Journal of Pediatric Surgery (2009) **44**, 695–701

ELSEVIER

Journal of Pediatric Surgery

www.elsevier.com/locate/jpedsurg

The extent of biliary proliferation in liver biopsies from patients with biliary atresia at portoenterostomy is associated with the postoperative prognosis☆

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Received 4 May 2008; revised 12 September 2008; accepted 12 September 2008

Key words:
Biliary atresia;
Prognosis;
Histopathology;
CK7;
Biliary proliferation;
Age;
Portoenterostomy;
Liver fibrosis

Abstract

Background/Purpose: In biliary atresia (BA), a derangement in the biliary system remains, despite portoenterostomy performance. Many factors can influence the disease progression rate. This study aimed to analyze the association between biliary proliferation extent in biopsies from BA patients and postoperative prognosis.

Methods: Biliary proliferation was evaluated by a morphometric analysis of the cytokeratin 7 positivity percentage (PCK7) in wedge liver biopsies from 47 BA patients. The extent of fibrosis was evaluated by a fibrosis score (FS). The outcome 1-year native liver survival was correlated, using a multivariable regression analysis, with PCK7, FS, and age at portoenterostomy.

Results: The PCK7 ranged between 0.80% and 14.79% (M ± SD = 7.36% ± 4.15%). Patients who died or underwent transplantation had higher PCK7 than survivors with their native livers ($P < .001$). The area under the receiver operating characteristic curve for PCK7 in relation to the outcome was 0.845 ($P < .001$). The cutoff point of PCK7 for the maximal effect on postoperative prognosis was 10.18% (sensitivity = 0.71, specificity = 0.88). The PCK7 was the only studied variable associated with 1-year native liver survival, independently of age and FS ($P = .002$).

Conclusion: The extent of biliary proliferation at portoenterostomy, evaluated by PCK7, was associated with 1-year native liver survival of BA patients.

Biliary atresia (BA) is an infantile disorder characterized by the complete obstruction of a portion or the entirety of the extrahepatic biliary ducts. Since 1959, after the description of portoenterostomy by Dr Morio Kasai, the removal of the impediment to biliary flow has become feasible in all types of the disease, including the previously "uncorrectable type" [1]. However, regardless of timely performance of a portoenterostomy, all (or almost all) patients with BA experience progressive derangement of the intrahepatic biliary system, leading to increasing fibrogenesis and eventually to cirrhosis [2].

☆ Sponsor: Fundo de Incentivo à Pesquisa e Eventos, Hospital de Clínicas de Porto Alegre.

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0022-3468/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jpedsurg.2008.09.013

The causal events of this progressive cholangiopathy remain elusive, and BA is the main indication for liver transplantation in children [3]. The time interval between portoenterostomy and native liver failure is variable; it may occur within 2 years if surgery is ineffective or after many years with compensated biliary cirrhosis. Biliary atresia histopathology is characterized by dynamic age-dependent changes [4]. Biliary proliferation (BP) is evident at around the sixth week of life and is associated with increasing peribiliary fibrogenesis. At approximately the 16th week of life, because of continuous biliary epithelial degeneration, BP is followed by disappearance of interlobular bile ducts; and over time, BA becomes a disorder of paucity of bile ducts [5,6].

Several clinical, surgical, histopathologic, and laboratory variables at the time of portoenterostomy may affect native liver survival. Age at the time of the operation and the extent of fibrosis in the liver biopsy obtained during the procedure have been considered critical factors in determining patients' postoperative prognosis [7]. However, there is not yet a consensus in this regard. Considering a hypothetical association between the severity of BP at portoenterostomy and postoperative prognosis, we aimed to evaluate the association between its extent, studied by morphometric analysis, and native liver survival, considering factors of age at portoenterostomy and severity of liver fibrosis.

1. Methods

1.1. Patients

The study group consisted of 47 infants (22 male and 25 female) with BA who were admitted to our institution. The diagnosis of BA was based on laparotomy findings, operative cholangiography, and histology of portal bile duct remnants. In this study, BA types were categorized according to the Japanese classification system [8]. The major extrahepatic anomalies associated with BA were classified according to Carmi et al [9] in 3 groups: (1) BA-associated splenic malformation (BASM), including splenic abnormalities associated with *situs inversus*, digestive tract anomalies, and/or cardiac defects; (2) nonsyndromic type malformation; and (3) isolated intestinal malrotation. This study includes patients seen over the last 3 decades; most (n = 24, 51.1%) were admitted after 2000, 16 patients (34.0%) were admitted between 1990 and 2000, and 7 patients (14.9%) were admitted between 1980 and 1990. The median duration of follow-up was 966 days, ranging from 106 to 8184 (25-75 interquartile interval = 405-3525) days. Wedge liver biopsies obtained during portoenterostomy were subjected to morphometric analysis to determine the extent of BP. Biopsies were taken from the anterior margin of segment IV using standard methods.

Formalin-fixed paraffin-embedded liver specimens were examined. Five-micrometer–thick sections were obtained and subjected to immunohistochemistry to label cytokeratin 7 (CK7), a marker of biliary epithelium in outlining biliary structures. Sections were incubated with rabbit anti-CK7 primary antibody (Dako, Glostrup, Denmark; dilution, 1:100). Immunolabeling was amplified using the avidin-biotin-peroxidase complex, as described previously [10]. We used a multispecies reagent (EasyPath; Erviegas Ltd, São Paulo, Brazil) as the secondary antibody. Alternate 5-μm–thick sections were stained with picrosirius red to evaluate the fibrosis extent. All patients included in the study group were operated on by the same surgical team, and they were followed during hospitalization and in the outpatient clinic of our institution by the same clinicians. The clinical variables and outcome were evaluated prospectively.

1.2. Morphometric image analysis

From each slide, 10 images were captured from randomly selected high-power fields (magnification, 200×) containing CK7-positive structures and saved in TIFF format for later analysis. The halogen lamp voltage was kept constant through voltage stabilization. Cytokeratin 7 staining was examined quantitatively. Morphometric measurements were performed using the Adobe Photoshop CS3 Extended 10.0 (Adobe Systems Inc, San Jose, CA) computer program on every biliary structure in each captured image. Every image exhibited on the monitor was adjusted to the same threshold level, and the area of CK7-positive structures was measured in pixels using the "magic wand" tool. The total amount of pixels per image remained constant in all fields. The percentage of CK7-positivity (PCK7) in each image was then calculated using the ratio of the CK7-positive area to the total amount of pixels per image. For each patient, the average of PCK7 was calculated in the 10 images. Values obtained in each measurement were registered, and the mean of each case was calculated. The histologic assessor in the CK7 study was blinded to the clinical data.

1.3. Evaluation of the extent of fibrosis

The evaluation of fibrosis extent in the slides stained with picrosirius red was carried out by the first author and a pathologist (LM), both blinded concerning the other clinical and morphologic variables. Fibrosis extent in the biopsies was evaluated according to the fibrosis score (FS) for BA developed by Weerasooriya et al [11], in which fibrosis was defined as follows: *mild* (FS1), fibrosis ranging from portal fibrous expansion to bridging fibrosis involving less than 50% of portal tracts; *moderate* (FS2), bridging fibrosis with more than 50% of portal tracts involved without nodular architecture; and *severe* (FS3), bridging fibrosis with more than 50% of portal tracts involved and nodular architecture.

1.4. Outcome evaluation

In this study, the outcome of interest was 1-year native liver survival, which was correlated with PCK7, age at the time of portoenterostomy, and FS.

1.5. Statistical analysis

Findings were expressed as mean ± SD and compared using Student's *t* test or analysis of variance followed by the Tukey procedure. Correlations were evaluated using the Pearson test. Aiming at analyzing the relative influence of the studied factors over native liver survival, multivariable regression analysis was carried out. In addition, the area under the receiver operating characteristic (ROC) curve was calculated; and with the help of the Youden Index, the cutoff point of PCK7 for the maximal effect on prognosis was determined. A Kaplan-Meier test followed by the log-rank (Mantel-Cox) test was used, comparing native liver survival between the groups of patients with PCK7 less than and PCK7 greater than the established cutoff point. *P* less than .05 was accepted as significant. Microsoft Excel 2007 (Microsoft Corp, Redmond, WA) and SPSS 15.0 (SPSS Inc, Chicago, IL) were used for data processing and statistical analysis.

1.6. Ethics

The Research and Postgraduation Group Ethics Committee of the Hospital de Clínicas de Porto Alegre approved this study.

2. Results

At the time of portoenterostomy, the age of the study group ranged between 25 and 155 (74.8 ± 25.9) days of life. Patient age group distribution was as follows: 17 of 47 (36.2%) were younger than 60 days, 19 of 47 (40.4%) were between 60 and 90 days old, and 11 of 47 (23.4%) were older than 90 days. Forty-five patients had type III BA, 1 patient had type I, and 1 patient had type II BA. The PCK7 ranged between 0.80% and 14.79% (7.36% ± 4.15%). Table 1 shows the characterization of PCK7 according to FS and age group.

Most patients in the study group (64.83%) already presented cirrhosis (FS3) by the time of portoenterostomy. The FS1 patients differed significantly from patients with FS3 with respect to PCK7 (Table 1, *P* = .001). Regarding the association between PCK7 and age groups, patients younger than 60 days differed from the other age groups (Table 1, *P* = .003).

In this study, 14 of 47 (29.8%) patients died or underwent liver transplantation within 1 year after portoenterostomy. In 7 patients, deaths were caused by liver failure and decompensated cirrhosis; and 3 patients died while awaiting liver transplantation. One patient died because of a Kasai postoperative infection, and another one died from sepsis associated with ascending cholangitis. In another patient, the cause of an early death was heart failure secondary to congenital cardiac malformation. This last patient was included in the study group because, just before death, he still presented cholestasis (total serum bilirubin = 13.5 mg/dL, direct reacting bilirubin = 7.0 mg/dL, gamma-glutamil transpeptidase (GGT) = 1880 U/L). Four patients underwent liver transplantation in the first year of life; 2 have died and the other 2 are alive 678 and 970 days after transplant, respectively. The PCK7 from the patients who died or underwent transplantation in the first year of life was significantly higher than that in the other 33 patients (11.04% ± 3.71% vs 5.80% ± 3.28%, *P* < .001). The area under the ROC curve for PCK7 in relation to native liver survival in the first postoperative year was 0.845 (*P* < .001). Fig. 1 shows the area under the ROC curve for PCK7 in relation to 1-year native liver survival.

Table 1 Characterization of PCK7 according to FS and age group

Variable	PCK7 (%)	*P*
FS		
FS1 (n = 8)	2.99 (1.60)[a]	.001
FS2 (n = 9)	6.92 (3.74)	
FS3 (n = 30)	8.66 (3.98)[b]	
Age group (d)		
<60 (n = 17)	4.77 (2.95)[a]	.003
60-90 (n = 19)	9.15 (3.88)[b]	
>90 (n = 11)	8.28 (4.47)[b]	

Statistics: analysis of variance.
[a,b]Means are different at *P* less than .05 (Tukey post hoc test).

The PCK7 cutoff point of the maximal effect on 1-year native liver survival was 10.18%, presenting 0.71 sensitivity and 0.88 specificity. Comparison of native liver survival between groups with PCK7 less than and PCK7 greater than 10.18% is displayed in Fig. 2. In the group with low PCK7 (<10.18%), 87.9% ± 5.7% survived with their native livers until the end of the first postoperative year, in comparison with only 28.6% ± 12.1% of the group with PCK7 greater than 10.18% (*P* < .001).

In this study, CK7 positivity was observed in proliferating periportal and/or periseptal ductules, as well as in scattered cells distributed in the liver parenchyma, with variable degrees of severity among different cases. Fig. 3 demonstrates CK7-positive structures from the livers of BA patients.

In the multivariable regression analysis, PCK7 was the only variable in study to be independently associated with 1-year native liver survival (*P* = .002). An odds ratio (OR) of 1.5 was observed in the prediction of death or transplantation in the first postoperative year for each increase of 1 percentage point in PCK7. Bivariate association between 1-year native liver survival and age at portoenterostomy, when analyzed as a continuous variable (*P* = .08), disappeared after adjusting for the other variables

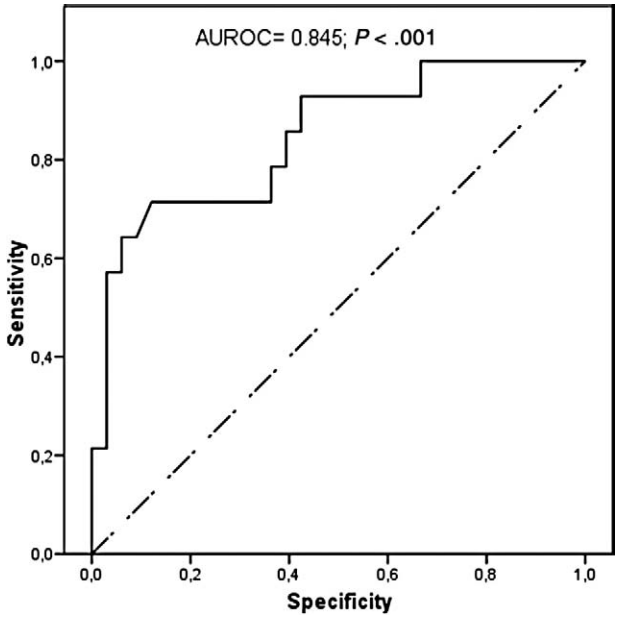

Fig. 1 Area under the ROC curve for PCK7 in relation to 1-year native liver survival.

(P = .746). Fibrosis score did not show association with 1-year native liver survival, not even in bivariate analysis. Table 2 presents the association of the studied variables with postoperative 1-year native liver survival, analyzing age as a continuous variable.

Considering the age groups and using the older than 60 days cohort as a cutoff point, PCK7 remained the only studied variable to be independently associated with

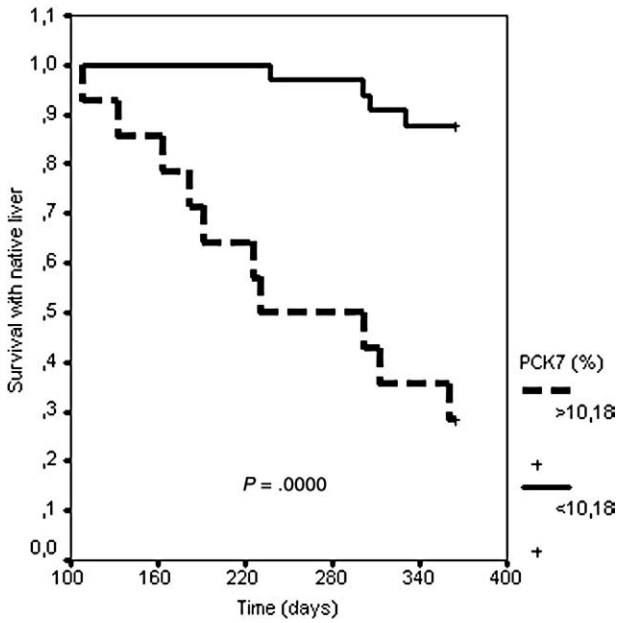

Fig. 2 Comparison of 1-year native liver survival according to the groups of patients with high and low PCK7. The cutoff point used for this comparison (PCK7 = 10.18%) was obtained by the area under the ROC curve for PCK7 in relation to 1-year native liver survival.

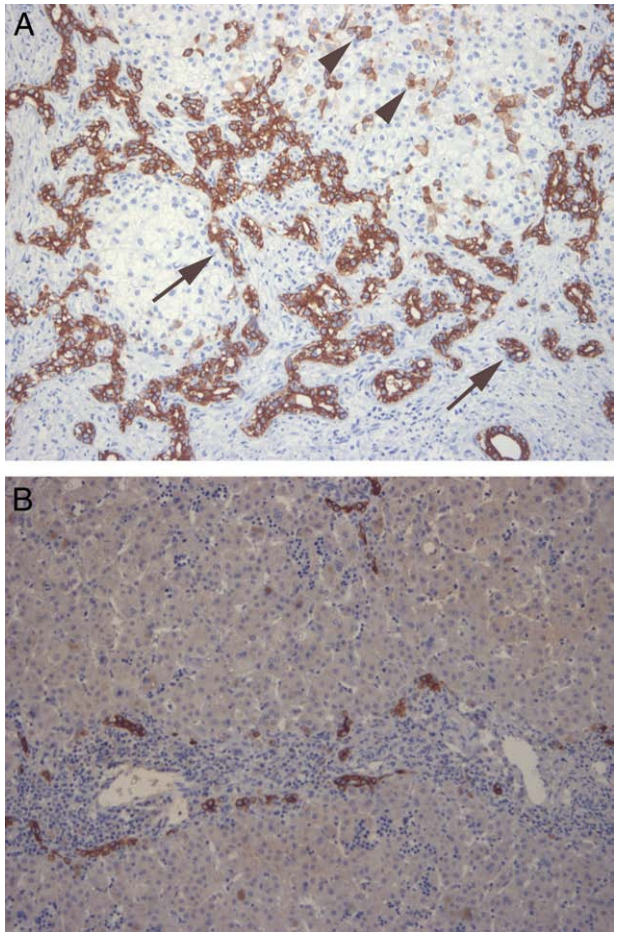

Fig. 3 The CK7-positive structures in livers from BA patients. A, Liver with high PCK7 (PCK7 = 15.5%). Age at portoenterostomy = 109 days. Observe the ductular reaction involving periportal/periseptal ductules (arrows) and cells distributed in liver parenchyma (arrowheads). B, Liver with low PCK7 (PCK7 = 1.23%). Age at portoenterostomy = 68 days. Immunohistochemistry, CK7, 200×.

1-year native liver survival (P = .004). The older than 60 days cohort presented an association with 1-year native liver survival in the bivariate analysis (P = .020); however, it did not show association with that outcome after adjusting for the other variables (P = .238). Fibrosis

Table 2 Association of the variables in study with the post-Kasai 1-year native liver survival

Variables	Crude OR (CI 95%)	P	Adjusted OR (CI 95%)	P
Age at PE (d)	1.02 (1.00-1.05)	.08	1.00 (0.97-1.04)	.746
PCK7	1.50 (1.20-1.80)	.001	1.50 (1.20-1.97)	.002
FS				
FS1	0.21 (0.02-1.97)	.174	3.00 (0.15-60.00)	.472
FS2	0.19 (0.02-1.70)	.136	0.22 (0.02-3.30)	.278
FS3 [a]	1	.160	1	.398

Statistics: multivariable regression. PE indicates portoenterostomy; CI, confidence interval.

[a] Cirrhosis.

Table 3 Association of the variables in study with the post-PE 1-year native liver survival using the age group older than 60 days as a cutoff point

Variable	Crude OR (95% CI)	*P*	Adjusted OR (95% CI)	*P*
Age at PE (>60 d)	12.2 (1.40-104.60)	.020	4.50 (0.40-54.20)	.238
PCK7	1.50 (1.20-1.80)	.001	1.50 (1.10-1.90)	.004
FS				
FS1	0.21 (0.02-1.97)	.174	3.50 (0.20-66.40)	.410
FS2	0.19 (0.02-1.70)	.136	0.25 (0.02-3.91)	.320
FS3 [a]	1	.160	1	.420

Statistics: multivariable regression.
[a] Cirrhosis.

score was not associated with outcome, not even in the bivariate analysis. Table 3 shows the association of the studied variables with postoperative 1-year native liver survival, considering age group cutoff point.

In respect to the results of laboratory tests performed before portoenterostomy, GGT values ranged between 11 and 2262 (700.71 ± 530.61) U/L and total serum bilirubin ranged between 5.30 and 24.00 (11.32 ± 4.31) mg/dL. The PCK7 presented weak positive correlations with GGT (r = +0.44, P = .009) and total serum bilirubin (r = +0.37, P = .013).

The inclusion of 2 other clinical factors in this study, decade of patient admission and BASM presence, was precluded by the small number of patients. Liver transplantation became available in our unit in 1995, and 16 patients (31.1%) underwent portoenterostomy before that period. This group included 11 long-term survivors with their native livers and 1 patient who died in the first year of life. Four other patients would perhaps have been included in the transplant list at the present time. Taking this presumed bias into account and reclassifying these 4 patients in the group with bad prognosis, PCK7 was nevertheless the only variable in study independently associated with 1-year native liver survival (adjusted OR = 1.29 [1.0-1.6], P = .030). Major extrahepatic anomalies associated with BA were observed in 13 patients (27.6%), including BASM in 4 cases and nonsyndromic phenotype in 9 cases. The BASM group seemed to present lower PCK7 (4.37% ± 2.07%) in comparison with the nonsyndromic group (7.55% ± 3.36%) and the group with isolated BA (7.67% ± 4.44%). However, there was no significant difference when comparing those 3 groups (P = .326).

3. Discussion

The frequency of successful Kasai operations, preventing the liver transplant within 5 years, reaches 42% in some series [3] and decreases in the long term [2]. A successful portoenterostomy has been associated with several variables at the moment of operation, including clinical factors, such as age [7,12], decade of patient admission [12], and presence of BASM [13,14], or histopathologic factors, such as extent of fibrosis [15] and presence of ductal plate malformation [16,17]. We have obtained negative results previously when analyzing the association between ductal plate malformation and Kasai prognosis [7]. In this study, instead of taking into consideration bile duct morphologic patterns, we investigated the relation between postoperative prognosis and the absolute amount of BP. We report the influence of BP extent observed in wedge biopsies obtained during portoenterostomy, expressed by PCK7, on 1-year native liver postoperative survival. *Ductular reaction* means biliary epithelial cell proliferation in liver diseases [18] that may arise from ductular proliferation of preexisting cholangiocytes, progenitor cells, or hepatocytic biliary metaplasia [19-21]. In this study, we observed CK7 positivity in proliferating periportal and/or periseptal ductules, as well as in scattered cells in the liver parenchyma, with variable degrees of severity among different cases (Fig. 2). The PCK7 was associated with FS and age group. Infants with the lowest FS differed from cirrhotic patients in terms of BP severity (Table 1). This association was expected, as BP induces fibrogenesis [22]. Conversely, patients younger than 60 days at portoenterostomy had less BP than the other age groups (Table 1). However, when comparing with FS and age in a multivariable analysis, PCK7 was the only independent variable associated with 1-year native liver survival (Tables 2 and 3). Age at the time of surgery is thought to have a detrimental effect on the post-Kasai prognosis [2,23-29]. Altman et al [12] found equal outcomes in the younger than 49 days and the 50- to 70-day age groups, which represent an improved prognosis compared with that of the older than 70 days age group. In our previous study, the younger than 60 days age group was associated with better prognosis compared with the older than 90 days cohort [7]. However, other studies have failed to find this association [15,30,31]. In a study analyzing the follow-up of more than 1000 patients, Nio et al [32] observed no influence of age up to 90 days on the clearance of jaundice. Moreover, a considerable percentage of patients who undergo late portoenterostomy (after 3 months of life) can survive with their native livers for 5 and 10 years [33,34]. Although the time-dependent effect of uncorrected cholestasis on liver function is intuitive, disease severity in BA appears not to be based solely on age [35]; and the association of age and Kasai postoperative prognosis is nonlinear [30]. Other variables at the time of portoenterostomy seem to affect the rate of disease progression. The extent of fibrosis is probably one such influencing factor. However, its assessment as a method for BA prognostic determination has become controversial [11,15,35,36]. The presence of cirrhosis in the liver periphery does not imply surgical failure because regenerative hilar nodules containing patent bile ducts can sustain an adequate bile flow [37]. In addition, there are inherent difficulties in histopathologic assessment of fibrosis

extent, involving the liver sample itself, such as biopsy size and staining methods [38]. The use of morphometric evaluation of collagen surface density has been presented as a promising quantitative method [39].

Architectural changes, such as nodularity and bridging, and the collagen deposition pattern, as measured by a specific score, cannot be evaluated through image analysis. Such measures may be more reflective of liver disease severity than the absolute amount of collagen per se [40]. Previously, we were not able to find an association between Kasai postoperative prognosis and the collagen surface density [7]; and in this study, fibrosis extent evaluated by a BA specific score [11] remained nonassociated with 1-year native liver survival. Wildhaber et al [15] described the influence of a "bridging fibrosis" factor on postoperative outcome, observing that variable in only 49% of their patients. The lowest fibrosis score (FS1) from the classification used in this study [11], however, included "bridging fibrosis involving <50% of portal tracts." In our study group, 39 of 47 (82.98%) presented bridging fibrosis involving more than 50% of the portal spaces, with or without cirrhosis; and 30 of 47 (63.83%) presented "bridging fibrosis with nodular architecture" (FS3), that is, cirrhosis. Patients with BA displaying just portal expansion in liver biopsies at portoenterostomy constituted, in our experience, a rare finding. We cannot specify whether the difference of fibrosis extent between their study and ours was related to the age of our patients, an average 10 days older than theirs, or to any phenotypic disparities between the samples. Nevertheless, the absence of association between postoperative results and FS in this study was probably influenced by the great number of patients already presenting cirrhosis at the time of portoenterostomy.

Ito et al [41] observed in 1983 that the pathologic alteration in intrahepatic bile ducts was one of the main factors determining BA prognosis. Subsequently, Kinugasa et al [42], evaluating 25 patients who underwent portoenterostomy, described an association between CK7-positive cell density and postoperative prognosis. However, Kinugasa et al used an outcome measure of total serum bilirubin level (threshold <1.0 mg/dL) in the first postoperative year, a rather restrictive variable, for prognostic determination. In this study, we included a greater number of patients and used native liver survival as the outcome in a multivariable analysis that compared the strength and independence of factors of age, FS, and PCK7. Specifically, with regard to assessment of the CK7-positive area, we did not restrict its measurement to ductular structures in the portal limiting plates as had been done by Kinugasa et al. Rather, we included CK7-positive hepatocytes distributed in liver parenchyma because they may be important ductular reaction constituents [43]. With this method for assessing BP extent, we were able to characterize a cutoff point (PCK7 = 0.18%) highly predictive of 1-year native liver survival (Fig. 1).

In conclusion, our data indicate that the extent of BP in the liver of BA patients at the time of portoenterostomy, evaluated by PCK7 morphometric analysis, is associated with postoperative prognosis and may be considered as a prognostic factor for this group of patients. Specifically, PCK7 values greater than 10.18% may predict a bad prognosis in the first postoperative year. The PCK7 measures may reflect the severity of biliary derangement at the time of portoenterostomy.

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