis of PBC. In this study, we evaluated the diagnostic value of ATX for PBC and analysed the relationship between ATX and other clinical indicators.

2. Methods

2.1. Study population

Plasma samples were collected from 103 patients with PBC who were treated at the Department of Rheumatology and Clinical Immunology, the Affiliated Hospital of Qingdao University, Qingdao, China, between September 2020 and September 2021. The inclusion criteria of PBC were based on the diagnosis and treatment specifications in the Guide of the British Gastroenterology Association [7]: (1) serological AMA-positive, or anti-gp210/anti-sp100 positive; (2) unexplained repeated elevation of serum alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT); (3) pathological liver biopsy showing non-suppurative cholangitis. PBC can be diagnosed if two of the three are met, while other liver diseases such as viral hepatitis, fatty liver, alcoholic liver, and drug liver were excluded. The plasma of the 74 healthy controls (HC) were collected from blood donors who had a healthy physical examination. The biochemical test of patients with PBC was primarily based on the data at the time of the first admission.

2.2. Specific antibody detection

AMA, anti-nuclear antibody, and anti-centromere antibody were measured via indirect immunofluorescence (IIF). AMA positivity was determined when specific fluorescence appeared in tissue cells and serum dilution >1:100. Anti-gp210/sp100 antibody was detected by immunoblot assay (Euroimmun AG, Lübeck, Germany).

2.3. Pathological staging

Pathological liver puncture in patients with PBC was evaluated according to Ludwig's classification by two double-blinded pathologists [8]. Four stages were included in this classification method.

I. Portal Stage. Portal hepatitis, with little or no periportal inflammation or piecemeal necrosis. The biopsy evidence at this stage may be indistinguishable from that of chronic persistent or various other types of hepatitis. Although granulomas and inflammatory destruction of bile ducts may be identifiable, their presence or absence does not affect the staging.
II. Periportal Stage. Periportal hepatitis; absence of bridging necrosis and of septal fibrosis. Usually, piecemeal necrosis is present. The biopsy evidence at this stage may be indistinguishable from that of chronic active or various other types of hepatitis. Granulomas, inflammatory destruction of bile ducts, and ductular proliferation, in multiple combinations, are often identifiable; but the presence or absence of these features does not affect the staging.
III. Septal Stage. Fibrous septa (" active septa") or bridging necrosis (" passive septa"), or both. The same comments apply that were made above for stage II.
IV. Cirrhotic Stage (" true" primary biliary cirrhosis): fibrous septa and nodular regeneration. In a few instances, the biopsy evidence at this stage may be difficult to distinguish from the other types of cirrhosis.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Fresh blood (5–10 ml) from patients with PBC or HC was collected in an anticoagulant tube containing EDTA, transferred to a 15-ml centrifuge tube, and centrifuged at 1000 rpm for 5 min. After centrifugation, the upper layer was taken as plasma and then stored in a refrigerator at −80 °C.

ATX levels were detected by competitive inhibition ELISA (CUSABIO, China). Aliquoted PBC and HC plasma were added to suitable microtitre plate wells along with ATX-specific antibodies and horseradish peroxidase (HRP)-conjugated ATX. HRP-labelled ATX and unlabelled ATX were used for competitive inhibition reactions with the antibody. Colour development was discontinued, and the intensity of the colour was evaluated. The binding amount of labelled ATX was inversely related to the amount of ATX in the plasma sample. Assays following the manufacturer's instructions were executed.

2.5. Histological evaluation

Pathological liver biopsy of PBC patient was evaluated by two pathologists at double-blinded according the Ludwig's classification

[8]. Four stages were included in this classification method (Fig. 1.).

2.6. Statistical analysis

The one-sample Kolmogorov–Smirnov test was used to verify the distribution trend of the data. Continuous variables are expressed as median and interquartile range (IQR). The difference in biochemical indexes between females and males of PBC and HC was tested using the One-Way ANOVA, and $P < 0.05$ was considered significant. Receiver operating characteristic curve (ROC) can detect experimental effectiveness. SPSS 25 (USA, SPSS) was used for statistical analysis, and GraphPad Prism 8.0 (USA, GraphPad Software) was used for correlation analysis. CURVE EXPERT 1.4 was used to create a standard curve, and the ATX concentration was calculated based on the OD value.

2.7. Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University. Informed consent was obtained from patients with PBC and HCs for blood sample collection for this study. committee's reference number: QYFY WZLL 26797. We declare that all methods were carried out in accordance with relevant guidelines and regulations.

3. Results

3.1. Analysis of laboratory indicators

A total of 103 patients with PBC and 74 HCs were included in this study. There was no difference between the age of patients with PBC and HCs (53 ± 12 vs. 55 ± 11 years, $P > 0.05$, Table 1). Clinical data included data from 94 females and 9 males out of 103 patients with PBC, and 65 females and 9 males out of 74 HCs. Significant difference of γ-GT can be observed between men and women

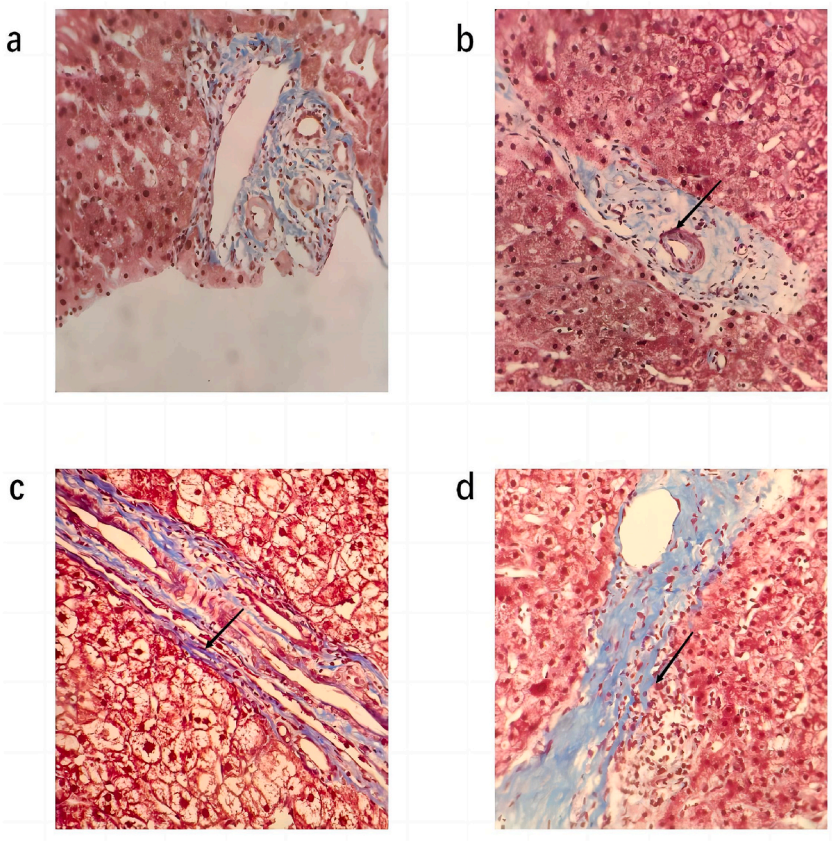

Fig. 1. The pathophysiology of the liver at various stages according to Ludwig's classification, Masson, x 400. **a** Periportal Stage: Normal cells can be found in the area around the portal vein, nevertheless some of them have intracellular steatosis. No fibrous tissue that can be found in this stage. **b** Periportal Stage: Lymphocyte aggregates and celluller edema can be seen in the portal vein area. The figure shows mild cellular punctate necrosis. **c** Septal Stage: Groups of lymphocytes as well as some fibrous bridging necrosis are showing in the picture. Certain portions of the cells are necrotic with loss of nuclei. **d** Although the hepatocyte loss is not extensive, fibrous bridges have formed, indicating that it is Septal Stage.

of PBC (P'< 0.05). However, there was no difference in the clinical data between men and women in other subjects (P > 0.05). Albumin (P < 0.001), ALT (P < 0.001), AST (P < 0.001), γ-GT (P < 0.001), ALP(P < 0.001), TBA (P < 0.001),IgM(P < 0.05), IgG (P < 0.05) were significantly different between female PBC, male PBC and HC. The evidence elaborates that the levels of ATX are independent of age and gender.

3.2. ATX levels in PBC and HC

ATX levels in patients with PBC were significantly higher than those in HCs (median: 60.7 vs. 32.6 ng/ml, P < 0.001). To balance sensitivity and specificity, cut-off value is required. Here, cut-off value was used as the threshold to count the positive rate of ATX. The cut-off value of ATX was 38.5 ng/ml, and the sensitivity, specificity, positive predictive value (PPV), and positive predictive value (NPV) were 54.3 %, 93.1 %, 84.4 %, and 74.8 %, respectively. The positivity rate was 33.9 %, calculated twice from the cut-off value. The AUC was 0.73, indicating that the assay used in this study is applicable. (Figs. 2 and 3a).

3.3. Correlation analysis of ATX and clinical characteristics

The correlations between ATX and other characteristics were assessed. ATX was correlated with IgM in patients with PBC (r = −0.22, P < 0.05, Fig. 3b). In addition, 31 patients with PBC, including 13 AMA-positive and 18 AMA-negative, were definitively diagnosed in this study by hepatic biopsy pathology, with 3, 10, 13, and 5 patients in stages I, II, III, and IV of Ludwig's classification, respectively [Fig. 1a-d]. A correlation was observed between ATX levels and Ludwig's classification (r = 0.76, P < 0.01, Fig. 3c).

3.4. Diagnostic value of ATX for AMA-negative PBC

The positivity rates of ATX, anti-sp100 antibody, and anti-gp210 antibody for AMA-positive and AMA-negative PBC were calculated. The positivity rates of ATX, anti-sp100, and anti-gp210 antibodies were 50 %, 16.7 %, and 11.1 %, respectively, in AMA-negative patients with PBC and 32.9 %, 11.8 %, and 11.8 %, respectively, in AMA-positive patients with PBC (Table 2). ATX showed a higher positive rate compared with anti-sp100/gp210 antibody in both AMA-positive and AMA-negative conditions, especially patients in AMA-negative.

4. Discussion

PBC has always been considered a rare disease in Europe and the Americas [9]; however, previous studies had shown higher morbidity and mortality of Chinese PBC patients [10,11], with increased focus on PBC and changes in environmental factors. It is latent and progressive in most patients, with the destruction of the intrahepatic bile ducts ultimately leading to cholestasis and cirrhosis. Currently, treatment for PBC is primarily based on UDCA [12]. Early diagnosis can help patients manage disease progression and provide proper treatment [13]. AMA is a hallmark of PBC; however, it does not indicate the prognosis or clinical course of the disease [14], and AMA-negative patients are often easily missed. In addition, AMA may be detected in other immune diseases and,

Table 1
Biochemical test results and antibody levels of patients with PBC and healthy controls.

	PBC(N = 103)		HC(N = 74)		
Characteristics	Female	Male		*P'* value	*P* value
Age (yr)	56 (49–66)	53 (47–60)	55 (48–62)	0.726	0.487
Albumin (g/L)[a]	39.5 (32.9–42.9)	38.0 (33.8–42.1)	44.2 (41.4–46.0)	0.637	< 0.001***
TBIL (μmol/L)[b]	70.4 (66.5–73.2)	70.1 (62.8–77.2)	70.9 (67.3–74.0)	0.625	0.79
Bilirubin (μmol/L)	15.5 (8.5–29.0)	12.5 (9.3–18.6)	16.5 (13.3–20.4)	0.418	0.672
ALT (U/L)[c]	71 (47–94)	42 (17–69)	15 (12–19)	0.100	< 0.001***
AST (U/L)[d]	59.5 (19.7–79.1)	36.2 (19.2–58.0)	18 (16–20)	0.364	< 0.001***
γGT (U/L)[e]	44 (17–170)	194 (124–264)	14 (11–19)	< 0.05*	< 0.001***
ALP (U/L)[f]	78 (55–123)	143 (82–213)	65 (53–85)	0.058	< 0.001***
TBA (μmol/L)[g]	6.5 (3.2–16.3)	8.3 (6.1–32.0)	1.7 (1.1–2.6)	0.193	< 0.001***
TC (mmol/L)[h]	4.6 (4.0–5.4)	4.8 (4.6–6.1)	4.8 (4.3–5.3)	0.305	0.53
PLT (10–9/L)[i]	185 (142–233)	190 (141–239)	196 (172–220)	0.455	0.432
IgM (g/L)[k]	2.0 (1.0–3.3)	2.3 (2.1–3.3)	1.0 (0.7–1.5)	0.078	< 0.05*
IgG (g/L)[l]	14.9 (12.0–19.0)	12.4 (8.8–17.6)	12.8 (10.1–13.9)	0.342	< 0.05*
AMA (%)	85/94 (90.4 %)	6/9 (66.6 %)	0/74		
ANA (%)	79/94 (84.0 %)	9/9 (100 %)	0/74		
Anti-gp210 (%)	10/94 (10.6 %)	1/9 (11.1 %)	0/74		
Anti-sp100 (%)	10/94 (12.6 %)	3/9 (33.3 %)	0/74		
Anti-SSA/Ro52 (%)	51/94 (54.3 %)	2/9 (22.2 %)	0/74		
ACA (%)	19/94 (20.2 %)	1/9 (11.1 %)	0/74		

Notes: [a]n.v. 65–85 g/L; [b]n.v. 3–22 g/L; [c]n.v. 7–40 U/L; [d]n.v. 13–35 U/L; [e]n.v. 7–45 U/L; [f]n.v. 35–100 U/L; [g]n.v. 0–12 U/L; [h]n.v. 2.32–5.62 mmol/L; [i]n.v. 125–350; [j]n.v. 0.7–4 g/L; [k]n.v. 0.4–2.3 g/L; [l]n.v. 7–16 g/L.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

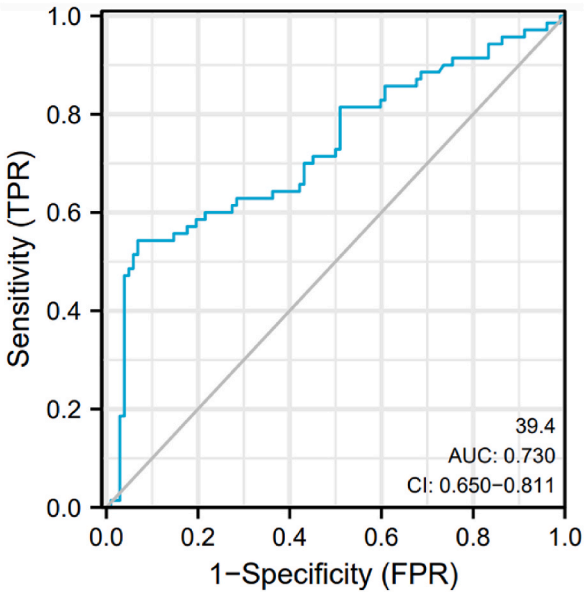

Fig. 2. Receiver operating characteristic curve (ROC) of autotaxin for predicting PBC.

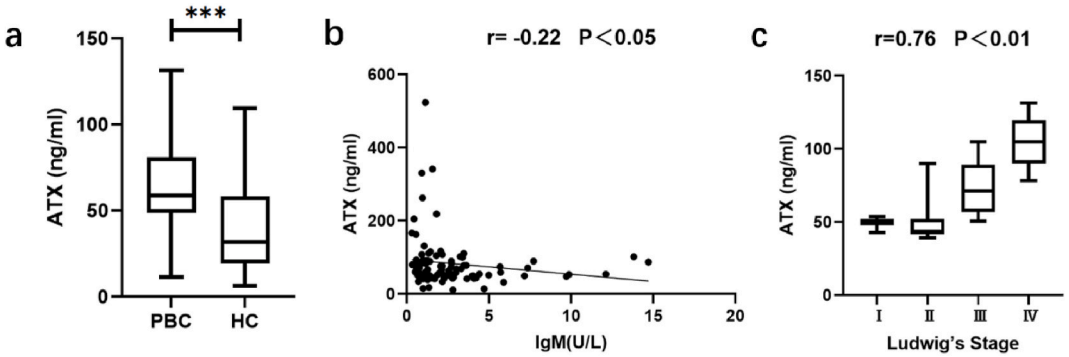

Fig. 3. A Box-and-whisker plot of autotaxin (ATX) levels in patients with PBC and healthy controls. Median values are indicated by the middle line, and statistical comparisons were done using the Mann-Whitney test. **a** Correlation between ATX and alanine aminotransferase (ALT). **c** Correlation between ATX and Ludwig stages.

Table 2
Positivity ratio of ATX and anti-sp100/gp210 antibodies in AMA-negative and AMA-positive patients with PBC.

	AMA-negative	AMA-positive
ATX	9/18 (50.0 %)	28/85 (32.9 %)
Anti-sp100-positive	3/18 (16.7 %)	10/85 (11.8 %)
Anti-gp210-positive	2/18 (11.1 %)	10/85 (11.8 %)

occasionally, in less than 1 % of healthy people. Thus, early and precise diagnosis remains challenging for PBC.

Approximately 10 % of patients with PBC test negative for AMA. In fact, the AMA-negative rate may be higher than 10 %, depending on ethnicity, admission department, and case selection [15]. AMA is primarily detected via IIF, ELISA, and immunoblotting. As the evaluation of AMA use different detection methods and scoring systems, AMA-negativity changes over time. The disadvantage of IIF is that it allows AMA to be incorrectly determined as liver–kidney microsomes, leading to false AMA-negative results [16,17]. Realistically, AMA negativity rates could be more than 10 % [18], and there may be up to 20 % patients with PBC who lack detectable AMA in IIF. Compared to IIF, ELISA offers a higher degree of automation and includes multi-parameter membranes which can assist in the detection of other specific antibodies. However, different antibodies encapsulated in ELISA may lead to false negative results, and ELISA is not as widely used as IIF [19]. Immunoblotting is not conventionally used for clinical diagnosis because it is highly prone to

errors in the judgment of AMA and is overly labour-intensive. Because IIF is the most commonly used method to detect AMA, we applied it in our study. The results showed that AMA-positivity accounted for 82.5 % of patients with PBC, which might be attributed to ethnicity, admission department, and detection methods. In this cohort, AMA-negative patients with PBC were confirmed by liver histopathology with other antibodies, such as anti-gp210 and anti-sp100.

Anti-gp210 and anti-sp100 antibodies are complementary antibodies of PBC, particularly in AMA-negative cases, and might be associated with disease progression. However, both have low sensitivity for diagnosing patients with PBC [20,21]. Two novel autoantigens, kelch-like 12 (KLHL12) and hexokinase 1 (HK1), were recently discovered [22,23]. However, they are not relevant to prognosis, and their diagnostic value for AMA-negative PBC requires further validation [24].

ATX is an autologous toxin, hemolysin D, which is encoded by the ENPP2 gene. The ATX-LPA signalling axis plays a role in the autophagy and migration of liver cells. Steatosis, insulin resistance [25], and common stimulants that cause liver damage can activate ATX [26], indicating that ATX is related to the pathogenesis and progression of liver diseases [6]. A retrospective study suggested that the concentration of ATX decreased as the degree of pruritus decreased in patients with PBC [27]. In addition, the ATX-LPA signaling axis plays a role in the process of liver fibrosis [28,29], which suggests that there might be some connections between ATX and PBC. Our results showed that the ATX levels in patients with PBC were significantly higher than those in HCs. ROC were plotted to determine the validity of the experiment demonstrated that ATX was valuable for the diagnosis of PBC. Our results also showed that IgM levels were correlated with ATX levels, which suggests that ATX might affect liver metabolism and disrupt liver immunity through the ATX–APL pathway. We discovered that ATX has high specificity and a reliable diagnostic significance for PBC, particularly in AMA-negative cases. Compared with the positivity rate of anti-gp210/sp100 antibodies in AMA-negative PBC, ATX had a significant positivity rate. Moreover, ATX levels correlated with the severity of liver injury in PBC. ATX level had a better diagnostic yield than anti-gp210/sp100 antibodies, which provides new insights for understanding the diagnosis and progression of PBC. ATX might be an effective biomarker for the diagnosis of PBC, particularly in AMA-negative patients.

Previous studies have revealed that the loss of ENPP2 can ameliorate liver fibrosis [30]. Some studies have shown that ATX plays an essential role in the progression of PBC. A longitudinal study in 2018 confirmed a significant correlation between ATX levels and disease progression [31]. A cohort study conducted by Ewa et al. found that patients with a long PBC disease had higher ATX activity [32]. In this study, an apparent correlation was observed between ATX and pathological stage, which suggests that ATX might be related to disease severity.

ATX, a common secreted protein in plasma, is readily accessible and can be rapidly detected. Notably, ATX has high specificity for the diagnosis of PBC. Compared to anti-gp210/sp100 antibodies, ATX had a higher positivity rate in AMA-negative patients. In addition, ATX was associated with disease severity, and ATX concentration was positively correlated with disease progression. Despite the high diagnostic value of ATX, its sensitivity is lower than its specificity; therefore, the combination of ATX, AMA, and other antibodies might be a reasonable approach for diagnosis.

Despite the high diagnostic value of ATX, its sensitivity is lower than its specificity, therefore, combination of ATX, AMA and other antibodies might be a reasonable approach. In addition, the expression of mRNA and protein in liver specimens with different stages of fibrosis will be the next part of our study.

In conclusion, ATX may be a valid diagnostic biomarker for PBC, particularly in AMA-negative patients. Moreover, ATX may indicate disease prognosis and severity of liver injury.

Data availability statement

The data are not publicly available due to privacy, morality and ethics. Their containing information that could compromise the privacy of research participants.

Ethics statement

This research project has been approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (No. QYFY WZLL 26797) and was conducted in strict accordance with ethical guidelines. In this study, we respected and protected the rights and privacy of the participants and ensured the confidentiality of their personal information. We will avoid misinterpretation and misuse of the data as much as possible and will only use the data for research purposes. This statement is intended to safeguard the ethical compliance of the research project and to protect the rights and privacy of the participants. If you have any further questions or concerns, please do not hesitate to contact us.

CRediT authorship contribution statement

Yifei Yang: Writing - review & editing, Writing - original draft, Formal analysis, Data curation. **Bingqian Liu:** Formal analysis. **Bo Zang:** Investigation, Formal analysis. **Qixuan Liu:** Project administration, Methodology. **Chenyang Zhao:** Methodology. **Yuan Yao:** Data curation. **Bin Liu:** Writing - review & editing, Project administration, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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