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# Divergent expression of liver transforming growth factor superfamily cytokines after successful portoenterostomy in biliary atresia ☆

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ABSTRACT

*Background:* Pathogenesis of progressive liver fibrosis in biliary atresia after successful portoenterostomy remains unclear. We related hepatic expression of transforming growth factor beta (TGF-$\beta$) superfamily cytokines to histologic liver injury after successful portoenterostomy.

*Methods:* Enrolled in our study were 28 patients with biliary atresia who had liver biopsies obtained during and after successful portoenterostomy, which normalized serum bilirubin (<20 μmol/l). Biopsies were evaluated for cholestasis, inflammation, ductal reaction, and fibrosis and were stained immunohistochemically for transforming growth factor beta 1, transforming growth factor beta 2, connective tissue growth factor, and decorin. Respective gene expression (*TGFB1, TGFB2, TGFB3, CTGF, DCN*) was analyzed at follow-up using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Results were compared with fibrotic and healthy control livers.

*Results:* After median follow-up of 3.0 years, histologic cholestasis resolved, whereas fibrosis had progressed only in isolated biliary atresia. Liver protein expression of transforming growth factor beta 1 and connective tissue growth factor ($P < .001$ for both), but not that of transforming growth factor beta 2 or decorin, decreased after successful portoenterostomy, although expression of all four cytokines remained elevated. In accordance with postportoenterostomy changes in protein expression, follow-up ribonucleic acid expression of *TGFB2* and *DCN,* but not that of *TGFB1* and *CTGF,* was upregulated when compared with the controls. Both protein and gene expression of transforming growth factor beta 1 and protein expression of transforming growth factor beta 2, connective tissue growth factor and decorin correlated with METAVIR fibrosis stage. Syndromic patients ($n = 12$) showed milder fibrosis and lower transforming growth factor beta 1 expression than patients with isolated biliary atresia.

*Conclusion:* These findings support a central role of transforming growth factor beta superfamily in mediating continuing liver fibrogenesis after successful portoenterostomy. Transforming growth factor beta pathway cytokines responded divergently to clearance of jaundice, which was reflected by differential progression of fibrosis between syndromic and isolated patients.



## Introduction

Biliary atresia (BA) is an obliterative fibroinflammatory cholangiopathy of infancy, with an incidence of 1 in 18,000–20,000 live births in Europe.[1–3] Genetic predisposition, developmental defects, and environmental factors, such as viral infection, play a part in its multifactorial etiology.[1,3,4] Up to 20%–30% of patients have associated anomalies and presumably different pathogenesis from the isolated disease.[1,4–6] Liver histology at diagnosis is characterized by cholestasis, ductular proliferation, inflammation, and fibrosis.[3] Despite successful restoration of bile flow and normalization of serum bilirubin by portoenterostomy (PE), the great majority of patients develop progressing liver fibrosis and liver failure.[2,3] Although BA is the leading indication for pediatric liver transplantation (LTx) worldwide,[2] the molecular mechanisms of liver fibrosis after PE remain unclear.[4]

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The transforming growth factor beta (TGF-$\beta$) superfamily consists of several cytokines that play important roles in the regulation of hepatic fibrogenesis, growth, development, and immunity.[7–9] TGF-$\beta$ is a potent activator of portal fibroblasts and hepatic stellate cells into $\alpha$-smooth muscle acting ($\alpha$-SMA) expressing myofibroblasts, which mediate liver fibrogenesis by producing extracellular matrix proteins.[10,11] Previous studies in BA have found an increased hepatic messenger ribonucleic acid (mRNA) and a protein expression of TGF-$\beta$ at the time of diagnosis and in explants with advanced stages of the disease after development of liver failure.[12–22] Connective tissue growth factor (CTGF) synergizes the profibrotic actions of TGF-$\beta$, and its expression has been found to increase in BA.[23–25] Decorin is a proteoglycan involved in the maturation of collagen and possibly in attenuation of TGF-$\beta$1 activity in hepatic fibrogenesis.[26]

We investigated how the expression of TGF-$\beta$ superfamily members evolves and relates with histologic liver injury in BA patients after normalization of serum bilirubin by successful PE before development of clinical and biochemical signs of advanced liver dysfunction. This knowledge is essential for the development of novel management strategies to delay or prevent the progression of liver fibrosis after successful PE.

## Materials and Methods

### *Patients and ethics*

Of 51 BA patients operated in Helsinki University Hospital (Finland) between 1991 and 2013, 30 patients (59%) cleared their jaundice after PE, and 28 of them (93%) were enrolled. They underwent liver biopsy at PE. An ultrasound-guided core-needle liver biopsy was obtained during routine follow-up, including endoscopic variceal surveillance under anesthesia, abdominal ultrasound examination, and blood sampling.[27] Follow-up liver biopsies were part of the routine follow-up protocol not warranted by patients' clinical status.[28] Splenomegaly and portal hypertension were defined as described elsewhere.[27] Associated congenital anomalies were recorded from medical records and BA splenic malformation (BASM) was defined as having polysplenia or asplenia.[29]

Study protocol was approved by Ethics Committee of the Hospital District of Helsinki and Uusimaa (number 345/13/03/03/2008) and ethical guidelines of the 1975 Declaration of Helsinki were followed. Informed consent was obtained from all legal guardians of participating children and from adults.

### *Controls*

Healthy nonfibrotic control liver biopsies were obtained from 19 pediatric donor livers (median age 14.2 years [interquartile range 8.0–16.2 years]) and from 10 children (age 11.4 years [7.8–14.8 years]) undergoing cholecystectomy for cholecystolithiasis (research protocol 434/13/03/03/2008). Fibrotic control liver tissue was obtained from 11 patients with intestinal failure (age 4.7 years [3.5–9.7 years]). Control blood samples were obtained from 47 generally healthy ambulatory-surgery patients (age 6.5 years [4.2–12.6 years]) with no liver disease.

### *Liver biopsies and immunohistochemistry*

In total, 24 liver biopsies obtained at PE and 28 at follow-up were available for analyses. After fixation in formalin, embedding in paraffin, slicing, and staining, liver biopsies were analyzed semiquantitatively by two experienced pediatric pathologists for METAVIR fibrosis stage (0–4), portal inflammation (0–3), and intracanalicular cholestasis (0–3).[27]

Immunohistologic studies were performed with antibodies for TGF-$\beta$1 (clone TGFB17; Leica Novocastra, Leica Biosystems, Nussloch, Germany), TGF-$\beta$2 (clone MGC116892; Abnova Corporation, Taipei City, Taiwan), CTGF (clone L-20; Santa Cruz Biotechnology Inc, Dallas, TX, USA), and decorin (clone NBP1-84970, Novus Biologicals, LLC, Littleton, CO, USA). Dilutions were 1:50, 1:1000, 1:400, and 1:750, respectively. Blinded to clinical data, primary author (A.K.) performed semiquantitative immunohistologic gradings with a Leica DM RXA microscope (Leica Microsystems GmbH, Wetzlar, Germany), as follows:

- TGF-$\beta$1 and CTGF: 0 = negative, 1 = mild, 2 = moderate, and 3 = strong staining
- TGF-$\beta$2: 0 = negative, 1 = mild, and 2 = moderate staining
- decorin: 0 = slight expression at portal areas, 1 = expanded expression at portal areas, 2 = with portal-to-portal septaes, 3 = with portal-to-central septaes, 4 = expanded nodular expression (cirrhosis)

Staining of bile duct epithelial cells (BECs) was graded dichotomously for TGF-$\beta$2. The expression of TGF-$\beta$1 and CTGF were also evaluated for localization (most staining in fibrotic = 0, parenchymal = 1, or both areas = 2). The periportal staining pattern was graded dichotomously for TGF-$\beta$1, TGF-$\beta$2, and CTGF.

Cytokeratin-7 (CK-7) expression was used to visualize ductal reaction because it stains proliferating bile ductules, metaplastic hepatocyte-cholangiocyte intermediate cells, and bile ducts.[30,31] Immunostaining for CK-7 was performed using SP52 monoclonal antibody and ultra View Universal DAB Detection KIT (Ventana, Tuscon, AZ, USA). Immunostaining for $\alpha$-SMA, a marker of activated myofibroblasts, was performed as described in detail elsewhere.[32] The proportion of the antibody-positive area to the entire biopsy surface area (area fraction) was calculated for CK-7 and $\alpha$-SMA, using ImageJ Image Analysis Software (SciJava Common open source software; Rasband, WS, ImageJ, US National Institutes of Health, Bethesda, MD, USA; http://imagej.nih.gov/ij/; 1997–2014).

### *RNA expression analyses*

RNA expression was analyzed from 24 BA patients with BA who had a liver RNA specimen available (Table 1). Liver tissue specimens were embedded in RNAlater-solution (Ambion, Life Technologies, Thermo Fischer Scientific Inc, Waltham, MA, USA) and frozen RNA was extracted with RNeasy Mini Kit (QIAGEN, Frederick, MD, USA). RNA integrity was determined spectrophotometrically, and the expression was analyzed in triplicate by quantitative real-time polymerase chain reaction using Human Fibrosis RT$^2$ Profiler PCR Array (QIAGEN SABiosciences, Frederick, MD, USA) on an ABI 7700 Sequence Detection System (Perkin-Elmer Lifer Sciences, Boston, MA, USA), according to the manufacturer's directions. Quantification of target gene mRNA expression was performed using the $\Delta\Delta$Ct method and expressed after normalization to housekeeping genes (*B2M, HPRT1, RPL13A, GAPDH, ACTB*) and relative to control subjects.

### *Analyses of serum TGF-$\beta$2 and decorin*

The serum concentrations of TGF-$\beta$2 and decorin were determined using commercially available enzyme-linked immune sorbent assay (ELISA) kits by Biovendor R&D (Bratislava, Slovakia). The analyses for human-TGF-$\beta$2 and human decorin were carried out according to the manufacturer's protocol. The interassay coefficient of variations (CV%) for TGF-$\beta$2 and decorin were 10.0% and 5.8 ng/mL, and detection limits were 6.6 and 56 pg/mL, respectively.

**Table 1**
Hepatic gene expression of transforming growth factor-beta (TGF-$\beta$) superfamily in biliary atresia patients after a median follow-up of 3.0 years in relation to controls

| Gene | Biliary atresia at follow-up $n=24$ | Intestinal failure controls $n=11$ | Cholelithiasis controls $n=10$ | $P$ value |
|---|---|---|---|---|
| *TGF-β* | | | | |
| *TGFB1* | 1.02 (0.89–1.36) | 0.86 (0.65–0.96) | 1.04 (0.76–1.20) | .051 |
| *TGFb2* | 3.73 (3.01–8.16)*,† | 0.88 (0.67–1.23) | 1.00 (0.68–1.44) | < .001 |
| *TGFB3* | 2.04 (1.49–2.40)*,† | 1.18 (0.78–1.37) | 1.01 (0.91–1.32) | < .001 |
| Receptors | | | | |
| *TGFBR1* | 1.55 (1.30–2.08)*,† | 0.69 (0.59–0.84) | 1.04 (0.72–1.37) | < .001 |
| *TGFBR2* | 0.98 (0.90–1.26) | 0.84 (0.80–1.01) | 0.96 (0.93–1.15) | .107 |
| Signaling pathway | | | | |
| *SMAD2* | 1.42 (1.14–1.73)*,† | 0.80 (0.72–0.96) | 0.92 (0.72–1.46) | < .001 |
| *SMAD6* | 0.57 (0.40–0.93)† | 0.52 (0.36–0.68)‡ | 1.00 (0.79–1.27) | .001 |
| Interactors and mediators | | | | |
| *CTGF* | 1.06 (0.66–1.64)* | 0.48 (0.44–0.79) | 0.90 (0.60–1.46) | .025 |
| *DCN* | 1.43 (1.21–2.00)*,† | 1.07 (0.87–1.17) | 0.98 (0.92–1.11) | < .001 |

* $P < .018$ compared with fibrotic intestinal failure controls.
† $P < .02$ compared with nonfibrotic cholelithiasis controls.
‡ $P < .001$ between intestinal failure controls and cholelithiasis controls. *Note:* Data are median (interquartile range). The RNA expression is expressed after normalization to housekeeping genes as fold change relative to cholelithiasis control subjects. Significance was evaluated for multiple groups, using the Kruskall-Wallis $H$ test, and for pairs of groups, using the Mann Whitney $U$ test.

### *Statistical analyses*

SPSS software v 22.0 for Windows (IBM Corp, Armonk, NY, USA) was used for statistical analyses. Data are medians (interquartile range [IQR]), unless otherwise stated. Kruskall-Wallis test was used for multiple comparisons. Pairwise comparisons of continuous variables were performed using the Wilcoxon signed rank test or the Mann-Whitney $U$ test, where appropriate, and the Fisher exact test for dichotomous variables. Correlations were calculated with the Spearman rank correlation. A $P$ value below .05 was considered statistically significant.

## Results

### *Patient characteristics*

Median patient (50% males) age at PE was 61 (interquartile range 40–84) days and 3.0 (2.1–6.7) years at follow-up. A total of 16 patients (57%) had isolated BA, and 12 patients (43%) had associated congenital anomalies (syndromic BA), including splenic malformations (BASM) in 8 patients (29%). At follow-up, splenomegaly was recorded in 9 patients (32%), and 14 patients (50%) had clinical signs of portal hypertension. After PE, bilirubin level had decreased from 159 (116–204) μmol/L to 10 (4–17) μmol/L, conjugated bilirubin from 115 (83–159) to 4 (2–8) μmol/L (both $p<0.001$), and alanine aminotransferase from 87 (42–164) U/L to 45 (24–94) U/L, ($P=.012$).

### *Evolution of histology and immunohistochemistry after PE*

After successful PE, histologic liver fibrosis progressed in isolated BA patients, but remained unchanged in syndromic BA, and cholestasis disappeared and portal inflammation reduced in both groups (Table 2). CK-7 expression, which reflects the extension of ductal reaction, decreased significantly after PE in syndromic patients (Table 2). The CK-7 area fraction correlated with METAVIR fibrosis stage both at PE ($r=0.611$, $P=.004$) and at follow-up ($r=0.467$, $P=.014$). METAVIR fibrosis stage was unrelated to follow-up age ($r=0.035$, $P=.859$).

The hepatic expression of TGF-$\beta$1 and CTGF, but not that of TGF-$\beta$2 or decorin, significantly decreased after successful PE (Table 2, Figs 1 and 2). However, the protein expression of all four cytokines remained significantly higher when compared with nonfibrotic control liver biopsies (0 [0–0], $P < .003$ for all, see Table 2). TGF-$\beta$1 was expressed around lobular hepatocytes and in fibrotic areas. The CTGF staining was perceived mainly as spindle-shaped periportal cells, but especially at follow-up also in lobu-

**Table 2**
Histology and immunohistochemistry at portoenterostomy (PE) and after a median follow-up of 3.0 years

| | All patients | $n$ | Syndromic BA | $n$ | Isolated BA | $n$ |
|---|---|---|---|---|---|---|
| Metavir stage (0–4) | | | | | | |
| PE | 2 (2–3) | 24 | 2 (2–3) | 9 | 2 (2–3) | 15 |
| Follow-up | 2 (1–4) | 28 | 2 (1–2) | 12 | 3 (2–4) | 16 |
| $P$ value | $P=.170$ | | $P=.579$ | | $P=.026$ | |
| Portal inflammation (0–3) | | | | | | |
| PE | 2 (2–3) | 24 | 2 (2–3) | 9 | 2 (2–3) | 15 |
| Follow-up | 1 (0–1) | 28 | 0 (0–1) | 12 | 1 (1–2) | 16 |
| $P$ value | $P < .001$ | | $P=.01$ | | $P=.002$ | |
| cholestasis (0–3) | | | | | | |
| PE | 2 (1–3) | 24 | 2 (1–3) | 9 | 2 (1–3) | 15 |
| Follow-up | 0 (0–0) | 28 | 0 (0–0) | 12 | 0 (0–0) | 16 |
| $P$ value | $P < .001$ | | $P=.007$ | | $P=.001$ | |
| Ductal reaction (%)* | | | | | | |
| PE | 5.0 (3.3–7.0) | 20 | 5.3 (3.4–7.0) | 8 | 4.3 (2.9–7.8) | 12 |
| Follow-up | 2.4 (1.8–4.9) | 27 | 1.9 (1.5–4.1) | 11 | 3.1 (1.8–8.6) | 16 |
| $P$ value | $P=.227$ | | $P=.028$ | | $P=.937$ | |
| TGF-β1, grade (0–3) | | | | | | |
| PE | 2 (2–3) | 22 | 2 (2–2) | 9 | 2 (2–3) | 13 |
| Follow-up | 1 (0–1) | 27 | 0 (0–1) | 11 | 1 (0–1) | 16 |
| $P$ value | $P < .001$ | | $P=.014$ | | $P=.003$ | |
| TGF-β2, grade (0–2) | | | | | | |
| PE | 1 (1–1) | 23 | 1 (1–1) | 8 | 1 (1–1) | 15 |
| Follow-up | 1 (0–1) | 28 | 0.5 (0–1) | 12 | 1 (0–1) | 16 |
| $P$ value | $P=.09$ | | $P=.414$ | | $P=.132$ | |
| CTGF, grade (0–4) | | | | | | |
| PE | 2 (2–3) | 23 | 2.5 (2–3) | 8 | 2 (2–2) | 15 |
| Follow-up | 1 (0–1) | 27 | 1 (0–1) | 12 | 1 (0–1) | 15 |
| $P$ value | $P < .001$ | | $P=.019$ | | $P=.003$ | |
| Decorin, grade (0–4) | | | | | | |
| PE | 2 (2–3) | 17 | 2.5 (2–3) | 6 | 2.5 (2–4) | 11 |
| Follow-up | 2 (1.5–2.5) | 25 | 2 (1–2) | 11 | 2 (2–3) | 14 |
| $P$ value | $P=.409$ | | $P=.279$ | | $P=.891$ | |

*TGF-β*, transfroming growth factor-beta; *CTGF*, connective tissue growth factor.
* Ductal reaction was analyzed using cytokeratin-7 immunostaining (area fraction).
*Note:* Data are median (interquartile range). Significance was evaluated using Wilcoxon sign rank test.

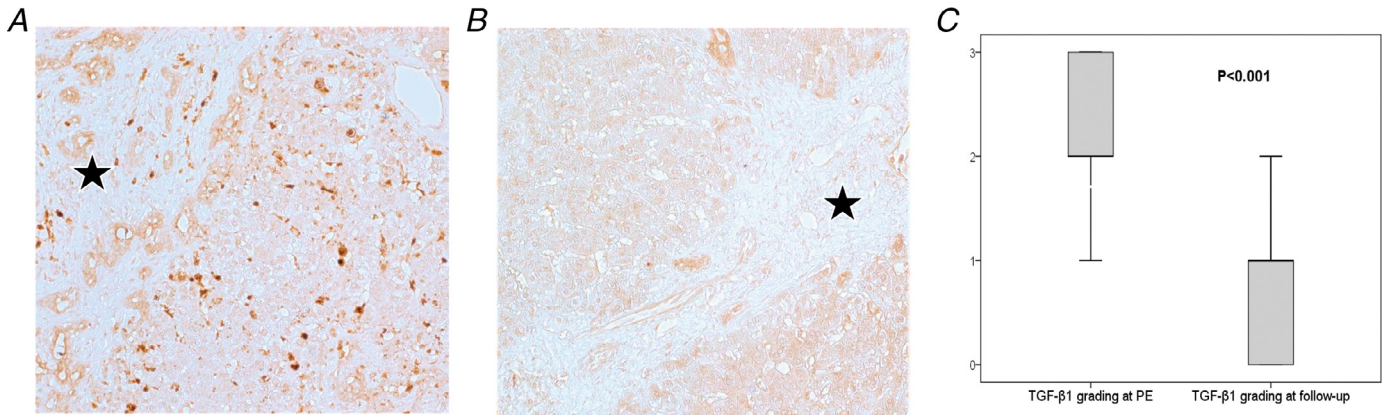


**Fig 1.** Hepaticv transforming growth factor beta 1 (TGF-$\beta$1) protein expression reduced significantly after portoenterostomy (PE). (*A*) PE of a patient 105 days old, hepatic TGF-$\beta$1 protein expression was grade 3 and seen both in fibrotic (*asterisk)* and parenchymal areas, with METAVIR stage and portal inflammation were grade 2 and intracanalicular cholestasis grade 3 ( $\times$ 200). (*B*) Liver biopsy at 2.4 years of the same patient shows reduced TGF-$\beta$1 protein expression and resolved cholestasis to grade 0, with METAVIR stage and portal inflammation grade 1 ( $\times$ 200; portal area [*asterisk*]). (*C*) Hepatic TGF- $\beta$1 expression reduced after portoenterostomy ($n = 27$). Box plots display median (*bold transverse line*), interquartile range (*rectangle*), and range. Significance was evaluated using the Wilcoxon sign rank test.

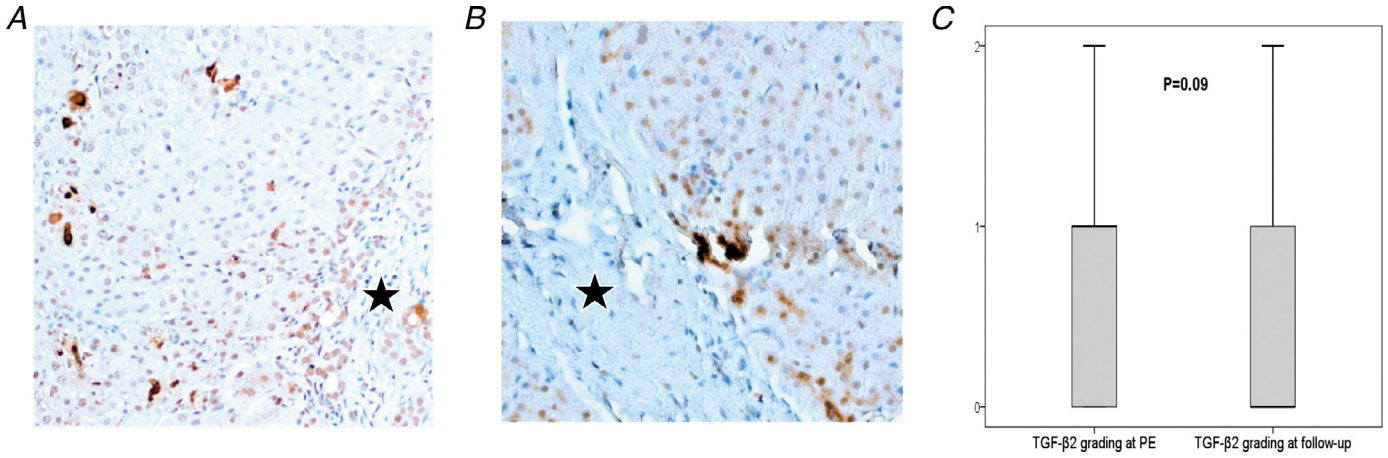


**Fig 2.** Hepatic transforming growth factor beta 2 (TGF-$\beta$2) protein expression persisted after portoenterostomy (PE). (*A*) PE of a patient 104 days old, hepatic TGF-$\beta$2 protein expression was grade 2 and expressed around lobular hepatocytes, METAVIR stage was 4, portal inflammation grade 2, and intracanalicular cholestasis grade 3. The fibrotic area is indicated by an *asterisk* ( $\times$ 200). (*B*) Liver biopsy of the same patient 18.9 years later shows reduced TGF-$\beta$2 protein expression and portal inflammation to grade 1, resolved cholestasis to grade 0, with METAVIR stage 4. TGF-$\beta$2 expression concentrated in periportal areas. The fibrotic area is indicated by an *asterisk* ( $\times$ 200). (*C*) Hepatic TGF- $\beta$2 persisted after portoenterostomy ($n = 28$). Box plots display the median (*bold transverse line*), interquartile range (*rectangle*), and range. Significance was evaluated using Wilcoxon sign rank test.

lar hepatocytes. The periportal CTGF positivity was more frequent at PE (74%) than at follow-up (4%, $P = .003$). No such change was observed for periportal TGF-$\beta$1 (45% versus 20%, $P = .50$). TGF-$\beta$1 and CTFG staining concentrated more to the fibrotic areas at follow-up than at PE (0 [0–0] versus 2 [0–2], $P = .025$ and 0 [0–2] versus 2 [2–2]), $P = .005$). TGF-$\beta$2 immunostaining was detected mainly around lobular hepatocytes both during and after PE (Table 2 and Fig 2). Frequency of periportal TGF-$\beta$2 expression was significantly higher at follow-up (50%) than at PE (14%, $P = .039$; Fig 2). At follow-up in 25% of samples, BECs expressed TGF-$\beta$2, which was not observed at PE (0%, $P = .125$). Expression of decorin was observed in portal and fibrotic areas similarly at PE and follow-up.

### *RNA expression of TGF-$\beta$-superfamily cytokines in BA patients compared with fibrotic and nonfibrotic controls*

As presented in Table 1, the follow-up RNA expression of *TGFB2, TGFB3*, and *DCN*—but not that of *TGFB1* and *CTGF*—was upregulated in BA patients compared with both control groups. Controls with intestinal failure had comparable liver METAVIR fibrosis stage (2 [2–2], $P = .370$) versus BA, and nonfibrotic controls were operated for cholelithiasis. The RNA expression of *TGFBR1* and *SMAD2* was also upregulated compared with both control groups. In contrast, the RNA expression of *SMAD6* was significantly downregulated compared with nonfibrotic controls.

### *TGF-$\beta$1 expression correlated with liver fibrosis*

In follow-up specimens both protein and RNA expression of TGF-$\beta$1 correlated positively with the METAVIR fibrosis stage (Fig 3). In addition, TGF-$\beta$2 (Fig 3), CTGF ($r = 0.478$, $P = .012$) and decorin ($r = 0.825$, $P < .001$) protein expression associated with fibrosis (Fig 3). At follow-up, the extension of CK-7 staining marking ductal reaction correlated with the decorin ($r = 0.542$, $P = .006$) and CTGF ($r = 0.554$, $P = .003$) expression.

### *Expression of TGF-$\beta$ cytokines coupled with myofibroblast activation*

Table 3 presents relationships between $\alpha$-SMA expression, denoting myofibroblast activation, and the expression of TGF-$\beta$

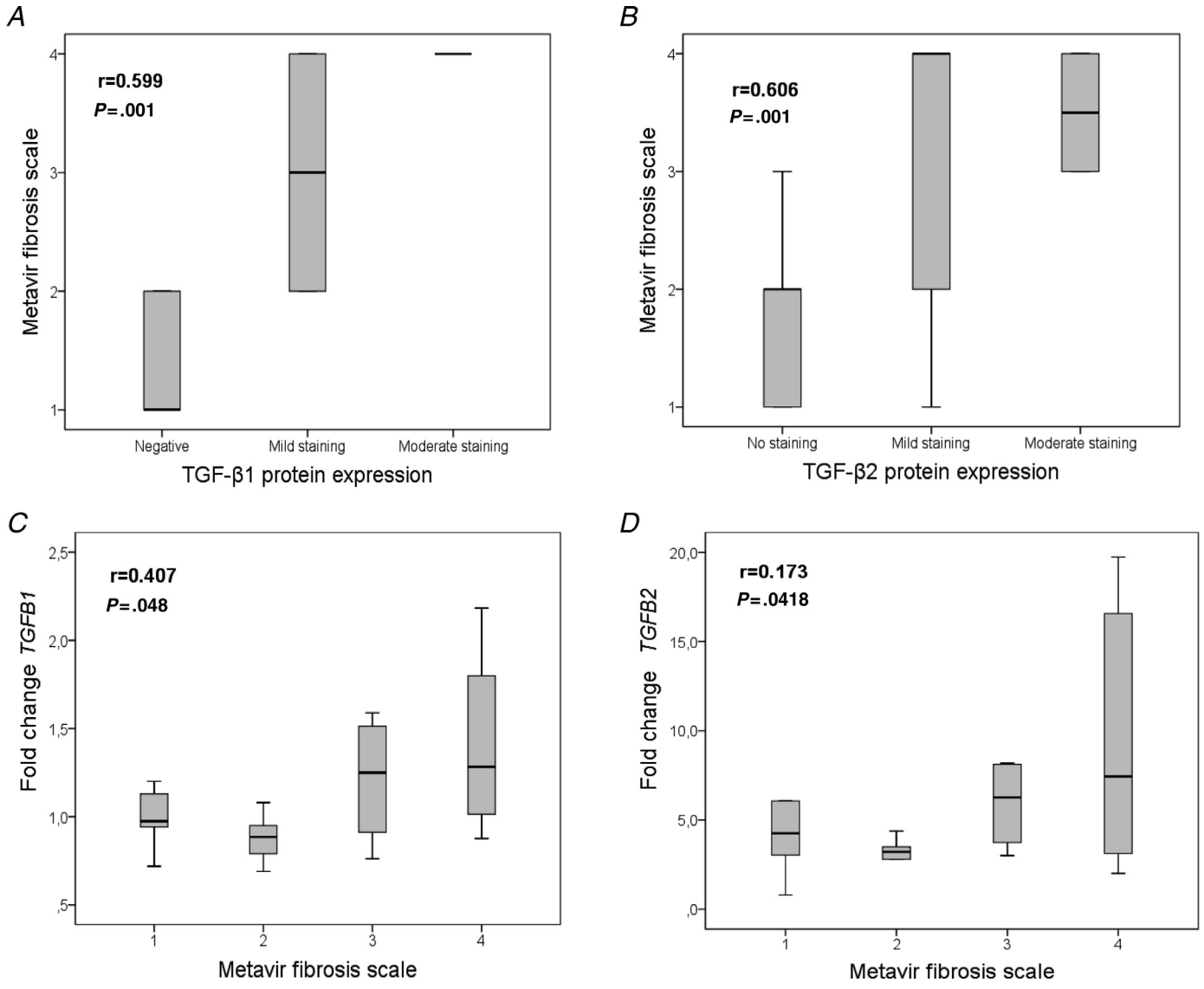


**Fig 3.** Histologic fibrosis correlated positively with hepatic transforming growth factor beta 1 (TGF-$\beta$1) and beta 2 (TGF- $\beta$2) protein and *TGFB1* gene expression after median 3.0 years of follow-up. (*A, B*) TGF-$\beta$1 and TGF- $\beta$2 protein expression correlated positively with METAVIR fibrosis stage. (*C, D*) *TGFB1*, but not *TGFB2*, gene expression correlated positively with METAVIR fibrosis stage. Box plots display the median (*bold transverse line*), interquartile range (*rectangle*), and range. Significance was evaluated using the Spearman rank correlation.

superfamily cytokines. Protein or RNA expression of all studied TGF cytokines correlated with $\alpha$-SMA protein expression, and the RNA expression of *TGFB2,* CTGF, and *DCN* correlated with *ACTA* encoding for $\alpha$-SMA.

### *Syndromic BA patients exhibited milder fibrosis and lower expression of TGF-$\beta$1 than isolated patients*

As presented in Table 4, histologic variables, age, and protein expression of TGF-$\beta$1 and 2, CTGF, and decorin were similar between syndromic and isolated BA patients at PE. In follow-up specimens obtained at a similar age, syndromic patients showed milder fibrosis and lower protein expression of TGF-$\beta$1 and decorin than isolated patients (Tables 2 and 4). Patients with BASM tended to have a lower METAVIR fibrosis stage (2 [1–2] versus 3 [2–4], $P = .072$) and TGF-$\beta$1 protein expression at follow-up (0 [0–1] versus 1 [0–1], $P = .205$], but the differences did not reach statistical significance.

### *Serum TGF-$\beta$2 levels were increased in BA patients compared with controls*

Patients showed increased serum TGF-$\beta$2 levels at follow-up (925 [833–1,590] pg/mL) compared with healthy controls (871 [599–1,200] pg/mL, $P = .016$). Serum decorin levels did not differ between patients (4,750 [4,090–6,170] pg/mL) and controls (4,630 [3,880–5,850] pg/mL), $P = .834$.

## Discussion

This is the first study to explore the evolution of hepatic TGF-$\beta$ superfamily cytokine expression after successful PE and resolution of histologic and biochemical cholestasis. We found that the expression of TGF-$\beta$1 and CTGF—but not that of TGF-$\beta$2 or decorin—decreased after successful PE. Both the RNA and protein expression of TGF-$\beta$1 correlated with the progression of fibrosis, which was more vigorous, along with a higher TGF-$\beta$1 expression, in isolated rather than in syndromic BA patients. These findings suggest that

**Table 3**
Correlation of $\alpha$-smooth muscle actin protein ($\alpha$-SMA area fraction) and gene (*ACTA* fold change) expression with transforming growth factor (TGF) superfamily cytokines

| Cytokines | $\alpha$-SMA area fraction | *n* | *ACTA* fold change | *n* |
|---|---|---|---|---|
| TGF-$\beta$1, grade | r = 0.272 | 24 | r = 0.028 | 23 |
| | *P* = .179 | | *P* = .899 | |
| *TGFB1*, fold change | r = 0.577 | 21 | r = 0.401 | 24 |
| | *P* = .004 | | *P* = .052 | |
| TGF-$\beta$2, grade | r = 0.545 | 25 | r = 0.222 | 24 |
| | *P* = .003 | | *P* = .298 | |
| *TGFB2*, fold change | r = 0.496 | 21 | r = 0.663 | 24 |
| | *P* = .016 | | *P* < .001 | |
| CTGF, grade | r = 0.484 | 25 | r = 0.484 | 23 |
| | *P* = .011 | | P = .011 | |
| *CTGF*, fold change | r = 0.427 | 21 | r = 0.650 | 24 |
| | *P* = .042 | | *P* = .001 | |
| Decorin, grade | r = 0.661 | 22 | r = 0.318 | 21 |
| | *P* < .001 | | *P* = .160 | |
| *DCN*, fold change | r = 0.405 | 21 | r = 0.680 | 24 |
| | *P* = .055 | | *P* < .001 | |

*TGF-$\beta$*, transforming growth factor-beta; *CTGF*, connective tissue growth factor.
*Note:* Correlations for immunohistochemical expression (grade) and respective gene expression (fold change) are provided for TGF cytokines.

downregulation of hepatic TGF-$\beta$1 and CTGF may have a central role in delaying the progression of liver fibrosis after successful PE.

The TGF-$\beta$ superfamily forms one of the most potent driving forces for hepatic fibrogenesis through hepatic stellate cell activation and epithelial-to-mesenchymal transition.[7,9,26] Three major isoforms of TGF-$\beta$, TGF-$\beta$1, TGF-$\beta$2, and TGF-$\beta$3 show similar biologic effects in vitro, and their in vivo expression patterns are very dissimilar from one another.[8,33] TGF-$\beta$1 is often described as the most abundant isoform in hepatic fibrosis.[8,26,34] We found that hepatic TFG-$\beta$1—but not TGF-$\beta$2—protein expression decreased after successful PE, and that TGF-$\beta$2 protein and RNA expression, together with TGF-$\beta$3 RNA expression, remained increased in follow-up specimens. Thus, TFG-$\beta$1 expression selectively decreased after a successful PE. In addition to a reduced TGF-$\beta$1 expression after a successful PE, both protein and RNA expression of TGF-$\beta$1correlated with a progression of histologic fibrosis in follow-up specimens, providing further support for involvement of TGF-$\beta$1 in the regulation of postoperative fibrogenesis. The most likely and obvious reason for downregulation of TFG-$\beta$1 expression after a successful PE is the resolution of cholestasis.[35] Interestingly, in syndromic patients the significant decrease in ductal reaction accompanied a lower TFG-$\beta$1 expression in relation to isolated patients. An alternative potential reason for the decreased TGF-$\beta$1 expression after PE may be a reduced TGF-$\beta$1–producing capacity when fibrosis progresses to cirrhosis and destroys TGF-$\beta$–storing cells.[7,17,19] This seems unlikely because most of our patients were not cirrhotic at follow-up, with a median METAVIR stage 2. In accordance with our results, earlier studies have described an increased TGF-$\beta$1 protein and RNA expression at the time of PE and again at LTx, when patients usually have end-stage cirrhosis.[16,18–22]

Our findings extend earlier observations by showing that, after a successful PE, liver expression of TGF-$\beta$ isoforms responded divergently to clearance of jaundice, which could be important in the surveillance and manipulation of postoperative liver fibrosis. In contrast with TGF-$\beta$1, liver protein and gene expression, and serum concentrations of TGF-$\beta$2, remained elevated, most likely reflecting active fibrogenesis despite a successful PE. TGF-$\beta$2 is usually expressed by BECs, but in BA also by hepatocytes.[12,15,34,36] We found TGF-$\beta$2 expression in hepatocytes and mesenchymal cells in fibrotic areas, but in BECs only in 25% of biopsies at follow-up and none at PE. This might be attributable to a less-advanced stage of the liver injury in our patients compared with end-stage liver disease in earlier reports. In line with the increased hepatic expression, serum TGF-$\beta$2 levels were also elevated in BA patients, but did not correlate with histologic fibrosis. This may be explained by the fact that the majority of circulating TGF-$\beta$ is in latent form.[9]

Alterations in the TGF-$\beta$ expression were accompanied with evocative changes in other superfamily members. The RNA expression of *TGFBR1* encoding for TGF-$\beta$ receptor 1 increased compensatorily, and *SMAD2* promoting intracellular TGF-$\beta$ signaling was upregulated. In contrast, the RNA expression of *SMAD6* with an inhibitory effect on the TGF-$\beta$ pathway was downregulated in BA patients compared with nonfibrotic controls.[8,9,33] Decorin has been reported to inhibit fibrogenesis by binding to TGF-$\beta$.[26] Accordingly, we found increased protein and gene expression of decorin after successful PE, suggesting that upregulation of a decorin expression may contribute to the deceleration of fibrosis after a successful PE by inhibiting TGF-$\beta$–driven fibrogenesis. Similar to TGF-$\beta$1,

**Table 4**
Differences between syndromic and isolated biliary atresia (BA) patients in relation to hepatic gene and protein expression and liver histology

| | Syndromic BA | *n* | Isolated BA | *n* | *P* value |
|---|---|---|---|---|---|
| At portoenterostomy | | | | | |
| age, days | 54 (39–77) | 12 | 64 (43–90) | *16* | .430 |
| Metavir stage (0–4) | 2 (2–3) | 9 | 2 (2–3) | *15* | .310 |
| TGF-$\beta$1, grade (0–3) | 2 (2–2) | 9 | 2 (2–3) | *13* | .126 |
| TGF-$\beta$2, grade (0–2) | 1 (1–1) | 8 | 1 (1–1) | *15* | .210 |
| CTGF, grade (0–3) | 2.5 (2–3) | 8 | 2 (2–2) | *15* | .296 |
| Decorin, grade (0–4) | 2.5 (2–3) | 6 | 2 (2–4) | *11* | 1.000 |
| At follow-up | | | | | |
| age, years | 4.2 (2.0–9.2) | 12 | 2.9 (2.1–5.2) | 16 | .642 |
| Metavir stage (0–4) | 2 (1–2) | 12 | 3 (2–4) | 16 | .019 |
| Ductal reaction, (%)* | 1.9 (1.5–4.1) | 11 | 3.1 (1.8–8.6) | 16 | .167 |
| TGF-$\beta$1, grade (0–3) | 0 (0–1) | 11 | 1 (0–1) | 16 | .015 |
| *TGFB1*, fold change | 1.08 (0.87–1.18) | 9 | 0.98 (0.88–1.48) | 15 | .905 |
| TGF-$\beta$2, grade (0–2) | 0.5 (0–1) | 12 | 1 (0–1) | 16 | .417 |
| *TGFB2*, fold change | 3.49 (2.91–4.25) | 9 | 4.47 (3.01–11.2) | 15 | .297 |
| *TGFB3*, fold change | 2.14 (1.64–2.20) | 9 | 2.01 (1.45–3.77) | 15 | .929 |
| CTGF, grade (0–3) | 1 (0–1) | 12 | 1 (0–1) | 15 | .411 |
| *CTGF*, fold change | 1.13 (0.70–2.19) | 9 | 0.99 (0.59–1.50) | 15 | .655 |
| Decorin, grade (0–4) | 2 (1–2) | 11 | 2 (2–3) | 14 | .024 |
| *DCN*, fold change | 1.40 (1.18–1.94) | 9 | 1.44 (1.18–2.00) | 15 | .770 |

*TGF-$\beta$*, transforming growth factor-beta; *CTGF*, connective tissue growth factor.
* Ductal reaction was analyzed using cytokeratin-7 immunostaining.
*Note:* Data are median (interquartile range). Significance evaluated using Mann-Whitney *U* test.

decorin expression is localized mainly in fibrotic areas and associated with the histologic fibrosis stage at PE and at follow-up. In our study, the protein expression of CTGF declined, the RNA expression remained unchanged, and the CTGF protein expression correlated with the fibrosis stage, paralleling the findings for TGF-$\beta$1. CTGF is produced by multiple mesenchymal and epithelial cell types and is recognized as a downstream mediator of TGF-$\beta$ by synergizing its actions.[9,23] In other studies, the hepatic CTGF expression has been found to be upregulated and to correlate with the degree of fibrosis at the time of PE.[20,24,25]

In our earlier study, a molecular signature of active fibrogenesis persisted after a successful PE, whereas the RNA expression of various Th1 and Th2 proinflammatory cytokines was low, suggesting that inflammation may have a less central role in the progression of fibrosis after the clearance of jaundice.[32] In the present study, the coupling of TGF superfamily cytokines with the expression of $\alpha$-SMA, a marker of extracellular matrix-producing myofibroblast, strengthens their role as active regulators of native liver fibrogenesis after the clearance of jaundice in BA.

Ductal reaction signifies a typical feature in cholangiopathies, where hepatocytes or progenitor cells transdifferentiate into CK-7 positive cell lineages and form immature biliary ductules.[30,37] In BA, the extent of ductular reaction at PE correlates with fibrosis and a worse prognosis.[31] We found that the extension of ductular reaction decreased after PE only in syndromic patients and correlated with fibrosis and the expression of decorin and CTGF at follow-up.

This study had several limitations, such as a relatively small sample size and various sources of control material, because of the rare incidence of BA and obvious limited availability of control liver biopsies. In addition, protein or RNA expression of growth factors does not necessarily reflect their biological activity.[7,26] However, this is one of the first studies to explore the TGF-$\beta$ pathway in BA before the development of liver failure after a successful PE.

In conclusion, our findings support a central role of the TGF-$\beta$ superfamily in driving liver fibrogenesis after a successful PE. The selective decline in TGF-$\beta$1 and CTGF expression and simultaneous upregulation of inhibitory decorin may contribute to unprogressive fibrogenesis after a successful PE, whereas the expression of TGF-$\beta$2 and TFG-$\beta$3 may have an opposite effect. Syndromic patients showed less progressive fibrosis, greater attenuation of ductal reaction, and a lower TGF-$\beta$1 expression than isolated ones, strengthening the possibility of different etiopathogenesis between these two subgroups. These findings may have important clinical implications for future development of antifibrogenic treatment strategies to delay or even prevent the need for LTx.

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