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Serum and tissue tumor growth factor β1 in children with biliary atresia

Fernanda dos Santos de Oliveira [b], Carlos Oscar Kieling [a], Jorge Luiz dos Santos [a], Patrícia Ponce de Leon Lima [b], Sandra Vieira [a], Luise Meurer [a,b], Themis Reverbel da Silveira [a,b], Ursula Matte [a,b,*]

[a] *Hospital de Clínicas de Porto Alegre, CEP-90035-903, Brazil*
[b] *Universidade Federal do Rio Grande do Sul, CEP-90035-903, Brazil*

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Abstract

Background: Biliary atresia (BA) is an infantile disorder characterized by the obstruction of a portion or the entirety of the extrahepatic bile ducts, leading to hepatic fibrosis and loss of liver function. The gold standard for diagnosing and grading fibrosis is liver biopsy, but there are many groups searching for noninvasive biomarkers that could replace and/or complement this procedure.

Methods and Materials: In this study, we evaluated serum and tissue transforming growth factor β1 (TGFβ1) and aspartate aminotransferase [AST]-to-platelet ratio index (APRI) in patients with BA at the time of diagnosis and at liver transplantation and correlated these data with tissue collagen density, to verify if they could act as biomarkers for BA.

Results: At the time of diagnosis, TGFβ1 levels were highly variable in BA patients. However, serum values at transplantation were significantly decreased (13.75 ± 3.68 ng/mL) as compared to controls (34.36 ± 9.35 ng/mL) ($P = .01$). No correlation was found between serum TGF1β1 and collagen density in both groups analyzed. Serum TGFβ1 showed no correlation with APRI at diagnosis. At the time of liver transplantation, all patients had low serum TGFβ1 and variable APRI, although all higher than 2.0. However, when platelet count was used, an inverse correlation with serum TGFβ1 was observed at the time of diagnostics ($r^2 = 0.749$; $P = .03$).

Conclusions: Our findings suggest that at the time of diagnosis the fibrogenic process is active, with higher levels of TGFβ1, whereas later on, there is scar tissue, with reduced TGFβ1 expression. Although our results should be confirmed in larger sets of patients with BA, the lack of TGFβ1 at the time of liver transplantation may have important consequences for the patient because it is a pleiotropic molecule, responsible for many functions in the body, mainly those related to immune response and cell growth.

Biliary atresia (BA) is an infantile disorder characterized by the complete obstruction of a portion or the entirety of the extrahepatic biliary ducts. The lapse of time between Kasai procedure and native liver failure is variable; it may occur within 2 years if surgery is ineffective or not performed or

* Corresponding author. Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos, 2350, CEP: 90035-903, Porto Alegre, Rio Grande do Sul, Brazil.
E-mail address: umatte@hcpa.ufrgs.br (U. Matte).

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after many years of compensated biliary cirrhosis [1]. The development of hepatic fibrosis in BA is more aggressive than in any other adult disorder, and many factors can influence the disease progression rate [2].

Transforming growth factor β1 (TGFβ1) is one of the main profibrotic cytokine involved in hepatic fibrosis, inducing differentiation of hepatic stellar cells (HSCs) into myofibroblasts. The activation of HSC leads to increased production of collagen, proteoglycans, structural glycoproteins, and hyaluronic acid [3].

The gold standard for diagnosing and grading fibrosis is liver biopsy, but there are many groups searching for noninvasive biomarkers that are less invasive and could replace and/or complement liver biopsy. Gressner et al [4] divided the serum fibrosis markers into 2 categories. Class I markers comprise mainly secretion products of activated HSC and portal myofibroblasts and reflect the disrupted extracellular matrix turnover (eg, TGFβ1, hyaluronic acid, and others). Class II markers comprise a variety of biochemical scores that are selected by various statistical models and mathematical algorithms, such as Fibrotest and aspartate aminotransferase [AST]-to-platelet ratio index (APRI) [4]. The latter is a test based on the ratio between aspartate aminotransferase (AST) and platelet count that showed high accuracy in predicting both significant fibrosis and cirrhosis in adult patients with chronic hepatitis C [5]. However, there are few reports regarding the noninvasive biomarkers of fibrosis in children with chronic liver disease [6].

In this study, we tested a class I (TGFβ1) and a class II biomarker (APRI) in 2 groups of patients at 2 stages of BA: at the time of diagnosis and at liver transplantation, comparing with tissue collagen density.

1. Material and methods

1.1. Patients

Liver samples were obtained from 17 patients with BA. The BA diagnosis was based on standard clinical laboratory, radiologic, and histologic findings. Only patients with isolated BA (and no other malformations) were included in the study. In 10 cases (mean age, 72.2 ± 19.12 days), the specimens were obtained by surgical biopsy before Kasai procedure. Specimens from the other 7 cases were obtained from the liver explants (mean age, 621 ± 492.25 days) (Table 1). At the time of liver transplantation, all patients had portal hypertension and cirrhosis was diagnosed based on clinical, biochemical, and ultrasound findings according to international criteria [7]. In 2 patients (13 and 15), liver transplantation was indicated because of bleeding of esophageal varices that were not responsive to endoscopic treatment. In 2 cases (1 and 2), samples could be obtained from the same patient both at diagnosis and at liver transplantation. Blood samples were collected between 1 week and 1 month before procedure (for liver biopsy and transplantation, respectively) for routine biochemical investigation. Samples were kept at −80°C until TGFβ1 quantification.

Blood samples from 9 children without liver disease or any inflammatory disorders (mean age, 418.67 ± 298.72

Table 1 Clinical, laboratory, and demographic characteristics of BA patients at the time of diagnosis and liver transplantation

Patient	Age (d)	Sex	INR	PELD	Child-Pugh	TB (mg/dL)	DB (mg/dL)	Albumin (g/dL)	AST (IU)	Platelets (1000/μL)	APRI	TGFβ1 (ng/mL)	Collagen density (%)
At the time of diagnosis													
1	Male	107	1.01	8	B7	12.5	9.0	3.7	247	410	1.5	87.27	38.72
2	Female	52	1.08	9	B7	11.6	8.6	3.5	165	485	0.9	23.62	38.49
3	Female	82	1.03	8	B7	10.9	6.1	3.6	191	256	1.9	63.72	37.39
4	Female	66	0.90	4	B7	12.9	9.5	4.5	274	430	1.6	54.30	24.11
5	Female	66	0.96	5	B7	11.5	4.8	4.6	307	536	1.4	13.04	14.33
6	Female	56	1.08	9	B7	16.6	11.8	4.3	216	385	1.4	90.00	19.76
7	Female	60	1.08	6	B7	7.7	4.4	4.3	92	530	0.4	10.51	25.3
8	Female	88	1.01	6	B7	10.3	6.8	4.1	285	650	1.1	14.41	32.26
9	Female	93	1.58	14	B8	10.0	6.7	4.3	158	350	1.1	38.95	12.19
10	Male	52	0.99	4	B7	7.3	5.6	3.9	100	385	0.6	55.47	8.57
At liver transplantation													
1	Male	275	2.06	26	C12	32.6	22.5	3.7	416	71	14.6	15.53	50.59
2	Female	268	1.54	19	B9	14.9	9.9	2.4	93	134	1.7	7.9	47.84
11	Female	404	2.16	31	C12	49.4	30.7	3.3	766	134	14.3	16.9	24.11
12	Male	635	1.14	15	B8	24.0	16.9	3.0	314	112	7.0	9.34	48.37
13	Male	815	1.02	3	B8	2.1	1.3	3.1	80	105	1.9	17.23	19.59
14	Male	317	2.24	28	C11	24.5	19.2	2.6	385	108	8.9	14.37	32.93
15	Female	1637	1.20	2	B7	4.7	2.8	3.8	91	50	4.6	14.9	14.83

INR indicates international normalized ratio; PELD, pediatric end-stage liver disease.

days) attending the hospital for routine evaluations were used as control for TGFβ1 levels. Clinical details of these patients can be found on Table 2.

1.2. Clinical and laboratory data

Laboratory data (platelets, international normalized ratio, AST, total bilirubin, direct bilirubin, and albumin) were obtained reviewing patient's charts from routine tests performed on the same serum used for TGFβ1 quantification. Clinical and laboratory information were used to calculate scores of liver disease severity: pediatric end-stage liver disease [8] and Child-Pugh [9].

1.3. TGFβ1 quantification

All sera were centrifuged at 3000 g within 30 minutes of collection and frozen at −80°C. A sandwich enzyme-linked immunosorbent assay (ELISA) for TGFβ1 (Duo Set ELISA; R&D Systems, Minneapolis, MN, USA) was performed according to the manufacturer's instructions. Previously, the ELISA plate wells were coated with a capture antibody. The samples were activated with 2.5N acetic acid/10 mol/L urea and neutralized with 2.7N NaOH/1 mol/L HEPES (N-2-Hydroxyethylpiperazine-N′-2′-ethanesulfonic Acid), then samples were diluted 10-fold and added to the plate and incubated for 2 hours. After a second incubation with the biotinylated detection antibody, the reaction was revealed with streptavidin conjugated to horseradish-peroxidase, and color was read in a microplate reader at 450 nm (Zenyth 200rt; Anthos Labtec, Wals, Austria). Results were calculated on a standard curve concentration and multiplied for the dilution factor.

Table 2 Clinical, laboratory, and demographic characteristics of control individuals

	Age (d)	Sex	Reasons for hospital referral	TGFβ1 (ng/mL)
C1	179	Female	Congenital toxoplasmosis investigation [a]	43.02
C2	510	Female	Anemia control [b]	37.64
C3	704	Female	Vertical exposure to human immunodeficiency virus [c]	33.61
C4	19	Female	Sibling with genetic disorder [d]	23.43
C5	238	Male	Anemia control [b]	25.88
C6	887	Female	Anemia control [b]	37.63
C7	201	Male	Anemia control [b]	30.93
C8	304	Male	Anemia control [b]	25.10
C9	726	Female	Vertical exposure to human immunodeficiency virus [c]	51.94

[a] Patients without active inflammatory disorder.
[b] Patients with red blood cells count within normal values.
[c] Patients with 2 negative viral loads.
[d] Patient tested negative for sibling's disease.

1.4. TGFβ1 immunohistochemistry

Formalin-fixed paraffin-embedded specimens were used. Wedge liver biopsy specimens obtained during diagnosis procedure or explant specimens were stained with rabbit anti-TGFβ1 primary antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) (1:50), and detection was performed using Picture Max Polymer Detection Kit (Zymed, Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. Liver biopsy specimens were taken from the anterior margin of segment IV using standard methods. Cecal appendix fragments were used as positive controls. Negative controls were performed in slides without the primary antibody. Slides were observed in low magnification (10×) in search for stained areas (brown areas). These were then observed in higher power fields to identify positive cells as hepatocytes, inflammatory cells, or other structures. The proportion of positive cells was calculated, as well as their location regarding the hepatic acinus.

1.5. Surface collagen density

Liver slides were stained with picrosirius red, and 5 images were captured from randomly chosen 200× fields. Evaluation was performed by the same individual, who had access to Hematoxylin-Eosin and picrosirius-stained slides. *Collagen density* was defined as the mean values of collagen percentage in 5 liver images, quantified by the morphometric method described by Masseroli et al [10]. Briefly, the images were captured in a physical device in TIFF format. The morphometric analysis, conducted on a computerized imaging system, allowed for the automatic quantification of the fibrotic area present in the surface fields. The analysis of the image was made with Image-Pro Plus version 4.1 software (Media Cybernetics, Bethesda, MD, USA).

1.6. APRI

Values for AST and platelet count were obtained from the patient's charts, as these tests are performed along with the routine biochemical tests. The AST-platelet ratio index was performed according to Wai et al [5], with the following formula:

$$\text{APRI} = \frac{\text{AST / upper limit of normal (ULN)}}{\text{platelets } (\times 10^9/\text{L})} \times 100.$$

The upper normal limit considered for AST was 40 IU, based on the laboratory reference values.

1.7. Statistics

Findings were expressed as mean ± SD and compared using Student's *t* test or analysis of variance followed by the Tukey post hoc test. Correlations were evaluated using Pearson's test. Microsoft Excel 2007 (Microsoft Corp,

Redmond, Wash) and SPSS 15.0 (SPSS Inc, Chicago, Ill) were used for data processing and statistical analysis.

2. Results

2.1. Serum TGFβ1

First, we evaluated TGFβ1 levels in the serum of patients with BA at the time of diagnosis and at the time of liver transplantation, comparing with those from normal children. At the time of diagnosis, TGFβ1 levels were highly variable, ranging between 90 ng/mL and 10.57 ng/mL (45.13 ± 28.28 ng/mL), as shown in Fig. 1. At the time of transplantation, BA patients had serum TGFβ1 levels significantly decreased (13.75 ± 3.68 ng/mL) as compared to controls (34.36 ng/mL ± 9.35 ng/mL) (P = .01).

2.2. APRI

Because APRI was calculated as a class II marker useful as an insight on the patient's clinical conditions, it was only calculated for BA patients. At the time of diagnosis, it ranged between 0.43 and 1.87 (1.20 ± 0.45), and at the time of transplantation, it was increased to values between 1.74 and 14.65 (7.58 ± 5.36) (P = .05).

2.3. TGFβ1 in the tissue

The expression of TGFβ1 was assessed in liver tissue by immunohistochemistry. At the time of diagnosis, most patients stained positively for TGFβ1, whereas liver explants were all negative, except for one patient. Moreover, marked TGFβ1 expression was observed in hepatocytes at the time of diagnosis, as shown on Fig. 2.

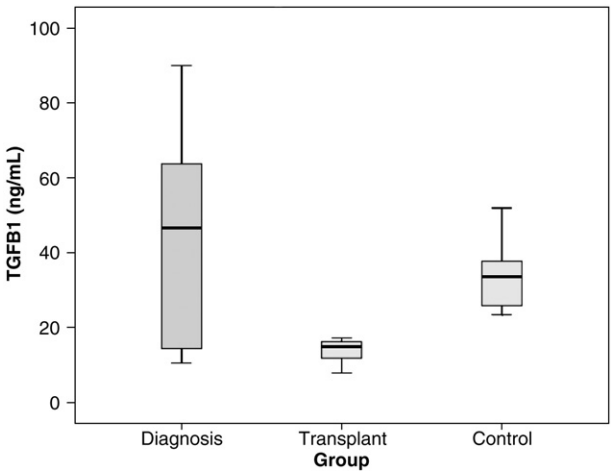

Fig. 1 Comparison of serum TGFβ1 levels between BA patients at the time of diagnosis (n = 10), at liver transplantation (n = 7), and normal children (n = 9).

2.4. Collagen density

Collagen density was used as a quantitative measure of liver fibrosis, and at the time of diagnosis, it was 25.11% ± 10.74%, whereas at the time of transplantation, it was 34.04% ± 15.00% (Fig. 3). Despite the expectation that patients undergoing liver transplantation would have higher values than patients at the time of diagnosis, no statistically significant difference was found between the 2 groups.

2.5. Serum as a surrogate marker of liver disease

Serum TGFβ1 showed no correlation with APRI, although at the time of diagnosis, all patients had APRI lower than 2.0, whereas at the time of liver transplantation, it was higher than 2.0 (Fig. 4A).

However, if platelet count is used, an inverse correlation with serum TGFβ1 is observed at the time of diagnosis (r^2 = 0.48; P = .025). At the time of liver transplantation,

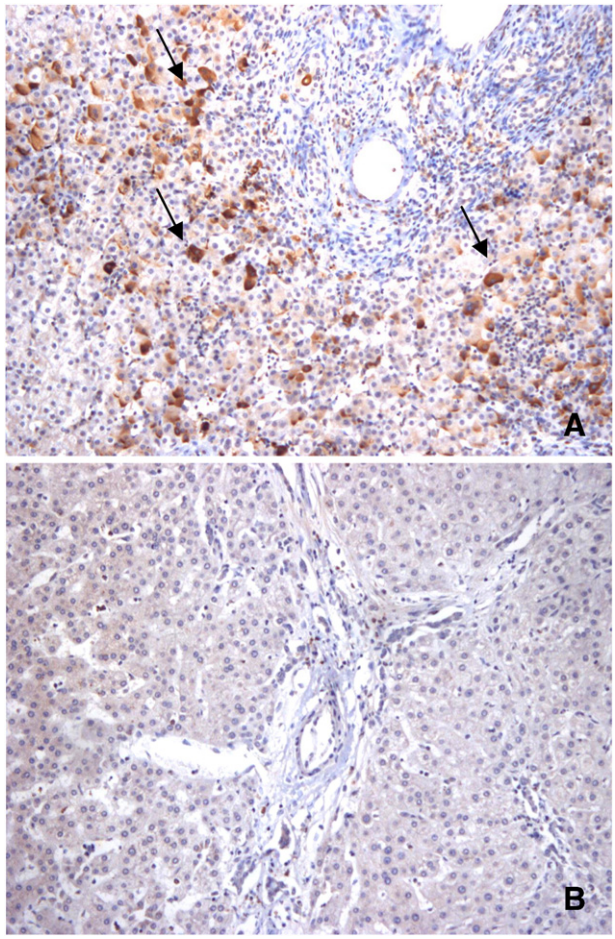

Fig. 2 Photomicrograph of immunohistochemistry for TGFβ1 in liver tissue from BA patients. (A) Liver biopsy of a patient at the time of diagnosis showing positive hepatocytes (arrows). (B) Liver explant, showing marked reduction of TGFβ1 in liver tissue. (A) Patient 6 and (B) patient 15. (Original magnification ×200.)

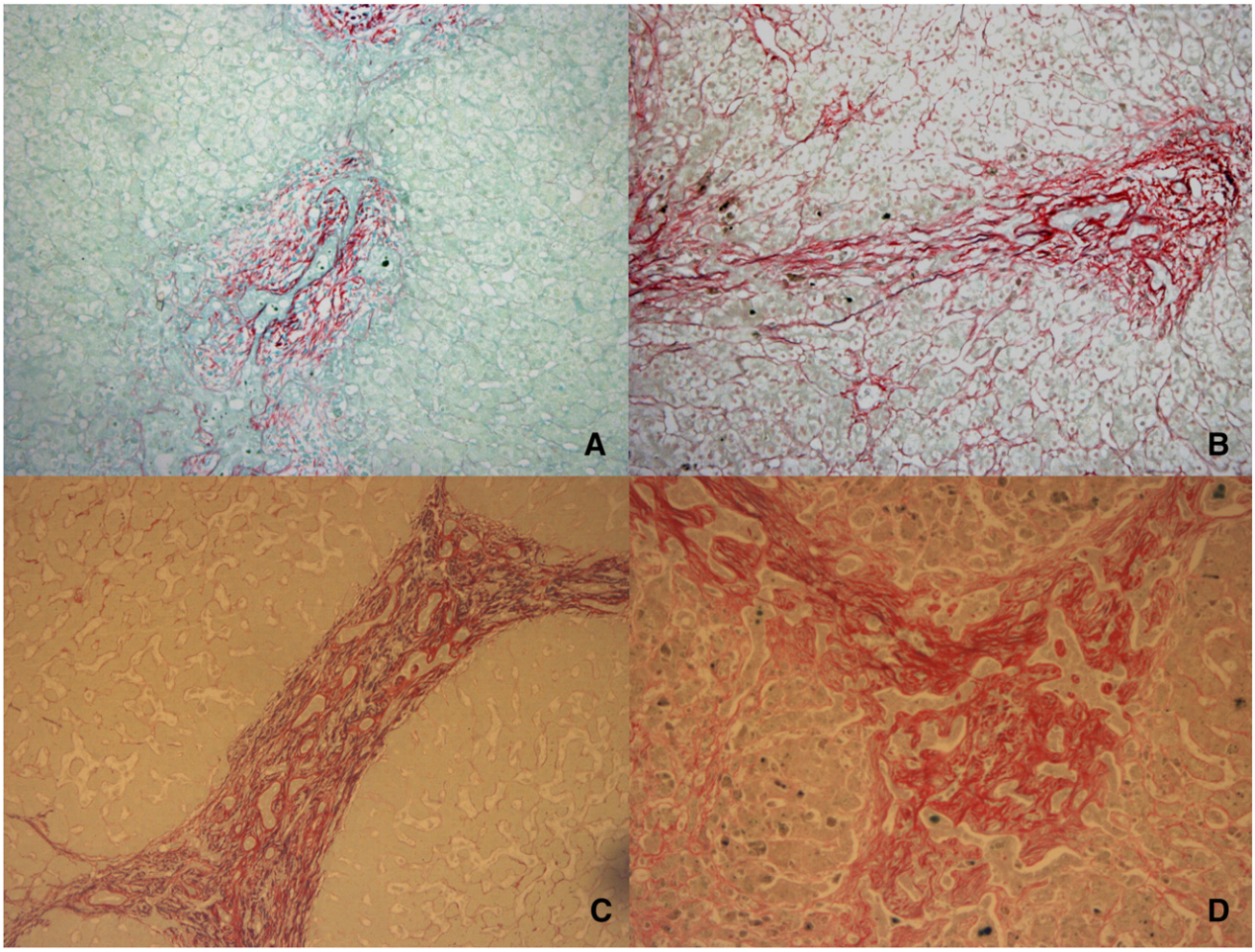

Fig. 3 Photomicrograph of picrosirius-stained slides of liver tissue from BA patients. Representative images of liver biopsy specimens at the time of diagnosis (A and B) and at liver transplantation (C and D) showing high (A and C) and low (B and D) collagen density. (A) Patient 10, (B) patient 1, (C) patient 13, and (D) patient 1. (Original magnification ×200.)

all patients had low TGFβ1 and platelet count below 150,000/uL (Fig. 4C).

No correlation was found between serum TGFβ1 and collagen density, even considering all patients as a single group ($r^2 = 0.03$; $P = .48$) (Fig. 4B).

3. Discussion

Tumor growth factor β1 is widely regarded as profibrogenic cytokine in liver injury [11]. It exists in mammals as 3 isoforms (TGFβ1, β2, and β3), which have very similar properties [12]. However, the isoform TGFβ1 has been described as the most important in the fibrogenic process by its ability to transform quiescent cells into activated collagen-producing myofibroblasts. Because of its important role in fibrogenesis, TGFβ1 has been considered a potentially useful biomarker of fibrosis and cirrhosis, but results using this cytokine are controversial [13-15].

In this study, we evaluated serum and tissue TGFβ1 in patients with BA at the time of diagnosis and at liver transplantation. As serum TGFβ1 values are inversely correlated with age in healthy children, it is important to point out that there was no significant difference of age in patients at the time of liver transplantation and controls in our study. However, patients at the time of diagnosis were significantly younger than the other groups. Indeed, in theory, all 3 groups are comparable because the decrease in TGFβ1 levels is more marked after 5 years of age [15].

No difference was found in serum TGFβ1 values at the time of diagnosis as compared to controls or patients at the time of transplantation because of the wide variation observed in this group. However, a marked decrease in serum TGFβ1 levels was observed in the patients at the time of liver transplantation.

To evaluate the reasons for such decline, that could include decreased production or release, and/or increased clearance or catabolism, we analyzed liver biopsy specimens at the time of diagnosis and liver explants. In liver explants,

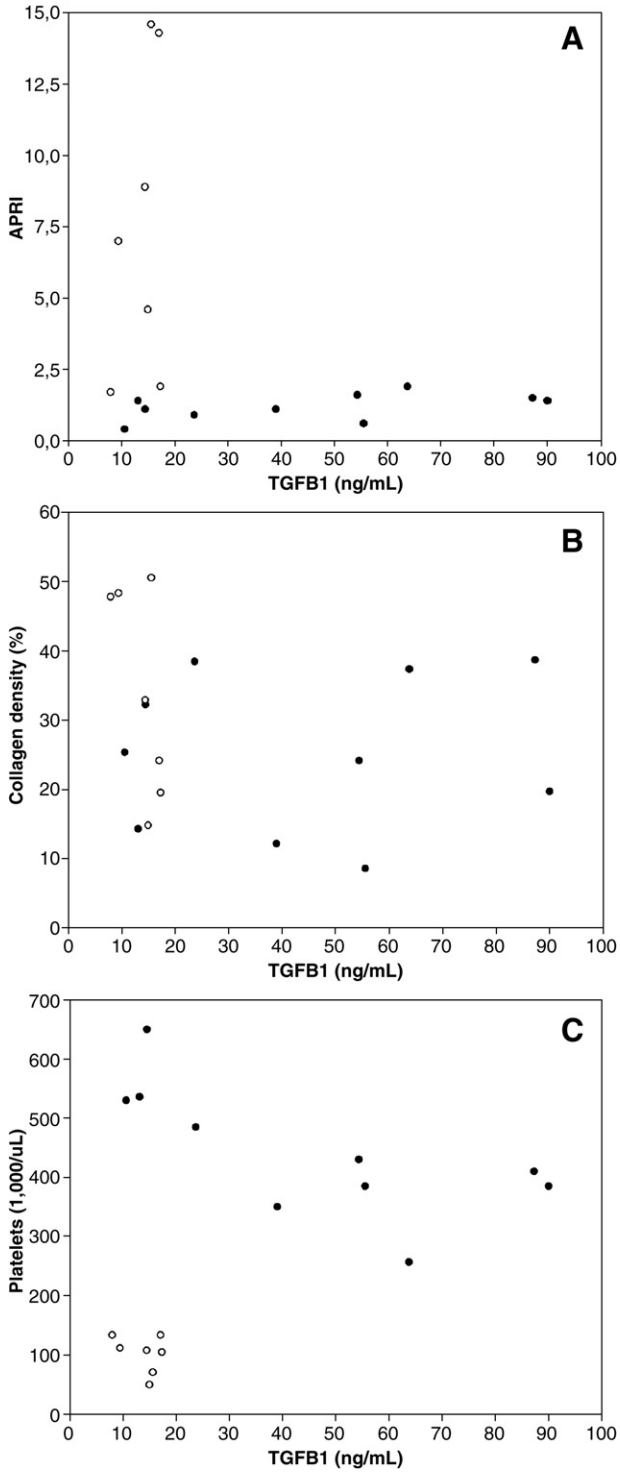

Fig. 4 Serum TGFβ1 correlation with (A) APRI, (B) collagen density, and (C) platelet count in patients with BA at the time of diagnosis (filled circles) and at liver transplantation (empty circles).

almost no expression of TGFβ1 was seen by immunohistochemistry, whereas variable expression was observed in liver biopsy specimens, but most samples had positive staining of medium intensity.

Our findings are in agreement with Rosensweig et al [15] that studied TGFβ1 in plasma and tissue of children with BA. In plasma, TGFβ1 levels in 9 children with BA undergoing liver transplantation were lower than the values of controls. Analysis of liver biopsies and explants showed increased expression of TGFβ1 in BA as compared with other causes of liver diseases. However, no correlation between plasma and tissue levels was observed, even though in the sample of Rosensweig et al [15] plasma and tissue samples were from different subjects.

The lack of expression of TGFβ1 in liver explants was also observed by Ahmed et al [16] who studied BA patients with early and late-stage fibrosis. They noted a global decrease of TGFβ1 in late-stage BA by immunohistochemistry. Lee et al [17], on the other hand, found an increase in TGFβ1 mRNA levels in liver explants compared to biopsy specimens obtained at Kasai procedure.

It is noteworthy that in our study, cells positive for TGFβ1 staining were clearly hepatocytes, except for one patient with faint bile duct positive staining. Ahmed et al [16] found marked TGFβ1 staining in spindle-shaped cells that could represent activated myofibroblasts. In our case, bile duct plugs may have made it difficult to visualize positive myofibroblasts, but as seen in Fig. 2, hepatocytes are expressing TGFβ1 at the time of diagnosis. This observation suggests that hepatocytes may play a role in fibrogenesis, as Kaimori et al [18] recently showed that hepatocytes treated with TGFβ1 in culture express collagen.

To use a clinically relevant surrogate marker to stage the liver disease, we chose APRI. All individuals at the time of transplantation had APRI greater than 2, and at the time of diagnosis, it was less than 2. Moreover, the 4 patients with lower TGFβ1 levels at the time of diagnosis had mean APRI of 0.95 (±0.42), whereas the ones with higher TGFβ1 levels had mean APRI of 1.36 (±0.42) that is consistent with marked fibrosis, and therefore maximum TGFβ1 activity [5].

Blood platelets are rich in TGFβ1, containing 40 to 100 times as much as other cells. Moreover, TGFβ1 is released when platelets are activated, as it happens during liver injury [19]. One important finding of this work is that there is no direct correlation between serum TGFβ1 and platelet count, thus, indicating that the major source of TGFβ1 in our patients was not platelet-derived. Rosensweig et al [15] also found that plasma TGFβ1 levels were independent of PF4 (platelet factor 4), hence, indicating that TGFβ1 decrease is not secondary to thrombocytopenia. Moreover, at the time of transplantation, there is no significant expression of TGFβ1 in liver tissue measured by immunohistochemistry.

At the time of liver transplantation patients are relatively homogenous: low platelet count, low serum TGFβ1, almost no TGFβ1 in the tissue, APRI elevated, and high collagen density. This reflects end-stage liver disease and does not account for the individual pathway that each patient took to get there. On the other hand, at the time of diagnosis, patients show great variability in TGFβ1 levels. This variation may

reflect that diagnosis is performed in patients with different stages of liver disease. Because there is more than one etiology for BA, some children may have the initial process in utero, whereas others will develop the disease at birth [20]. Although no patient in our sample presented associated extrahepatic malformations that are usually related to the in utero forms of BA, it is not possible to determine for how long the disease was active in our patients before diagnosis.

The analysis of liver biopsy specimens could provide an indication for the duration of disease, despite the individual variability in response to liver injury [21]. Although among our patients the 3 with higher collagen density were the oldest, no clear correlation between TGFβ1 and collagen density could be made at any time-point. This, in part, could be because of the small sample size, but it may also reflect that histologic grading of fibrosis (and more specifically cirrhosis) is not directly related to collagen density. Collagen area, evaluated by morphometry, can be very large without the presence of the architectural disorder characteristic of cirrhosis, it is to say, nodular formation. On the other hand, we can find characteristics of cirrhosis, evaluated by specific scores (portal-central bridges, nodular formation, disruption of vascular irrigation, and others) without a very large extent of fibrosis. According to Wanless [22], longstanding cirrhosis can even be associated with an apparent disappearance of collagen in tissue because of the reorganization of the hepatobiliary structures, associated with regeneration of hepatocytes.

In this study, we wanted to investigate if there was a correlation between the amount of collagen and TGFβ1, another product of the same collagen-producing cells. Patients were divided into 2 groups that reflect different stages of the disease (at diagnosis and at liver transplantation). Our findings suggest that at the time of diagnosis (ie, earlier) the fibrogenic process is active, with higher levels of TGFβ1, whereas later on, there is scar tissue, with reduced TGFβ1 expression.

Our results should be confirmed in larger sets of patients with BA. Anyway, the lack of TGFβ1 at the time of liver transplantation may have important consequences for the patient because it is a pleiotropic molecule, responsible for many functions in the body, mainly those related to immune response and cell growth [23,24]. The association of liver disease with hepatocellular carcinoma and immunosupression, although derived from many complex factors, may be also connected with low TGFβ1 levels given its pivotal role in all aspects of inflammation, immune surveillance, and neoplasia [25].

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