

RESEARCH ARTICLE

Serum autotaxin levels correlate with hepatic dysfunction and severity in postoperative biliary atresia

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Abstract

Objective: To investigate correlation of serum autotaxin and disease severity in biliary atresia (BA).

Methods: Eighty postoperative BA patients and 15 controls were recruited. Serum autotaxin levels were determined by enzyme-linked immunosorbent assay.

Results: BA patients had greater serum autotaxin and liver stiffness than controls. Serum autotaxin and liver stiffness were markedly elevated in BA patients with jaundice compared to those without jaundice. Furthermore, serum autotaxin was correlated with liver stiffness and biochemical parameters in BA.

Conclusions: Elevated serum autotaxin was correlated with hepatic dysfunction in BA. Accordingly, serum autotaxin is a promising biomarker reflecting the severity in BA.

Keywords

Autotaxin, biliary atresia, jaundice, liver stiffness, severity

History

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Introduction

Biliary atresia (BA) is a devastating cholestatic liver disorder in neonates characterized by progressive inflammatory cholangiopathy. It results from the fibrosclerotic destruction of the extrahepatic bile duct, leading to complete obliteration of the biliary tract at any point between the porta hepatic and duodenum. The obstruction of bile flow presents as a triad of jaundice, acholic stool, and hepatosplenomegaly. If left untreated, the majority of BA patients will develop severe hepatic fibrosis, biliary cirrhosis, portal hypertension, hepatic failure, and ultimately die by the age of 2 years (Hartley et al., 2009). Surgical treatment which remains the standard of care for first line intervention for infants with BA is the Kasai portoenterostomy (Davenport, 2012). Failure of the Kasai procedure leaves liver transplantation as the only hope for survival (Hartley et al., 2009). The precise etiology of biliary atresia remains a mystery; however, several possible theories have been proposed for pathogenesis of BA, including genetic defect, perinatal viral infection, abnormality

of bile duct morphogenesis, and immune-mediated bile duct injury (A-Kader et al., 2003).

Autotaxin, also known as ENPP-2 (ectonucleotide pyrophosphatases/phosphodiesterase-2), is a 125 kDa secreted glycoprotein that belongs to the ENPP family (Yuelling & Fuss, 2008). It was originally characterized as an autocrine motility-stimulating factor from the conditioned medium of A2058 melanoma cells (Stracke et al., 1992). Since then, the increased expression of autotaxin has been shown in various malignant tumor growth and metastasis (Stracke et al., 1997). Autotaxin uniquely exhibits a lysophospholipase D (LPD) activity through which it hydrolyzes lysophosphatidylcholine (LPC) into lysophosphatidic acid (LPA) (Tokumura et al., 2002). Autotaxin is widely expressed in tissues such as brain, placenta or high endothelial venules (Fotopoulou et al., 2010; Iwasawa et al., 2009, Nakasaki et al., 2008). In heterozygous autotaxin-null mice, both the lysoPLD activity and the LPA concentrations were about half of those observed in wild-type mice, whereas complete knock-out of autotaxin is embryonic lethal due to blood vessel abnormalities, showing that autotaxin is responsible for the bulk of LPA production in blood (Tanaka et al., 2006; van Meeteren et al., 2006).

Regarding its potential effect on hepatic stellate cells (HSCs), LPA was first shown to stimulate rat hepatic stellate cell proliferation, suggesting that LPA could be a pro-fibrogenic factor in liver (Ikeda et al., 1998). Development of liver fibrosis is coordinated by various cell types, including HSCs. In continuously injured livers hepatic stellate cells are activated and transdifferentiated into myofibroblasts, resulting

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in the production of abundant extracellular matrices (Wallace et al., 2008). Previous investigations have also suggested a connection between liver fibrosis and serum or plasma LPA and autotaxin was elevated in patients with chronic hepatitis C virus (HCV) infection (Nakagawa et al., 2011; Watanabe et al., 2007a). However, the origin and fate of serum autotaxin must be further studied and serum autotaxin should be investigated as a plausible liver fibrosis marker in not only patients with chronic hepatitis C, but also patients with liver fibrosis in general.

According to our knowledge, serum autotaxin in various clinical stages of BA and its potential role in BA patients have not yet been demonstrated. The present study is the first to evaluate the correlation of serum autotaxin, liver stiffness, and biochemical parameters in postoperative BA. We postulated that serum autotaxin would be elevated and associated with the disease severity and liver stiffness in BA patients, and to prove this hypothesis, we examined serum autotaxin and liver stiffness in BA patients compared with healthy controls. Therefore, the objective of this study was to analyze serum autotaxin levels collected from BA patients and to determine the possible correlations of serum autotaxin and biochemical parameters of postoperative BA patients.

Materials and methods

This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. All parents of children were informed of the purpose of the study and of any interventions involved in this study. Written informed consents were obtained from the participants' parents upon informing them about the protocol and procedures involved in the research.

Study population

Eighty BA patients (42 girls and 38 boys with mean age of 9.6 ± 0.7 years) who came for the follow-up visit to the Pediatric Liver Clinic and 15 healthy children (8 girls and 7 boys with mean age of 9.5 ± 0.7 years) were enrolled in this prospective study. All patients with type 3 (uncorrectable) isolated BA had undergone hepatic portojejunosotomy with Roux-en-Y reconstruction (original Kasai procedure), and they were generally in good health; no signs of suspected infection or bleeding abnormalities at the time of blood sampling. None of the participants had histories of liver transplantation or adjuvant steroid therapy, but the patients with serum total bilirubin exceeding 2 mg/dl had been treated with ursodeoxycholic acid.

Healthy controls attending the Well Baby Clinic at King Chulalongkorn Memorial hospital for vaccination had normal physical findings and no underlying disease. Serum samples were taken during their routine follow-up between January 2011 and December 2013. The duration of follow-up after the Kasai operation was 8.8 ± 0.9 years. BA patients were categorized into two groups according to serum total bilirubin (TB). Based on their jaundice status, BA children were divided into a non-jaundice group ($TB < 2$ mg/dl) and a persistent jaundice group ($TB \geq 2$ mg/dl). Subsequently,

portal hypertension (PH) was validated by the presence of ascites and/or esophageal varices as diagnosed by endoscopic screening. Twenty-eight patients had no evidence of PH whereas the rest of the 52 patients suffered from PH.

Laboratory methods

Samples of peripheral venous blood were collected from every participant, and were stored at -80°C for further measurement. Quantitative determination of autotaxin concentration in serum was performed using a commercially available enzyme-linked immunosorbent assay (ELISA) development kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Serum samples were first diluted in accordance with manufacturer's recommendation. Recombinant human autotaxin standards and serum samples were added into each well, which was pre-coated with a monoclonal antibody against autotaxin. After incubating for 2 h at room temperature, every well was washed thoroughly 4 times with wash buffer. Then, a horseradish peroxidase-conjugated polyclonal antibody specific for autotaxin was pipetted into each well and incubated for a further 2 h at room temperature. After 4 washes, substrate solution was pipetted into the wells and then the microplate was incubated for 30 min at room temperature with protection from light. Finally, the reaction was stopped by the stop solution and the optical density was measured with an automated microplate reader at 450 nm. The amount of colour generated is directly proportional to the amount of autotaxin in the sample. Autotaxin concentration was determined by a standard optical density-concentration curve. Twofold serial dilutions of recombinant human autotaxin with a concentration of 0.781–50 ng/mL were used as standards. The intra- and inter-assay coefficients of variation (CVs) were 2.6–3.7% and 2.9–4.7%, respectively. The sensitivity of this assay was 0.157 ng/mL.

The liver function tests including serum albumin, total bilirubin (TB), direct bilirubin (DB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT) were performed using a Hitachi 912 automated machine at the central laboratory of our hospital. The aspartate aminotransferase to platelets ratio index (APRI) was calculated as follows: $(\text{AST}/\text{upper limit of normal}) \times 100/\text{platelet count}$ ($10^9/\text{L}$) (Wai et al., 2003).

Liver stiffness measurement

Liver stiffness measurement was performed on the same day as blood collection. Transient elastography measured the liver stiffness between 25 and 65 mm from the skin surface, which is approximately equivalent to the volume of a cylinder of 1 cm diameter and 4 cm length. The measurements were performed by placing a transducer probe of FibroScan (Echosens, Paris, France) on the intercostal space at the area of the right lobe of the liver with patients lying in a dorsal decubitus position with maximum abduction of the right arm. The target location for measurement was a liver portion that was at least 6 cm thick, and devoid of major vascular structures. The measurements were performed until 10 validated results had been obtained with a success rate of

at least 80%. The median value of 10 validated scores was considered the elastic modulus of the liver, and it was expressed in kilopascals (kPa).

Statistical analysis

Statistical analysis was performed using the SPSS version 16.0 statistical software package (SPSS Inc., Chicago, IL). Comparisons of demographic and clinical parameters between groups were performed using Chi-square and Student's unpaired *t*-test when appropriate. Correlation between numerical data was acquired using Pearson's correlation coefficient (*r*). Data were expressed as mean \pm standard error of the mean. All the *p* values <0.05 based on a two-tailed test were considered statistically significant.

Results

Comparison between BA patients and healthy controls

A total of 80 BA patients and 15 healthy controls were prospectively recruited in the present study. The characteristics of participants in both groups are summarized in Table 1. Mean age, gender ratio, and body mass index (BMI) in BA patients and controls were not different, while liver stiffness scores in BA patients were considerably higher than those in controls (28.3 ± 2.6 versus 5.2 ± 0.7 kPa, $p < 0.001$). In addition, BA patients had significantly higher serum autotaxin levels than healthy controls (905.9 ± 53.6 versus 290.0 ± 37.1 ng/ml, $p < 0.001$), as shown in Figure 1.

Comparison between BA patients with and without persistent jaundice

We further classified BA patients into a persistent jaundice ($n = 42$) and non-jaundice group ($n = 38$). The demographic data and biochemical parameters including liver function tests, serum autotaxin, and liver stiffness values based on jaundice status are demonstrated in Table 2. BA patients with persistent jaundice had significantly lower albumin levels than those patients without jaundice. In contrast, serum

bilirubin, AST, ALT, ALP, GGT, and APRI were markedly elevated in BA patients with jaundice compared to those without jaundice. Moreover, the mean liver stiffness values of patients with persistent jaundice were remarkably higher than those of patients without jaundice (40.8 ± 3.7 versus 15.1 ± 2.3 kPa, $p < 0.001$). As presented in Figure 2, serum autotaxin levels in BA patients with jaundice were substantially greater than those in BA patients without jaundice (1144.4 ± 72.2 versus 642.3 ± 54.2 ng/ml, $p < 0.001$).

Further analysis showed that serum autotaxin levels were markedly elevated in BA patients with PH than those without PH (1078.5 ± 62.8 versus 585.4 ± 65.6 ng/ml, $p < 0.001$) (Figure 3). Additionally, serum autotaxin levels were positively correlated with serum TB ($r = 0.46$, $p < 0.001$), ALP ($r = 0.66$, $p < 0.001$), AST ($r = 0.67$, $p < 0.001$), ALT ($r = 0.35$, $p = 0.006$), and liver stiffness values ($r = 0.66$, $p < 0.001$). Conversely, serum levels of autotaxin were inversely correlated with serum albumin ($r = -0.57$, $p < 0.001$). Correlations between serum autotaxin, ALP,

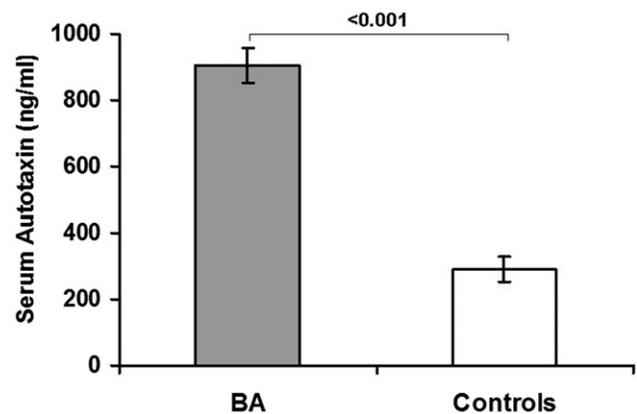


Figure 1. Comparison of serum autotaxin levels in postoperative biliary atresia patients and healthy controls. The data are expressed as mean \pm SEM.

Table 2. Comparison between biliary atresia patients with and without jaundice.

Table 1. Demographic data, biochemical characteristics, and liver stiffness scores of biliary atresia patients and healthy controls.

Variables	BA ($n = 80$)	Controls ($n = 15$)	<i>p</i> Value
Age (years)	9.6 ± 0.7	9.5 ± 0.7	0.9
Gender (Female: Male)	42:38	8:7	0.5
BMI (kg/m^2)	18.0 ± 0.6	18.0 ± 0.5	0.8
Albumin (g/dl)	4.3 ± 0.1	–	NA
Total bilirubin (mg/dl)	2.5 ± 0.5	–	NA
Direct bilirubin (mg/dl)	2.0 ± 0.5	–	NA
AST (IU/l)	128.1 ± 11.2	–	NA
ALT (IU/l)	117.8 ± 12.3	–	NA
ALP (IU/l)	430.0 ± 28.9	–	NA
GGT (IU/l)	205.3 ± 20.5	–	NA
Platelet count ($10^3/\text{mm}^3$)	162.2 ± 12.8	–	NA
APRI	3.0 ± 0.4	–	NA
Liver stiffness (kPa)	28.3 ± 2.6	5.2 ± 0.7	<0.001
Autotaxin (ng/ml)	905.9 ± 53.6	290.0 ± 37.1	<0.001

The data are expressed as mean \pm SEM. BA, biliary atresia; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; APRI, aspartate aminotransferase to platelets ratio index; NA, not applicable.

Variables	BA Patients with jaundice ($n = 42$)	BA Patients without jaundice ($n = 38$)	<i>p</i> Value
Age (years)	9.6 ± 1.0	9.5 ± 0.9	0.9
Gender (Female: Male)	21:21	21:17	0.5
BMI (kg/m^2)	17.0 ± 0.7	18.8 ± 0.9	0.1
Albumin (g/dl)	3.9 ± 0.1	4.6 ± 0.1	<0.001
Total bilirubin (mg/dl)	5.1 ± 1.0	0.5 ± 0.1	<0.001
Direct bilirubin (mg/dl)	4.3 ± 1.0	0.2 ± 0.1	<0.001
AST (IU/l)	187.7 ± 15.1	82.5 ± 10.8	<0.001
ALT (IU/l)	156.4 ± 21.2	88.2 ± 12.4	0.005
ALP (IU/l)	568.5 ± 34.0	317.5 ± 33.3	<0.001
GGT (IU/l)	265.0 ± 28.9	128.3 ± 25.8	<0.001
Platelet count ($10^3/\text{mm}^3$)	123.3 ± 17.2	192.0 ± 16.8	0.007
APRI	5.2 ± 0.6	1.5 ± 0.2	<0.001
Liver stiffness (kPa)	40.8 ± 3.7	15.1 ± 2.3	<0.001
Autotaxin (ng/ml)	1144.4 ± 72.2	642.3 ± 54.2	<0.001

The data are expressed as mean \pm SEM. BA, biliary atresia; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; APRI, aspartate aminotransferase to platelets ratio index.

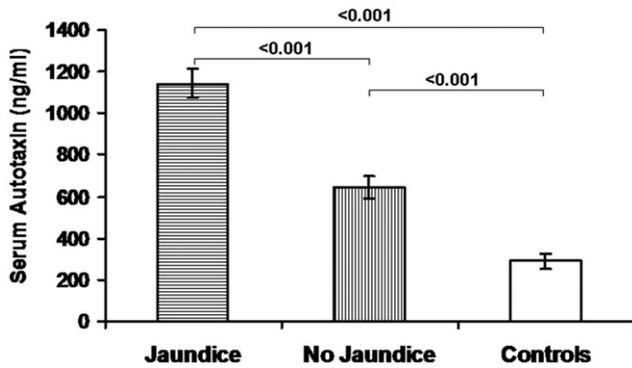


Figure 2. Comparison of serum autotaxin levels in biliary atresia patients with jaundice, biliary atresia patients without jaundice, and controls. The data are expressed as mean \pm SEM.

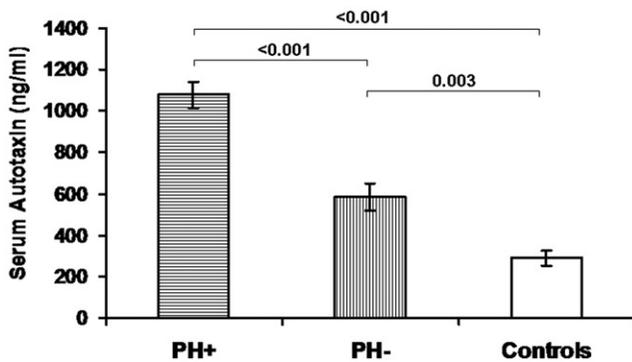


Figure 3. Comparison of serum autotaxin levels in biliary atresia patients with portal hypertension, biliary atresia patients without portal hypertension, and controls. The data are expressed as mean \pm SEM.

AST, ALT, liver stiffness, and serum albumin are illustrated in Figure 4.

Discussion

BA is an inflammatory obstructive cholangiopathy of unknown etiology, and therapeutic options are unsatisfactory. Despite early diagnosis and successful Kasai operation, a significant number of BA children inevitably develop progressive liver fibrosis, cirrhosis with concomitant portal hypertension, and end-stage liver disease. Liver transplantation is an effective treatment modality if the Kasai portoenterostomy fails and serious complications occur such as recurrent cholangitis, persistent jaundice, progressive ascites, and bleeding esophageal varices. Therefore, there remains a critical need for the assessment of fibrogenic progression in BA patients.

The functional basis for liver fibrosis and cirrhosis is activation of non-parenchymal cells, such as hepatic stellate cells. After HSCs are stimulated, these key effector cells in hepatic fibrogenesis are transformed into extracellular matrix-producing myofibroblasts. This process results in the production and the accumulation of collagen and other extracellular matrices in liver parenchyma, thus initiating and perpetuating the fibrosis (Gressner & Weiskirchen, 2006). Progression of liver fibrosis is associated with an increased number of HSCs (Yamaoka et al., 1993). LPA, which appears to be the major biological effector of autotaxin, inhibits apoptosis, stimulates, and contracts rat HSCs (Ikeda et al., 1998). Therefore, one

can speculate that elevated serum autotaxin levels could be a biochemical indicator for activation of HSCs during the development of liver fibrosis. This study has been aimed to evaluate the association between serum autotaxin, liver stiffness measurements, and biochemical parameters in BA patients after Kasai procedure.

In the present study, we demonstrated that serum autotaxin levels were significantly higher in BA patients compared with healthy controls. Furthermore, serum autotaxin levels were substantially higher in BA patients with persistent jaundice than those without jaundice. Subsequent analysis revealed that serum autotaxin was positively correlated with serum total bilirubin, suggesting that serum autotaxin was associated with jaundice status in BA patients. We further found that elevated serum autotaxin was positively correlated with serum TB, AST, ALT, and ALP in postoperative BA patients. Serum AST and ALT routinely serves as biochemical parameters of liver dysfunction reflecting hepatocellular damage. In addition, serum ALP is likely to be an indicator for the severity of biliary obstruction. Further analysis also showed a negative correlation between serum autotaxin and serum albumin. Thus, these findings indicate that autotaxin could be a useful biochemical marker in determining hepatic dysfunction and biliary obstruction in postoperative BA patients.

According to our knowledge, the present study is the first to show that serum autotaxin is elevated in BA patients compared with healthy controls. We also found that serum autotaxin was positively correlated with AST, ALP, TB, and liver stiffness, but negatively correlated with serum albumin. These results support that serum autotaxin is associated with jaundice status, hepatic dysfunction, and liver fibrosis in BA patients. Previous investigation has also indicated that autotaxin is a key enzyme for converting LPC to LPA and plasma LPA levels are correlated with the serum autotaxin activity in patients with chronic liver disease (Watanabe et al., 2007a). In agreement with our findings, Watanabe and colleagues demonstrated that serum autotaxin levels were elevated in patients with chronic hepatitis C (Watanabe et al., 2007a). A recent study using hepatectomized rats suggested that elevated autotaxin activity in rats with liver injury was caused by a decrease in autotaxin clearance (Watanabe et al., 2007b). In addition, Wu and coworkers further reported that the increased autotaxin expression was detected mainly in hepatocellular carcinoma (HCC) tissues compared to normal liver tissues and that autotaxin overexpression in HCC was specifically correlated with inflammation and liver cirrhosis (Wu et al., 2010).

It is notable that the more elevated serum autotaxin was observed in BA children with PH. PH is a consequence of advanced hepatic fibrosis that obstructs sinusoidal blood flow leading to the perpetuation of multiple varices. In this regards, portal-systemic shunting could affect the clearance of autotaxin. A reduced first-pass effect in the liver may be responsible for the greater serum autotaxin in the patients with PH. In line with our findings, Pleli et al. have documented that serum autotaxin was associated with the stage of liver cirrhosis, the prevalence of esophageal varices, and portal hypertensive gastropathy, suggesting that serum autotaxin could be an indicator for the severity of liver disease and the prognosis of cirrhosis patients (Pleli et al., 2014).

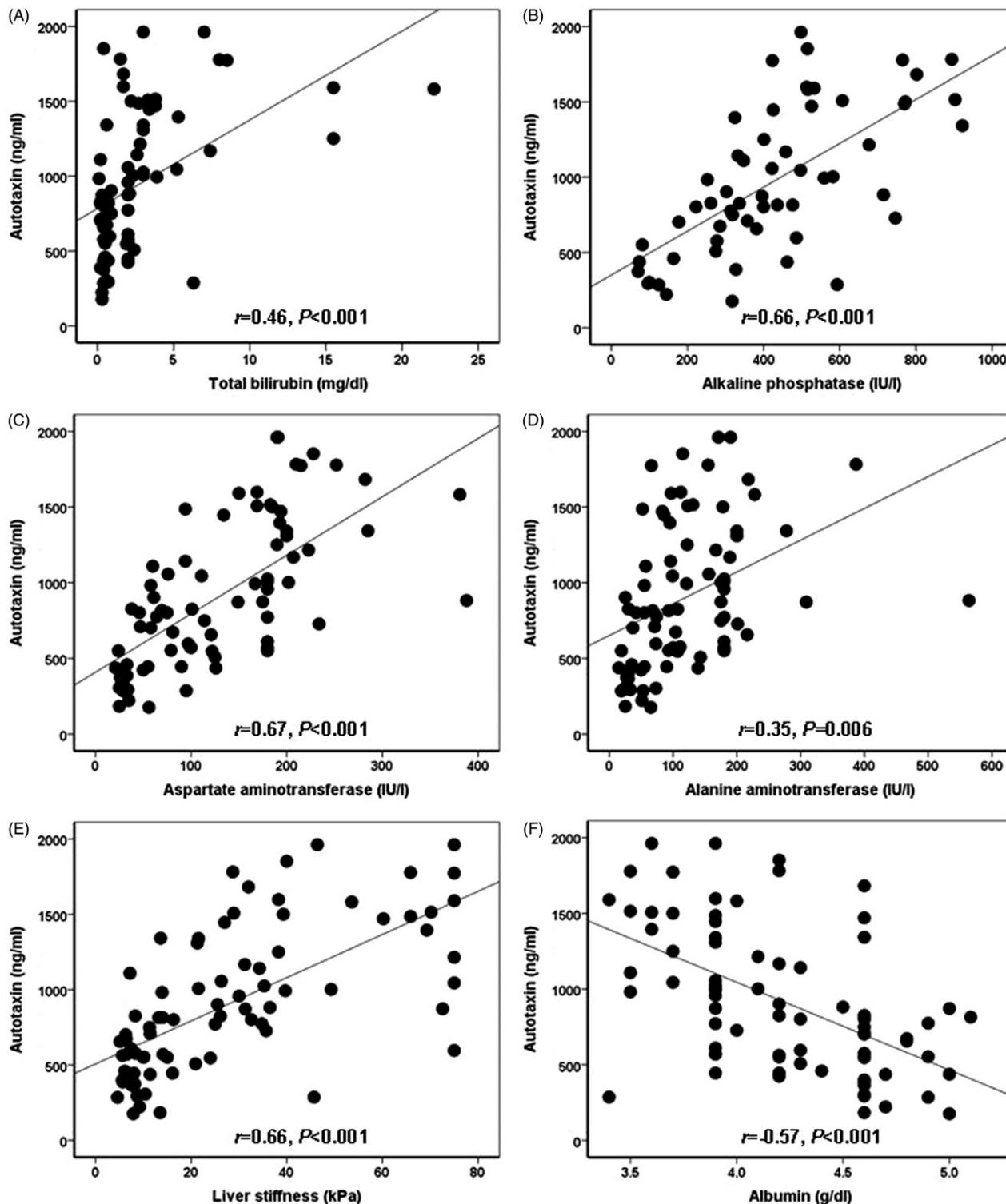


Figure 4. Scatter diagram and correlation analysis in biliary atresia patients. Serum autotaxin levels are correlated with total bilirubin (A), alkaline phosphatase (B), aspartate aminotransferase (C), alanine aminotransferase (D), liver stiffness (E), and albumin (F).

Several possible mechanisms may contribute to the significant elevation of serum autotaxin in BA patients, especially in those with a poor outcome. The elevated serum autotaxin is likely attributed to an increase in autotaxin production, a reduction in autotaxin clearance from the circulation or a combination of both. In the advanced BA patients with jaundice and/or PH, the decreased clearance could be caused by reduced uptake of autotaxin by liver sinusoidal endothelial cells (LSEC) (Jansen et al., 2009).

Lack of LSEC fenestration and formation of an organized basement membrane resulting in the capillarization of liver sinusoids, not only precedes fibrosis, but is also permissive for HSC activation and fibrosis (Muro et al., 1993). Thus, dysregulation of the LSEC phenotype is a critical step in liver fibrosis. This process may lead to a reduction in autotaxin clearance thereby increasing circulating autotaxin levels. Furthermore, other organs apart from the liver can produce and secrete autotaxin in systemic circulation. In recent years,

autotaxin expression has been evident in brain, lung, heart, liver, duodenum, adrenals, and skeletal muscle, indicating that autotaxin could be expressed in various tissues or organs (Stefan et al., 1999). The major sources of high serum autotoxin in this study may be extrahepatic organs. The higher autotaxin levels could be regarded as indicating hepatic damage and cholestasis in BA children.

A number of caveats need to be emphasized regarding the current study. First, the study is cross-sectional in design with relatively small numbers of patients and controls. Accordingly, cause-and-effect relationships cannot be concluded and require prospective longitudinal studies to elucidate any relationships. However, with a small sample size, caution must be applied, as the findings might not be transferable to other populations. Secondly, incomplete evaluation of possible confounding factors including medical comorbidities needs to be taken into account. Other limitations would be the lack of serum creatinine and pediatric end-stage liver disease (PELD) values. Future studies could evaluate whether serum creatinine correlates with serum autotaxin and further determine the PELD score for assessing the severity of chronic liver disease. Moreover, this study was limited to those patients who attended our hospital. As a result, the findings might not be directly applicable to subjects from other ethnic groups. Ultimately, tissue expression of autotaxin has not been determined. Additional immunohistochemical analysis of autotaxin hepatic expression could render more valuable information on the pathophysiologic role of autotaxin in BA.

In summary, the current evidence revealed that BA patients had significantly elevated serum autotaxin and liver stiffness values compared with healthy controls. Serum autotaxin and liver stiffness values were markedly higher in BA patients with persistent jaundice than in those without jaundice. Subsequent analysis showed that BA patients with PH had substantially greater serum autotaxin than those without PH. Furthermore, serum autotaxin was associated with status of jaundice, hepatic dysfunction, and liver stiffness in post-operative BA. Based on these findings, serum autotaxin and liver stiffness measurements could serve as possible non-invasive biomarkers reflecting the disease severity and the development of liver fibrosis in the post Kasai BA patients. Further studies will be needed to determine the exact mechanisms resulting in increased serum autotaxin in BA. Although underlining mechanisms of the cause-and-effect relationships are not entirely elucidated, there is abundant room for further research regarding the potential role of autotaxin in the pathogenesis of biliary atresia.

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Declaration of interest

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