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Relapsing features of bile salt export pump deficiency after liver transplantation in two patients with progressive familial intrahepatic cholestasis type 2

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Background & Aims: PFIC2 is caused by mutations in ABCB11 encoding BSEP. In most cases affected children need liver transplantation that is thought to be curative. We report on two patients who developed recurrent normal GGT cholestasis mimicking primary BSEP disease, after liver transplantation.

Methods: PFIC2 diagnosis was made in infancy in both patients on absence of canalicular BSEP immunodetection and on ABCB11 mutation identification. Liver transplantation was performed at age 9 (patient 1) and 2.8 (patient 2) years without major complications. Cholestasis with normal GGT developed 17 and 4.8 years after liver transplantation, in patient 1 and patient 2, respectively, during an immunosuppression reduction period.

Results: Liver biopsies showed canalicular cholestasis, giant hepatocytes, and slight lobular fibrosis, without evidence of rejection or biliary complications. An increase in immunosuppression resulted in cholestasis resolution in only one patient. Both patients developed atrial fibrillation, and one melanonychia. The newborn of patient 1 developed transient neonatal normal GGT cholestasis. Immunofluorescence staining of normal human liver sections with patient's sera, collected at the time of cholestasis, and using an anti-human IgG antibody to detect serum antibodies, showed reactivity to a canalicular epitope, likely to be BSEP. Indeed, Western blot analysis showed that patient 2 serum recognized rat Bsep.

Conclusions: Allo-immune mediated BSEP dysfunction may occur after liver transplantation in PFIC2 patients leading to a PFIC2 like phenotype. Extrahepatic features and/or offspring transient neonatal cholestasis of possible immune mediated mechanisms, may be associated. Increasing the immunosuppressive regimen might be an effective therapy.

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Introduction

Progressive familial intrahepatic cholestasis type 2 (PFIC2) is a recessive hereditary cholestasis of childhood caused by mutations in the *ABCB11* gene, which encodes the canalicular bile salt export pump (BSEP) only expressed in hepatocytes. PFIC2 is characterized by infancy onset of jaundice with severe pruritus and failure to thrive, persistently normal serum gamma-glutamyl transpeptidase (GGT) activity, elevated serum bile acids, lobular cholestasis with a diffuse giant cell transformation of hepatocytes, absence of canalicular BSEP staining, and profound reduction of biliary secretion of bile salts [1–4]. Progression of liver injury towards end-stage liver disease, lack of an effective medical treatment, and increased risk of hepatic malignancy make liver transplantation necessary for patients affected by the severe form, usually before adolescence [1–3].

So far, liver transplantation (LT) has been considered as curative for patients with PFIC2 [2,5]. Recently, four pediatric patients, successfully transplanted for PFIC2 were reported to develop cholestasis recurrence with the histological and biochemical features of primary BSEP deficiency [6,7]. Patients had no immunodetectable BSEP in their native livers. Anti-BSEP antibodies causing impairment of biliary bile acid secretion were identified in their post-LT sera. Among 20 children transplanted for PFIC2 at the pediatric liver centre of Bicêtre Hospital, two developed episodes of normal GGT cholestasis with features resembling primary BSEP deficiency [2, E. Jacquemin, personal data]. Here we describe their clinical, biochemical, and histological evolution after LT. We provide evidence that these two children who had no immunodetectable BSEP in their native livers,

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likely did not develop tolerance to this protein and have developed an antibody mediated immune response to donor BSEP.

Patients

Patient 1: This girl presented in 1981, at four months of age, with hepatocellular cholestasis, persistently normal serum GGT activity and numerous giant hepatocytes. Intractable pruritus, cirrhosis, and growth failure required a deceased donor LT that was performed at age nine. Diagnosis of PFIC2 was based on negative canalicular BSEP staining on liver tissue, very low concentration of primary bile acids in bile, and identification of biallelic *ABCB11* mutations (Table 1). Immune suppression was induced with prednisone and cyclosporine. No major problems were recorded following LT. Serum liver tests and liver ultrasonography was normal. At 13.5 years old, while undergoing decreased immune suppression, she developed subicterus and pruritus. Serum liver tests were abnormal except for GGT activity (Table 2). Increasing doses of prednisone (0.5 mg/kg/day) and cyclosporine (blood through levels of 200–250 ng/ml) led to disappearance of pruritus and jaundice and to normalization of serum liver tests within 2 months. This episode of normal GGT cholestasis remained unexplained. At age 24 years, she gave birth to a normal male newborn. During a second pregnancy at age 26 years, immune suppression was reduced. At 6th month of pregnancy she presented with normal GGT cholestasis, severe pruritus and jaundice (Table 2). Liver ultrasonography was normal. She was treated with ursodeoxycholic acid (600 mg/day) and cholestyramine (16 g/day) without any improvement and she underwent a premature delivery giving birth to a hypotrophic female newborn. After delivery, a liver biopsy was performed and showed lobular cholestasis with diffuse giant cell transformation of hepatocytes and absence of signs of rejection or of biliary obstruction. Cyclosporine and prednisone doses were increased for 8 months, but cholestasis did not improve. In the meantime her baby developed intrahepatic-cholestasis characterized by normal GGT serum activity, that spontaneously disappeared within 4 months. The patient was listed for a second LT. Intravenous immunoglobulins produced a transitory reduction of total serum bilirubin and bile acids (Table 3). Moreover, she developed melanonychia of hands and feet (Fig. 1). An atrial fibrillation was documented after a malaise and she died due to cardiac arrest 6 h after admission to the hospital (Table 3). Some pre-transplant data concerning patient 1 have been reported elsewhere (PFIC2 No. 25, [2]).

Patient 2: Born from consanguineous parents, this boy developed hepatocellular cholestasis at age 1 month, characterized by persistent serum normal GGT activity and by numerous giant hepatocytes. Unremitting pruritus, cirrhosis, and severe growth failure developed. PFIC2 diagnosis was based on negative BSEP canalicular immunostaining and identification of biallelic *ABCB11* mutations (Table 1). A deceased donor LT was performed at age 2.8 years with cyclosporine and prednisone regimen. An acute rejection was diagnosed three weeks post-LT and it was successfully treated with steroid boluses. Donor liver showed a normal canalicular BSEP staining (data not shown). An unexplained acute episode of normal GGT cholestasis with severe pruritus, discoloration of stools and subicterus, occurred 4.8 years after LT, with decreasing immune-suppression (Table 2). Liver ultrasonography was normal. Liver biopsy showed canalicular cholestasis and giant hepatocytes, with slight centrilobular fibrosis, and absence of signs of rejection or of biliary obstruction. Cyclosporine and prednisone doses were increased and azathioprine was introduced. Cholestasis resolved after three months of therapy (Table 3). Ten years post-LT, liver histology showed fibrosis and

Table 1. Characteristics at liver transplantation of the two patients with recurrent normal GGT cholestasis.

Patient Gender	ABCB11 mutations	Canalicular BSEP staining / Biliary bile acids (N >10 mmol/L)	Age at LT / Indication	Native liver histology	Serum liver tests at LT				
					PT (N >70%)	Total bilirubin (N <17μmol/L)	ALT (N <40 IU/L)	AFP	GGT (N <60 IU/L)
Patient 1 Female	Compound heterozygous c.301delCA p.Q101DfsX8 and c.2944G>A p.G982R with c.1331T>C p.V444A	Negative 0.1 mmol/L	9 years SC P Growth failure	Cirrhosis Hepatocellular cholestasis Giant hepatocytes Portal inflammation	100	36	234	N	17
Patient 2 Male	Homozygous c.77-19T>A leading to abnormal splicing*: p.Y26Ifs7X Homozygous c.1331T>C p.V444A	Negative NA	2.8 years SC P Growth failure	Cirrhosis Hepatocellular cholestasis Giant hepatocytes	76	139	420	2xN	20

SC, severe cholestasis; P, pruritus refractory to medical management; PT, prothrombin time; ALT, alanine aminotransferase; AFP; alpha fetoprotein; GGT, gamma-glutamyl transpeptidase; BSEP, bile salt export pump, LT, liver transplantation; NA, not available; *, in silico test predicts abnormal splicing [2].

Table 2. Clinical course of the two patients with recurrent normal GGT cholestasis after liver transplantation.

Patient	Circumstances and IS at cholestasis attack	First symptom and delay to onset of normal GGT cholestasis* after LT	Serum liver tests during cholestatic flare				
			PT (N >70%)	Total bilirubin (N <17μmol/L)	ALT (N <40 IU/L)	GGT (N <60 IU/L)	Bile acids (N <15 μmol/L)
Patient 1	**Episode n°1$:** Decrease of IS Cyclosporine (blood through levels: 150-200 ng/ml) and prednisone 0.25 mg/kg every other day	Pruritus 40 months post-LT No liver biopsy performed	90	32	160	35	107
	Episode n°2: Decrease of IS since third month of pregnancy Cyclosporine (blood through levels: 50-75 ng/ml) and prednisone 0.1 mg/kg every other day	Pruritus 17 years post-LT during the 6th month of a 2nd pregnancy. Liver biopsy performed after delivery	85	620	600	45	352
Patient 2	Decrease of IS Cyclosporine (blood through levels: 150-200 ng/ml) and prednisone 0.2 mg/kg every other day	Pruritus 4.8 years post-LT Liver biopsy performed 4.8 years post-LT	100	32	31	14	298

IS, immunosuppression regimen; PT, prothrombin time; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; LT, liver transplantation; *, cholestasis without rejection, infection, or biliary or vascular complications; NA, not available; $, Evolution of episode n°1 after adaptation of IS is described in the case report of patient 1.

Table 3. Evolution after recurrence of normal GGT cholestasis* on liver graft.

Patient	Adaptation of IS	Serum liver tests			Symptoms	Last follow-up
		PT (N >70%)	Total bilirubin (N <17μmol/L)	Bile acids (N <15 μmol/L)		
Patient 1	**Episode n°2:** Increase of cyclosporine dose (blood through levels: 200-250 ng/ml) and of prednisone 0.3 mg/kg every day Intravenous immunoglobulins: one injection of 1 g/kg	93	513	150	Unremitting P SC Hard hepatomegaly Hyperkeratosis of hands and feets Melanonychia	**At age 28 years:** SC with persistence of abnormal liver tests. Abrupt malaise. Atrial fibrillation. Cardiac arrest and death, while awaiting for a 2nd LT **Offspring:** Transient neonatal cholestasis with normal serum GGT and spontaneous resolution within 4 months
Patient 2	Increase of cyclosporine dose (blood through levels: 250-300 ng/ml) and of prednisone 0.5 mg/kg every day Azathioprine started: 1.2 mg/kg every day	100	1	5	Disappearance of pruritus without recurrence	**Ten years post-LT:** • Liver histology: no rejection but slight portal and lobular fibrosis with giant hepatocytes • Unexplained polyclonal hyper gamma-globulinemia (18.5 g/L) • Non organ specific autoantibodies: negative Alive at age 22 years with normal clinical exam and serum liver tests. Atrial fibrillation of unknown cause

IS, immunosuppression regimen; PT, prothrombin time; GGT, gamma-glutamyl transferase; LT, liver transplantation; *, cholestasis without rejection, infection, or biliary or vascular complications; SC, severe cholestasis; P, pruritus refractory to medical management.

persistence of numerous giant hepatocytes. Prednisone was stopped 17 years after LT. At age 22, the patient is alive with normal growth and sexual maturation, receiving azathioprine (0.8 mg/kg/day) and cyclosporine (1.3 mg/kg/day; blood through levels: 25–50 ng/ml). Serum liver tests and renal function are normal. Cholestasis did not recur. The patient developed an atrial fibrillation of unknown cause (Table 3).

Methods

ABCB11 sequence analysis, bile analysis

Sequence analysis was performed as described [2]. Mutation screening was done using PCR amplification and DNA sequencing of coding exons 2–28 and all splice junctions of ABCB11 (RefSeq NM_003742.2). Combined in silico splice tools were

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Fig. 1. Melanonychia involving nails of hands and feet that developed in patient 1 after liver transplantation.

used to test the potential effect on pre-mRNA splicing of the homozygous intronic variation found in patient 2 [2]. Biliary concentration of bile acids was measured using standard procedures [2].

Liver histology and immunohistochemistry

Native and post-transplant liver samples were studied in both patients. A polyclonal anti-BSEP and a monoclonal anti-MDR3 (Sigma–Aldrich) antibody were used as described on paraffin-embedded sections [2,3,8].

Immunofluorescence analysis

Indirect immunofluorescence staining was performed as described [7]. Post-transplant serum samples from the two patients obtained during cholestatic episodes were screened at dilutions ranging from undiluted to 1:500 with the use of normal human liver sections. For the shown experiments, patient sera were used diluted at 1:25. Bound antibodies were detected using fluorescein isothiocyanate conjugated rabbit anti-human IgG, IgA, and IgM antibodies (dilution: 1:100, DakoCytomation). Sera from two patients transplanted for biliary atresia and obtained 10 years after LT were used similarly, and served as a control.

Western blot analysis

Rat bile salt export pump (Bsep)-GFP-transfected HEK293 cells were used for Western blotting as previously described [2,6,8]. Serum of patient 2 (dilution: 1:50) was available and detected with a peroxidase-conjugated anti-human-

IgG-specific antibody (1:2000). Control sera were used similarly. Bsep-GFP was detected using anti-green fluorescent protein (GFP) antibody (dilution: 1:400, Roche, France).

Results

Native liver histology, native and post-LT liver immunohistochemistry, and immunofluorescence analyses

Native liver histology and immunohistochemistry studies showed a typical PFIC2 pattern, as shown in patient 2 (Fig. 2A and B) [2]. Post-transplant liver histology revealed a PFIC2 pattern, as shown in patient 1 (Fig. 2C). Canalicular BSEP immunostaining performed 17.3 years post-LT, during a bout of normal GGT cholestasis, was negative in patient 1 (Fig. 2D). In patient 2, canalicular BSEP immunostaining was faintly positive during a bout of normal GGT cholestasis, occurring 4.8 years post-LT (Fig. 2E). In both patients, canalicular MDR3 immunostaining used as a control was positive. In patient 2, giant hepatocytes were observed 10 years post-LT as well as fibrosis (Fig. 2F).

Immunofluorescence staining of normal human liver sections incubated with the serum of each patient collected at the time of the cholestasis bout, revealed, using an anti-human IgG antibody, clear staining of bile canaliculi (Fig. 2G and H). No staining was seen using anti-human IgA and IgM antibodies (data not shown). When normal liver sections were incubated with control sera, no canalicular staining was seen (Fig. 2G).

Western blot analysis

To test the hypothesis that antibodies reacting with epitopes in bile canaliculi were directed against BSEP, a serum sample from patient 2 was analyzed by immunoblotting using proteins from cells expressing Bsep. The anti-GFP antibody immunoreacted with a protein of approximately 180 kDa in Bsep-GFP-transfected cells. Patient serum reacted with a protein of the same molecular mass in Bsep-GFP-transfected cells but not in control GFP-transfected cells, revealing Bsep as the antibody target (Fig. 3) [6,7]. Control sera did not recognize Bsep (data not shown).

Discussion

PFIC2 is a common indication for pediatric LT. This procedure is thought to cure the genetic disease and recurrent disease related to immunization of the recipient against donor BSEP protein, but has, until very recently, not been considered [1,2,5–7]. This study reports the clinical course and the long term evolution of two patients, successfully transplanted for PFIC2, and who developed after LT, episodes of normal GGT cholestasis with features suggesting recurrence of BSEP dysfunction. Thus, our report reinforces the idea that recurrence of BSEP dysfunction after LT, secondary to an allo-immunization process in a patient immunologically "naive" for BSEP, may represent a novel clinico-pathological entity more than a rare and sporadic phenomenon. The chronology of this condition is variable with a wide range of time between liver transplantation and the development of cholestasis. The severity spectrum ranges from mild cases that can be reversed by increasing the immunosuppression regimen as in patient 2 and in the patients reported by Jara et al. [7], to severe episodes with very limited or no response to medical therapy,
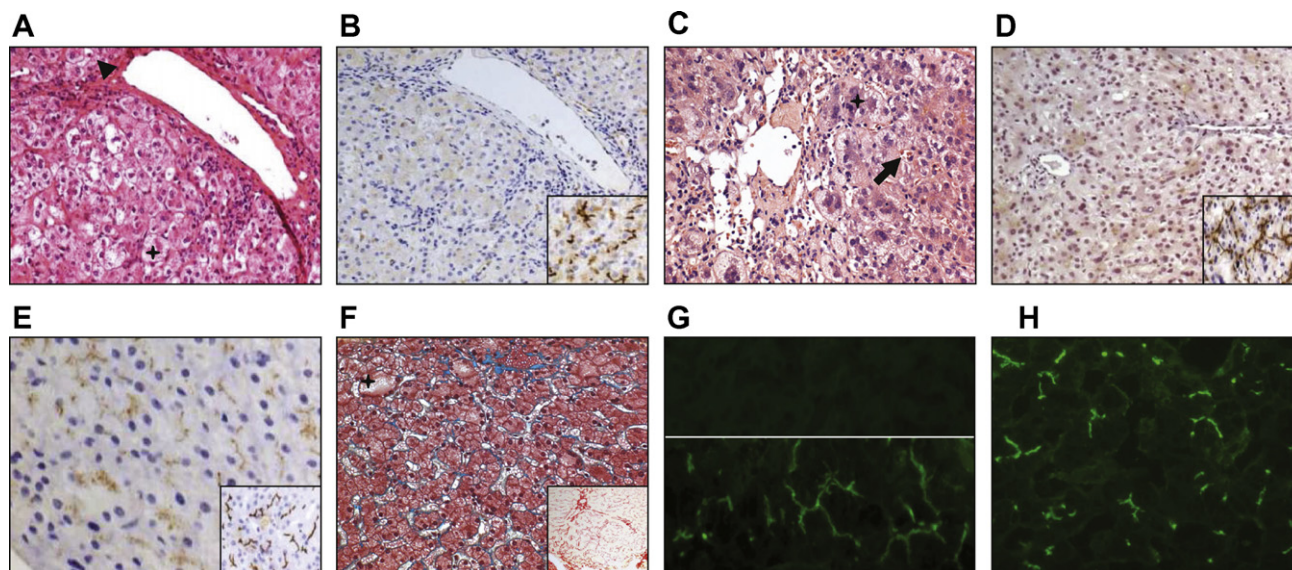
Fig. 2. Initial and/or post-transplantation liver histology in patients 1 and 2, transplanted for PFIC2 (A, B, E, F, and H: patient 2. C, D, and G: patient 1). (A) Native liver histology (HE stain) showing numerous giant hepatocytes (star), canalicular cholestasis (arrowhead), inflammation and septal fibrosis. (B) Negative immunohistochemical detection of canalicular BSEP in native liver. Note positive canalicular MDR3 immunostaining (Inset). (C) Liver histology (HE stain) 17.3 years post-transplantation showing numerous giant hepatocytes (star) and canalicular cholestasis (arrow). (D) Negative immunohistochemical detection of canalicular BSEP 17.3 years post-transplantation. Note positive canalicular MDR3 immunostaining (Inset). (E) Faint immunohistochemical detection of canalicular BSEP 4.8 years post-transplantation. Note positive canalicular MDR3 immunostaining (Inset). (F) Trichrome stain, and Sirius red stain (Inset) 10 years post-transplantation showing thin septal, perisinusoidal and centrilobular fibrosis and numerous giant hepatocytes (star). (G) Patient 1 serum collected 17.3 years post-transplantation was used for immunofluorescent staining of normal human liver. Antibodies within the serum sample were detected by an anti-human IgG antibody (lower part). Serum contained antibodies reactive to a canalicular epitope. A serum collected 10 years post-transplantation from a liver patient transplanted for biliary atresia was used as control and was not reactive to canaliculus (upper part). (H) Patient 2 serum collected 4.8 years post-transplantation was used for immunofluorescent staining of normal human liver as indicated in G. Serum contained antibodies reactive to a canalicular epitope. Original magnification: A, B, C, D, and F: 125×; E and H: 250×; G: 400×.
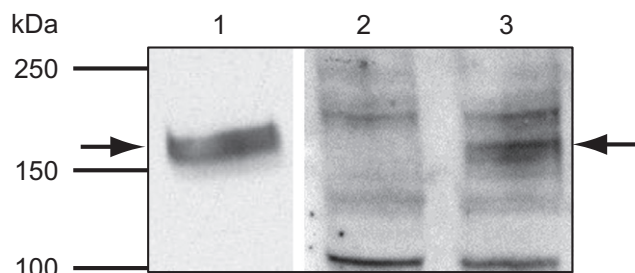
Fig. 3. Western blot showing reactivity of serum from patient 2 to rat bile salt export pump (Bsep). Lanes 1 and 3: Bsep-GFP-transfected HEK293 cells; lane 2: control GFP-transfected HEK293 cells. Protein lysates were incubated with anti-GFP antibody (lane 1), and patient 2 serum (lanes 2 and 3). Bsep (arrow, 180 kDa) could be detected with anti-GFP antibody (lane 1) or patient 2 serum (lane 3) in Bsep-GFP-HEK293 transfected cells, but not in GFP-HEK293 transfected cells (lane 2).

requiring liver retransplantation, as in patient 1 and in the patient reported by Keitel et al. [6]. Allo-immune induced BSEP dysfunction may be underdiagnosed in the post-transplant course because of its rapid resolution by increasing the immunosuppression regimen.

In the four previously reported patients, presence of anti-BSEP IgG antibody, suggested an allo-immune reaction of the recipient directed against BSEP of the donor liver [6,7]. It is assumed that these IgG allo-antibodies are internalized by hepatocytes, transported throughout the cell and reach the canaliculus membrane where they exert their blocking effect on BSEP [7,9–11]. The

mechanisms that lead to allo-immune reaction are not precisely known and one can only hypothesize. Allo-immune reaction implies a loss of tolerance by the host of the graft towards wild type BSEP [6,7,12]. This may occur if an antigenic part of BSEP is exposed to blood lymphocytes, under post-transplant circumstances such as hepatocyte necrosis due to infection or rejection, BSEP misrouting to the basal membrane of the hepatocytes, or regurgitation from bile to blood through increased paracellular permeability due to biliary obstruction [6,13,14]. Whether pregnancy may play a role in the loss of tolerance is not known. When performed, BSEP immunostaining showed no canalicular BSEP in mostly all native livers of PFIC2 patients [2–4]. In some cases (i.e. premature stop codon), BSEP is probably not expressed at all, explaining the lack of tolerance and the development of anti-BSEP antibodies following LT. In other instances (i.e. missense mutation), BSEP is retained in the endoplasmic reticulum, and peptides resulting from its degradation might be presented to immune cells and induce tolerance [15]. This might explain why disease recurrence after LT is not observed in all PFIC2 patients. Considering the genotypes of the 2 patients we report on, no or little protein expression was expected in their native livers [2,3,6,15]. Conditions favoring or triggering allo-immunization in patients transplanted for PFIC2 are unknown, but in our two patients as well as in two of the previously reported patients, reduction of immunosuppression was noted [7].

In both our patients, sera collected during the cholestatic episodes were tested against normal human liver tissue by immunofluorescence, and revealed an IgG specific canalicular reactivity. This suggests the presence of an antibody of IgG class specifically reacting against an epitope in bile canaliculus of normal liver

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[6,7]. Canalicular BSEP immunostaining of the transplanted liver of each patient, using an anti-BSEP antibody, was negative or faint. It is likely that allo-anti-BSEP IgG antibodies produced by the patient partially or completely mask the BSEP epitope recognized by anti-BSEP antibody. Western blot analysis showed strong evidence for BSEP being the target of allo-antibodies. Indeed, Bsep was recognized by patient serum and BSEP is highly identical to its rat ortholog [7,8]. In addition, the clinical and biochemical picture of a normal GGT cholestasis associated with features akin to PFIC2, together with absence of histological evidence of the most common causes of cholestasis in liver-transplanted patients, such as rejection or biliary complications, strongly support an allo-immune induced BSEP dysfunction in our patients. The presence of associated extra-hepatic phenomena, not yet reported in this condition, further supports the immune-mediated nature of this entity. Indeed, atrial-fibrillation may be caused by an immune-mediated mechanism [16], and melanonychia is known to be associated with immune-mediated disorders [17]. Interestingly, the baby from patient 1 developed neonatal normal GGT cholestasis that spontaneously resolved within 4 months. *ABCB11* heterozygous status has been found to favour transient neonatal cholestasis, characterized by slightly elevated serum GGT activity [18]. In this baby, likely harbouring a *ABCB11* mutated allele transmitted from his mother, transplacental transfer of anti-BSEP antibodies might have led to transient blockage of the function of the wild type BSEP encoded by the normal allele, and favored transient neonatal cholestasis with normal serum GGT activity.

These data confirm that allo-immune BSEP dysfunction may occur after LT in PFIC2 patients with no immunodetectable BSEP in their native livers. It may develop even many years after an apparently well-controlled transplantation. Allo-antibodies generated against BSEP of donor liver may inhibit BSEP function and cause cholestasis with PFIC2 like phenotype. It may be treatable or not by increasing immunosuppression and can lead to graft failure requiring re-transplantation. Extrahepatic features and/or offspring transient-neonatal normal GGT cholestasis of possible immune mediated mechanisms, can be associated. Liver transplanted PFIC2 patients should be carefully monitored for the appearance of anti-BSEP antibodies. An immunosuppression regimen increase before normal GGT cholestasis onset might avoid insidious progression of fibrosis in the liver graft. Prospective strategies should be established to prevent "disease recurrence" in high-risk patients.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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