Title: Significant hepatic expression of IL-2 and IL-8 in biliary atresia compared with other neonatal cholestasis disorders

Keywords: biliary atresia; interleukin 2, interleukin 8, etiopathogenesis, immunostaining, neonatal cholestasis

Corresponding Author: Dr. Mostafa Mohamed Sira, M.D.

Corresponding Author's Institution: National Liver Institute

First Author: Reda S Arafa, MD

Order of Authors: Reda S Arafa, MD; Omima M Abdel Haie, MD; Dina S El-Azab, MD; Amira M Abdel-Rahman, MSc; Mostafa Mohamed Sira, M.D.
Significant hepatic expression of IL-2 and IL-8 in biliary atresia compared with other neonatal cholestatic disorders

Running title: IL-2 and IL-8 immunostaining in biliary atresia

Reda Sanad Arafa, Omima M. Abdel Haie, Dina Shehata El-Azab and Amira Mohamed Abdel-Rahman, Mostafa M Sira

aDepartment of Pediatrics, Faculty of Medicine, Benha University, Egypt.
bDepartment of Pathology, National Liver Institute, Menofiya University, 32511 Shebin El-koom, Menofiya, Egypt.
cDepartment of Pediatric Hepatology, National Liver Institute, Menofiya University, 32511 Shebin El-koom, Menofiya, Egypt.

Correspondence to: Mostafa M Sira, Department of Pediatric Hepatology, National Liver Institute, Menofiya University, 32511 Shebin El-koom, Menofiya, Egypt. msira@liver-eg.org

Telephone: +2-048-222-2740 Fax: +2-048-223-4586
Abstract

Objectives: Although the exact etiology of biliary atresia (BA) is still elusive, inflammation plays a key role. Release of proinflammatory cytokines from activated immune cells perpetuates the injury and causes biliary destruction. We aimed to study interleukin (IL)-2 and IL-8 expression in liver tissue of BA patients compared with other neonatal cholestatic disorders. Methods: The study included 59 infants with neonatal cholestasis in two groups; BA group ($n=31$) and non-BA group ($n=28$) with cholestatic disorders other than BA as controls. Demographic, clinical, laboratory, and histopathological parameters were collected. IL-2 and IL-8 immunostaining was performed. Immunostaining in portal cellular infiltrate was scored as positive or negative and expressed as the mean cell count in three portal tracts.

Results: The mean value of IL-2 and IL-8 positive inflammatory cells was significantly higher in BA than in non-BA group ($P$-values of 0.004 and 0.002 respectively). IL-2 correlated significantly with IL-8 immunostaining in both BA and non-BA group ($P<0.0001$ for both). Furthermore, both cytokines in both groups correlated significantly with inflammatory activity in liver biopsy while there was no significant correlation with the other studied parameters. Yet, there was a trend of increased expression of IL-2 and IL-8 with increasing stage of fibrosis in BA group. This trend was not observed in non-BA group.

Conclusion: The significantly higher expression of IL-2 and IL-8 in patients with BA compared to non-BA suggests a potential role for these cytokines in the pathogenesis in therapy of this devastating neonatal hepatic disorder.

Keywords: biliary atresia; interleukin 2, interleukin 8, etiopathogenesis; immunostaining, neonatal cholestasis
1. Introduction

Biliary Atresia (BA) is a complex disorder for which the etiology is still far from clear. It constitutes about one third of all neonatal cholestasis and the most frequent cause for surgery for cholestatic jaundice in this age group [1]. It is a destructive inflammatory obliterative cholangiopathy that affects varying lengths of both intrahepatic and extrahepatic bile ducts [2]. If not treated, BA leads to cirrhosis, hepatic failure and death within the first two years of life [3, 4].

The precise etiology remains largely unknown [5]. Therefore, it hampers prevention strategies or therapies designed to stop progression of the necro-inflammatory process and bile duct injury. Viral infection as a trigger factor leads to development of an autoimmune response against antigens from the biliary epithelium, activation of resting auto-reactive T cells, and polyclonal activation of lymphocytes [6]. Recently, the association of defects of genes involved in biliary tract morphogenesis in a patient with BA has also been reported [7].

The immune system plays a major role in the progressive fibrosis and obliteration of bile ducts in BA [8]. The predominant cellular immune response in BA at diagnosis encompasses activated CD4⁺ and CD8⁺ T cells within portal tracts [9]. This is followed by release of proinflammatory cytokines, from activated immune cells, that perpetuates the injury and causes biliary destruction, followed by fibrosis, producing the atresia phenotype [10].

Evidence for a proinflammatory state came from a gene expression analysis of liver biopsy specimens which showed a coordinated activation of genes involved in T helper 1 (Th1) response at early stages of BA [11, 12]. Interleukin (IL)-8 is not only a potent recruiter of neutrophils and T cells but also a potent stimulator of the degranulation [13]. Neutrophilic infiltration around reactive bile ductules may be related to the IL-8 expressed in bile ductular
epithelia [14]. The proinflammatory immune response within the liver in BA may be
associated with upregulation of inflammatory cytokines such as IL-2, interferon gamma (IFN-
\( \gamma \)), and tumor necrosis factor-alpha (TNF-\( \alpha \)) [11, 15].

The aim of the current study was to investigate IL-2 and IL-8 expression in liver tissue in
BA patients compared with other neonatal cholestatic disorders.

The current study showed that the expression of IL-2 and IL-8 was significantly higher in
liver tissue of patients with BA compared to non-BA. This may suggest a potential role for
these cytokines in the pathogenesis of BA.
2. Patients and methods

2.1. Study population

This retrospective study included 59 infants with neonatal cholestasis in whom liver biopsy was indicated for etiological diagnosis. Primarily, 80 patients (40 with BA and 40 with non-BA cholestasis) were selected from the medical records of the Pediatric Hepatology department, National Liver Institute, Menofiya University, Egypt. Liver biopsy paraffin blocks of selected patients were retrieved from the archives of Pathology department, National Liver Institute, Menofiya University. Those with no available tissue paraffin blocks or with insufficient tissue for histological examination were excluded from the study. Finally, the study included BA group \((n = 31)\), aged between 30 and 105 days; and non-BA group \((n = 28)\), with cholestasis due to causes other than BA, aged between 15 and 191 days. Being a retrospective study, a written informed consent was not needed. The study was approved by the Research Ethics Committee of the National Liver Institute, Menofiya University, Egypt.

2.2. Etiological diagnosis

After full history taking, thorough clinical examination, routine investigations, the patients in each group were allocated as BA and non-BA by our newly developed BA diagnostic score [16] which has a higher sensitivity (100%) and specificity (97.67%) compared to previously developed scores of Chiba and Kasai [17] which has a sensitivity of 90% and that of Gupta et al [18] which has a sensitivity of 91.5%, a specificity of 76.3% in discriminating BA. Diagnosis of BA was confirmed by operative cholangiography and/or laparotomy prior to surgery. Routine investigations included total and direct bilirubin, total proteins, albumin, alanine transaminase, aspartate transaminase, alkaline phosphatase, gamma glutamyl transpeptidase, prothrombin time, complete blood count, viral antibodies (immunoglobulin [Ig]M and IgG for rubella, cytomegalovirus, herpes simplex virus type 1
and 2 and hepatitis B virus core), toxoplasma antibodies (both IgM and IgG), hepatitis B surface antigen, ultrasonography (US) and Doppler US, and liver biopsy. Follow up in the non-BA group, together with a set of specific laboratory tests according to the expected etiology, the diagnosis of BA was ruled out in the patients of this group. Their diagnoses were progressive familial intrahepatic cholestasis ($n = 8$), galactosemia ($n = 4$), tyrosinemia ($n = 1$), idiopathic neonatal hepatitis ($n = 5$), Alagille syndrome ($n = 3$), cytomegalovirus hepatitis ($n = 3$), Niemann-Pick type C ($n = 1$), ARC (Arthrogryposis, Renal dysfunction, Cholestasis) syndrome ($n = 1$), inborn error of bile acid metabolism ($n = 1$), congenital hepatic fibrosis, cholangitic type ($n = 1$).

2.3. Liver biopsy and IL-2 and IL-8 immunohistochemistry

Ultrasonography-guided liver biopsy was done for all patients using a Tru-Cut needle (GTA, Quistello, MN, Italy). A core of liver tissue containing at least 5 portal tracts was considered sufficient. Biopsy specimens were fixed in formalin and embedded in paraffin. Five-µm thick sections were cut, mounted on glass slide and stained with Hematoxylin and Eosin to evaluate pathological changes, with Mason-Trichrome that stains collagen fibers to assess fibrosis, and with Perls' Prussian blue stain which reveals iron deposits. Portal fibrosis and inflammatory activity were assessed using a semi-quantitative histopathological scores as described in Russo et al [19]. For comparative purpose, we further categorized Russo fibrosis grades arbitrarily into mild (Russo 1 and 2), moderate (Russo 3) and severe (Russo 4 and 5). The slides were blindly interpreted by a histopathologist who was unaware of the clinical data or the final diagnosis. For immunohistochemical staining of IL-2 and IL-8, 4-µm sections were cut from paraffin blocks and placed on positive charged slides then deparaffinized in xylene and rehydrated in descending grades of ethyl alcohol. The sections were pre-treated using heat-mediated antigen retrieval with EDTA buffer (pH9, epitope retrieval solution;
Dako, Carpinteria, CA). Endogenous peroxidase was blocked using a 3% H$_2$O$_2$ methanol solution, followed by blocking with 5% bovine serum albumin in phosphate buffer saline (PBS) at 37 °C for 30 minutes to prevent nonspecific binding of antibodies. Then the slides were incubated overnight with the primary antibody at 4°C, using rabbit polyclonal anti-IL-2 antibody (NBP2-16948; Novus Biologicals, Littleton, CO 80120, USA) at a dilution of 1:50 or rabbit polyclonal anti-IL-8 antibody (NBP2-16958; Novus Biologicals, Littleton, CO 80120, USA) at a dilution of 1:100. After several washing in PBS, the slides were incubated with biotin-conjugated secondary antibody using goat anti-rabbit immunoglobulins and then incubated with streptavidin- horseradish peroxidase (HRP). The reaction was visualized using diaminobenzidine (DAB) followed by Hematoxylin counterstaining (All were done using LSAB2 System-HRP, Dako, North America). Positive immunostaining was identified by brownish discoloration of inflammatory cells in the portal tracts. The expression of IL-2 and IL-8 was scored according to the presence or absence of immunostain as positive or negative for immunoreaction with no intensity variation. The expression was quantified by calculating the mean of immunoreactive (positive) inflammatory cells counted in the richest three portal tracts along the length of tissue core biopsy using Olympus Light Microscope (Tokyo, Japan).

2.4. Statistical analysis

Descriptive results were expressed as mean ± standard deviation (mean ± SD) or number and percentage. For quantitative data, significance was tested either by independent sample t-test or Mann–Whitney U-test according to the nature of the data. For qualitative and categorical data, significance was tested by χ$^2$-test or Fisher's exact test. Correlation was tested by Spearman's test. The diagnostic value of immunostaining was assessed by calculating the area under the receiver-operating characteristic (ROC) curves. The cut-off value for optimal clinical performance was determined from the ROC curves. The diagnostic
performance was measured as sensitivity, specificity, and balanced accuracy and expressed as percentage. Results were considered significant if \( P \leq 0.05 \). Statistical analysis was performed using SPSS software version 13 (SPSS Inc, Chicago, IL, USA).
3. Results

3.1. Study population's characteristics

The current study included 59 infants divided according to the final diagnosis into BA group \((n = 31)\) and non-BA group \((n = 28)\). Both were age and sex matched \((P > 0.05)\). The occurrence of clay-colored stool and serum levels of gammaglutamyl transpeptidase (GGT) and platelet count were significantly higher in BA than in the non-BA group \((P <0.0001, <0.0001\) and \(0.015\) respectively). On the other hand, prothrombin time (PT) and serum ferritin were significantly higher in non-BA group than in BA group \((P = 0.018\) and \(0.014\) respectively). Other baseline demographic, clinical, and laboratory parameters were comparable in both groups (Table 1).

3.2. Histopathological findings

The occurrence of ductular proliferation, bile plugs, higher grades of portal fibrosis and higher grades of inflammatory activity were significantly higher in BA group than the non-BA group \((P <0.0001, <0.0001, 0.003\) and \(0.001\) respectively), while the frequency of multinucleated giant hepatocytes was significantly higher in non-BA group \((P = 0.026)\). All BA patients had either mild or moderate/severe portal cellular infiltrate in liver biopsy and none had no/minimal portal cellular infiltrate. On the other hand 35.7\% of non-BA patients had no/minimal portal cellular infiltrate and the remaining 64.3\% had either mild or moderate/severe portal cellular infiltrate (Table 2).

3.3. IL-2 and IL-8 immunostaining in liver biopsies and their correlation with the studied parameters in both groups

The expression of both cytokines in the hepatic parenchyma was comparable in both groups while none of the bile ducts was positive in BA or non-BA group. The expression of IL-2 and IL-8 was evident in the inflammatory cells in the portal tract in both groups (Figure
1). The expression was significantly higher in BA than in non-BA group ($P = 0.004$, and 0.002 for IL-2 and IL-8 respectively) (Fig. 2a and 2b). Both cytokines correlated significantly with each other in both BA and non-BA group ($P < 0.0001$ for both) (Fig. 3a and 3b). The expression of both IL-2 and IL-8 correlated significantly with portal cellular infiltrate in liver biopsy in both BA ($P = 0.004$ for both cytokines) and in non-BA group ($P < 0.0001$ for both cytokines) while there was no significant correlation with other studied parameters in both groups (Table 3). Although there was no significant correlation between cytokine expression and portal fibrosis, yet, there was a trend of increasing expression with increasing fibrosis in BA group (Fig. 4a and 4b), but not in the non-BA group (Fig. 4c and 4d).
Figure 1: Immunostaining of IL-2 and IL-8 in liver tissue. (a) A case of biliary atresia showing positive cytoplasmic IL-2 expression in portal cellular infiltrate (arrows) as well as hepatic parenchyma whereas bile ducts (arrows head) showed negative IL-2 expression (original magnification x200). (b) A case of biliary atresia showing positive cytoplasmic IL-8 expression in portal cellular infiltrate (arrows) as well as hepatic parenchyma whereas bile ducts (arrows head) showed negative IL-8 expression (original magnification x200). (c) A case of progressive familial intrahepatic cholestasis showing negative IL-2 expression in portal cellular infiltrate (arrows) whereas hepatic parenchyma showed positive IL-2 expression (original magnification x200). (d) A case of idiopathic neonatal hepatitis showed marked giant cells transformation in positive IL-8 in hepatic parenchyma with decreased expression of IL-8 in portal cellular infiltrate (arrows) (original magnification x200).
Figure 2: Comparison of IL-2 (a) and IL-8 (b) expression in the studied groups. Box-and-whiskers plot for liver stiffness measurement. The top and bottom of each box are the 75\textsuperscript{th} and 25\textsuperscript{th} percentiles. The line through the box is the median and the error bars are the maximum and minimum. The horizontal bar represents the significance between the designated groups.

Figure 3: Correlation of IL-2 with IL-8 in the biliary atresia group (a) and non-biliary atresia group (b).
Figure 4: IL-2 and IL-8 expression in different fibrosis categories in biliary atresia group (a and b) and in non-biliary atresia group (c and d). The bar represents the mean and the error bar represents the standard deviation.
4. Discussion

The inflammatory process in BA is an ongoing one leading to progressive scarring of the liver and severe fibrosis ending in cirrhosis even after successful surgery. This is probably due to the persistent release of proinflammatory cytokine by the primed immune cells in the portal tracts around bile duct vicinity [8]. The induction and maintenance of systemic and local inflammatory responses plays a pivotal role in this process. The expression of proinflammatory cytokines induces the expression of adhesion molecules and production of chemokines by endothelial cells, which encourages the recruitment of inflammatory cells and perpetuates liver damage [20]. Similar to other studies [21, 22], we have previously demonstrated the significantly increased expression of adhesion molecules such as intercellular adhesion molecule-1 [23], neural cell adhesion molecule [24] and P-selectin [25] in BA patients compared to non-BA cholestatic disorders.

In the current study, we found that higher grades of portal cellular infiltrate (moderate/severe) were frequently observed in BA group (45%) while the majority of non-BA group had either no/minimal (35.7%) or mild (39.3%) portal cellular infiltrate. The degree of activity is a reflection of lymphocytic and neutrophilic infiltration. Mack et al and Pacheco et al [15, 26] described a prominent portal inflammation in BA. On the other hand, Lee and Loi [27] reported that lymphocytic and neutrophilic infiltrations were uncommon in BA ranging between 3% and 20%.

Serum ferritin is widely recognized as an acute phase reactant and a marker of acute and chronic inflammation, and is nonspecifically elevated in a wide range of inflammatory conditions, including chronic kidney disease [28], chronic liver injury [29] and autoimmune disorders [30]. In the current study, serum ferritin levels were higher in non-BA than in BA group. Ferritin is present in the circulation either via secretion in the form of serum ferritin or
through release from damaged hepatocytes or Kupffer cells [31]. This may explain the higher levels in non-BA group in which the injury is mainly hepatocellular compared to BA group in which the injury is mainly biliary.

Hyperferritinemia could be associated with a flood of cytokines. The absence of correlation between serum ferritin and both IL-2 and IL-8 IHC in the current study cannot be commented upon due to the small number of cases with measured serum ferritin. However, several studies reported a contradictory results regarding the correlation of serum ferritin with IL-2 and IL-8. Kawasumi et al 2014 [32] reported a significant direct correlation between serum ferritin and IL-8 but not with IL-2 in lung disease. Pardananiet al 2013 [33] found no correlation between serum ferritin with IL-2R or IL-8 in primary myelofibrosis. Abou-Shousha et al 2006 [34] reported a significant direct correlation between serum ferritin and IL-8 in patients with diabetes mellitus. DePalma et al 2010 [35] found no correlation between serum ferritin and IL-2 in patients with atherosclerosis. Very recently, Ruddell et al [29] proposed a new role for extracellular ferritin as a pro-inflammatory signaling molecule in hepatic stellate cells that drive fibrogenesis. A future study targeting serum ferritin in neonatal cholestasis is worthy.

The main aim of the current study is to investigate the hepatic expression of IL-2 and IL-8 in patients with BA compared to other neonatal cholestatic disorders. We found that IL-2 and IL-8 were expressed by hepatic parenchyma and the expression was comparable in both BA and non-BA group. On the other hand, biliary epithelial cells did not express any of these cytokines in both groups. Isse et al [14], reported that human biliary epithelial cells express IL-8 in adult patients with chronic liver disease including chronic hepatitis and extrahepatic biliary obstruction, while it was never studied in neonatal cholestatic disorders. Whether the biliary epithelial cells can express IL-2 was not reported before.
Kasprzak et al [36] using immunocytochemistry and in situ hybridization they examined cell sources of TNF-α, IL-1α, and IL-2 in control and hepatitis C virus-infected children and adults. A high proportion of hepatocytes demonstrated expression of TNF-α, IL-1α, and IL-2. The augmented expression of TNF-α, IL-1α, and IL-2 in liver with a similar proportion of involved cells (mainly hepatocytes) in children and in adults points to participation of the cytokines in the pathogenesis of chronic hepatitis C. In contrast, Rowell et al [37] studied the cytokine expression by hepatocytes in response to bacterial infection. They found that IL-8, TNF-α and macrophage colony stimulating factor were expressed by hepatocytes while IL-2, was not expressed by hepatocytes.

Human intrahepatic biliary epithelial cells function in innate immunity by producing IL-6 and IL-8 in culture when stimulated by lipopolysaccharide [38], and the IL-8 expressed in bile ductular epithelia, possibly induced by bacterial components and proinflammatory cytokines released locally in chronic liver diseases [14]. In the liver, cytokine secretion is usually associated with non-parenchymal cells, particularly Kupffer cells. Dong et al [39] reported the expression of IL-6 and IL-8 in hepatocyte cultures stimulated by TNF-α and H₂O₂. Similarly, Thornton et al [40] found that Kupffer cell-derived cytokines induce the synthesis of IL-8, in human hepatoma and primary hepatocyte cultures. In an animal model of colorectal liver metastasis, Bandapalli et al [41] reported the upregulation of IL-8 in the tumor cells of the invasion front compared to the cells in the inner part of the tumor. The hepatocyte origin of IL-2 and IL-8 in neonatal cholestasis needs to be further studied and verified by in situ hybridization.

We found that IL-2 expression in portal cellular infiltrate was significantly higher in BA compared to non-BA group (25.85 ± 22.92 vs. 10.75 ± 14.25; P = 0.004) and correlated
significantly with the portal cellular infiltrate in both groups ($P = 0.004$ and $<0.0001$ respectively). In agreement with our results, Shinkai et al [42] reported that the expression of IL-2 mRNA was significantly increased within the liver in BA compared with other cholestatic disorders. Although the cellular source of IL-2 production could not be identified, they presumed that cytotoxic T lymphocytes were increased in the liver with BA in the portal tracts, which in turn results in bile duct destruction. IL-2 might act as a driving force in portal inflammation by cytotoxic T lymphocytes. Mack et al [15] identified increased numbers of lymphocytes (both CD4+ and CD8+) and a Th1-type cytokine profile (IL-2, IFN-γ, and TNF-α) in cases of BA in contrast to normal liver controls and other neonatal cholestatic liver diseases.

Narayanaswamy et al [43] performed a thorough analysis of plasma Th1 (IL-2, IFN-γ), Th2 (IL-4, IL-10), and macrophage (IL-18, TNF-α) cytokines and intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in BA patients. Plasma levels were assessed at the time of the Kasai procedure and serially thereafter for 6 months. All cytokines (except IL-10) and adhesion molecules increased during the 6-month period, suggesting that the inflammatory process is progressive and not ameliorated by portoenterostomy.

In the current study, we found that IL-8 expression was significantly higher in BA compared to non-BA group ($24.39 \pm 20.07$ vs. $9.25 \pm 11.3; P = 0.002$) and correlated significantly with the portal cellular infiltrate in both groups ($P = 0.004$ and $<0.0001$ respectively). IL-8 is a chemokine produced by neutrophils, macrophages [44] and other cell types such as epithelial cells [14], and endothelial cells [45]. Endothelial cells store IL-8 in their storage vesicles, the Weibel-Palade bodies [46]. Since IL-8 is also a chemo-attractant for T lymphocytes, production of IL-8 may lead to the recruitment not only of neutrophils but also of T lymphocytes [47].
In hand with our results, Bezerra et al [11] found that IL-8 was overexpressed in BA livers. Similarly, Nobili et al [48] showed that serum IL-8 was higher in BA compared to controls and was significantly correlated with the histological activity index. Honsawek et al [49] reported that serum IL-8 increased significantly in BA particularly in the late stage in patients with persistent than in those without jaundice.

Both Nobili et al [48] and Honsawek et al [49] found that the serum levels of IL-8 was significantly correlated with the degree of fibrosis in patients with BA, and they indicated that the increased production of IL-8 could be responsible for the pathogenesis of fibrosis. In the current study, although the correlation of IL-8 expression with fibrosis stage was not significant, yet, there was a trend of increased IL-8 expression with higher stages of fibrosis. This difference may be due to the higher age of BA patients in Honsawek's study (6.3 ± 0.6 years) and Nobili's (8.75 ± 3.1 months) compared to (57.39 ± 1.51 days) in our study. Furthermore, in Honsawek's study, all BA patients were post-Kasai operation. Another difference is the use of serum IL-8 in Nobili's and Honsawek's studies compared to the use of IL-8 hepatic expression in ours.

El-Faramawy et al [50] found that the level of serum IL-8 positively correlated with the number of lymphocytes but negatively correlated with the level of albumin in BA patients, while there was no significant difference of serum IL-8 in BA patients with mild and severe fibrosis. Contrary to our patients, their patients had older age (14.5 ± 4.3 months) and were all recruited post Kasai.

In conclusion, IL-2 and IL-8 expression was significantly higher in BA compared to non-BA group and correlated significantly with portal cellular infiltrate in liver tissue. There was a trend of increased IL-2 and IL-8 expression with increased stage of fibrosis in BA patients. This trend was not observed in the non-BA group. This may be due to the
heterogeneous etiology in the non-BA group. The significantly higher expression of IL-2 and IL-8 in patients with BA compared to non-BA suggests a potential role for these cytokines in the pathogenesis of this devastating neonatal hepatic disorder.
Acknowledgments

Competing interest

All the authors declare that they have no competing interest.

The study was funded by: National Liver Institute, Menofiya University, Egypt, without any particular role in the study design, recruitment of individuals, data analysis or the writing of the manuscript.
References


<table>
<thead>
<tr>
<th>Characteristics</th>
<th>BA (%)</th>
<th>Non-BA (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 31)</td>
<td></td>
<td>(n = 28)</td>
<td></td>
</tr>
<tr>
<td>Age at liver biopsy (days)</td>
<td>57.39 ± 17.51</td>
<td>81.18 ± 54.57</td>
<td>0.28</td>
</tr>
<tr>
<td>Male n (%)</td>
<td>17 (54.8)</td>
<td>16 (57.1)</td>
<td>0.859</td>
</tr>
<tr>
<td>Clay stool</td>
<td>29 (93.5)</td>
<td>8 (28.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>25 (80.6)</td>
<td>23 (82.1)</td>
<td>0.883</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>13 (41.9)</td>
<td>16 (57.1)</td>
<td>0.059</td>
</tr>
<tr>
<td>Ascites</td>
<td>3 (9.7)</td>
<td>3 (10.7)</td>
<td>1.0</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>11.97 ± 4.54</td>
<td>11.26 ± 5.69</td>
<td>0.499</td>
</tr>
<tr>
<td>Direct bilirubin (mg/dl)</td>
<td>8.61 ± 3.4</td>
<td>7.85 ± 3.91</td>
<td>0.59</td>
</tr>
<tr>
<td>Alanine transaminase (U/L)</td>
<td>131.68 ± 92.34</td>
<td>196.79 ± 246.01</td>
<td>0.82</td>
</tr>
<tr>
<td>Aspartate transaminase (U/L)</td>
<td>221.65 ± 143.46</td>
<td>279.36 ± 279.76</td>
<td>0.976</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.12 ± 0.47</td>
<td>3.1 ± 0.7</td>
<td>0.881</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>468.77 ± 233.15</td>
<td>436.0 ± 304.98</td>
<td>0.316</td>
</tr>
<tr>
<td>γ-glutamyl transpeptidase (U/L)</td>
<td>817.13 ± 526.49</td>
<td>259.96 ± 442.02</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Prothrombin time (Sec.)</td>
<td>12.08 ± 1.26</td>
<td>13.05 ± 1.79</td>
<td>0.018</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>9.96 ± 1.34</td>
<td>10.25 ± 1.92</td>
<td>0.493</td>
</tr>
<tr>
<td>White blood cells (x10³/µl)</td>
<td>12.12 ± 2.79</td>
<td>10.86 ± 3.76</td>
<td>0.148</td>
</tr>
<tr>
<td>Platelets (x10³/µl)</td>
<td>480.65 ± 201.57</td>
<td>361.93 ± 201.06</td>
<td>0.015</td>
</tr>
<tr>
<td>Serum ferritin * (ng/ml)</td>
<td>522.38 ± 191.71</td>
<td>1175.67 ± 494.38</td>
<td>0.014</td>
</tr>
</tbody>
</table>

BA: biliary atresia

*Serum ferritin was recorded only in 8 BA patients and in 3 non-BA patients
Table 2: Histopathological characteristics of the studied groups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>BA (%) (n = 31)</th>
<th>Non-BA (%) (n = 28)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ductular proliferation</td>
<td>30 (96.8)</td>
<td>6 (19)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Bile plugs</td>
<td>27 (87.1)</td>
<td>4 (14.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Multi-nucleated giant hepatocytes</td>
<td>6 (19.4)</td>
<td>13 (46.4)</td>
<td>0.026</td>
</tr>
<tr>
<td>Grade of liver fibrosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent or fibrous expansion of some portal tracts</td>
<td>6 (19.4)</td>
<td>18 (64.3)</td>
<td>0.003</td>
</tr>
<tr>
<td>Fibrous expansion of most portal tracts</td>
<td>4 (12.9)</td>
<td>5 (17.9)</td>
<td></td>
</tr>
<tr>
<td>Focal porto-portal bridging</td>
<td>11 (35.5)</td>
<td>2 (7.1)</td>
<td></td>
</tr>
<tr>
<td>Marked bridging</td>
<td>8 (25.8)</td>
<td>2 (7.1)</td>
<td></td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>2 (6.5)</td>
<td>1 (3.6)</td>
<td></td>
</tr>
<tr>
<td>Portal cellular infiltrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No/Minimal</td>
<td>0.0</td>
<td>10 (35.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>Mild</td>
<td>17 (54.8)</td>
<td>11 (39.3)</td>
<td></td>
</tr>
<tr>
<td>Moderate/Severe</td>
<td>14 (45.2)</td>
<td>7 (25)</td>
<td></td>
</tr>
</tbody>
</table>

BA: biliary atresia
Table 3: Correlation of IL-2 and IL-8 immunostaining in both BA and non-BA groups with age and laboratory data, and histopathological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BA</th>
<th>IL-2</th>
<th>P-value</th>
<th>IL-8</th>
<th>P-value</th>
<th>Non-BA</th>
<th>IL-2</th>
<th>P-value</th>
<th>IL-8</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>-0.076</td>
<td>0.685</td>
<td>-0.083</td>
<td>0.658</td>
<td>-0.031</td>
<td>0.877</td>
<td>-0.127</td>
<td>0.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>0.092</td>
<td>0.622</td>
<td>0.127</td>
<td>0.495</td>
<td>-0.018</td>
<td>0.926</td>
<td>0.14</td>
<td>0.479</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Direct bilirubin (mg/dl)</td>
<td>0.081</td>
<td>0.666</td>
<td>0.09</td>
<td>0.632</td>
<td>-0.055</td>
<td>0.781</td>
<td>0.058</td>
<td>0.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>-0.004</td>
<td>0.985</td>
<td>0.068</td>
<td>0.717</td>
<td>-0.006</td>
<td>0.977</td>
<td>-0.213</td>
<td>0.277</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>-0.038</td>
<td>0.839</td>
<td>-0.067</td>
<td>0.722</td>
<td>0.131</td>
<td>0.506</td>
<td>0.135</td>
<td>0.492</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td>0.015</td>
<td>0.935</td>
<td>-0.006</td>
<td>0.972</td>
<td>0.111</td>
<td>0.757</td>
<td>0.157</td>
<td>0.425</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>0.005</td>
<td>0.978</td>
<td>0.094</td>
<td>0.614</td>
<td>-0.054</td>
<td>0.783</td>
<td>-0.192</td>
<td>0.329</td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-glutamyl transpeptidase (U/L)</td>
<td>0.067</td>
<td>0.722</td>
<td>0.038</td>
<td>0.837</td>
<td>-0.115</td>
<td>0.562</td>
<td>-0.042</td>
<td>0.831</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prothrombin time (seconds)</td>
<td>-0.086</td>
<td>0.644</td>
<td>-0.073</td>
<td>0.695</td>
<td>-0.024</td>
<td>0.905</td>
<td>-0.018</td>
<td>0.928</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>-0.211</td>
<td>0.254</td>
<td>-0.288</td>
<td>0.116</td>
<td>0.123</td>
<td>0.533</td>
<td>0.074</td>
<td>0.708</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White blood cells (x10³/mm³)</td>
<td>-0.064</td>
<td>0.733</td>
<td>-0.158</td>
<td>0.395</td>
<td>0.117</td>
<td>0.554</td>
<td>0.247</td>
<td>0.206</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelets (x10³/mm³)</td>
<td>-0.191</td>
<td>0.303</td>
<td>-0.233</td>
<td>0.207</td>
<td>0.092</td>
<td>0.642</td>
<td>0.116</td>
<td>0.558</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade of liver fibrosis</td>
<td>0.149</td>
<td>0.424</td>
<td>0.079</td>
<td>0.672</td>
<td>-0.017</td>
<td>0.933</td>
<td>-0.075</td>
<td>0.705</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Necroinflammatory activity</td>
<td>0.5</td>
<td>0.004</td>
<td>0.499</td>
<td>0.004</td>
<td>0.755</td>
<td>&lt;0.0001</td>
<td>0.725</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum ferritin * (ng/ml)</td>
<td>-0.405</td>
<td>0.319</td>
<td>-0.335</td>
<td>0.417</td>
<td>0.50</td>
<td>0.667</td>
<td>0.0</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BA: biliary atresia; r: correlation coefficient

*Serum ferritin was recorded only in 8 BA patients and in 3 non-BA patients.