Cell-based BSEP trans-inhibition: A novel, non-invasive test for diagnosis of antibody-induced BSEP deficiency

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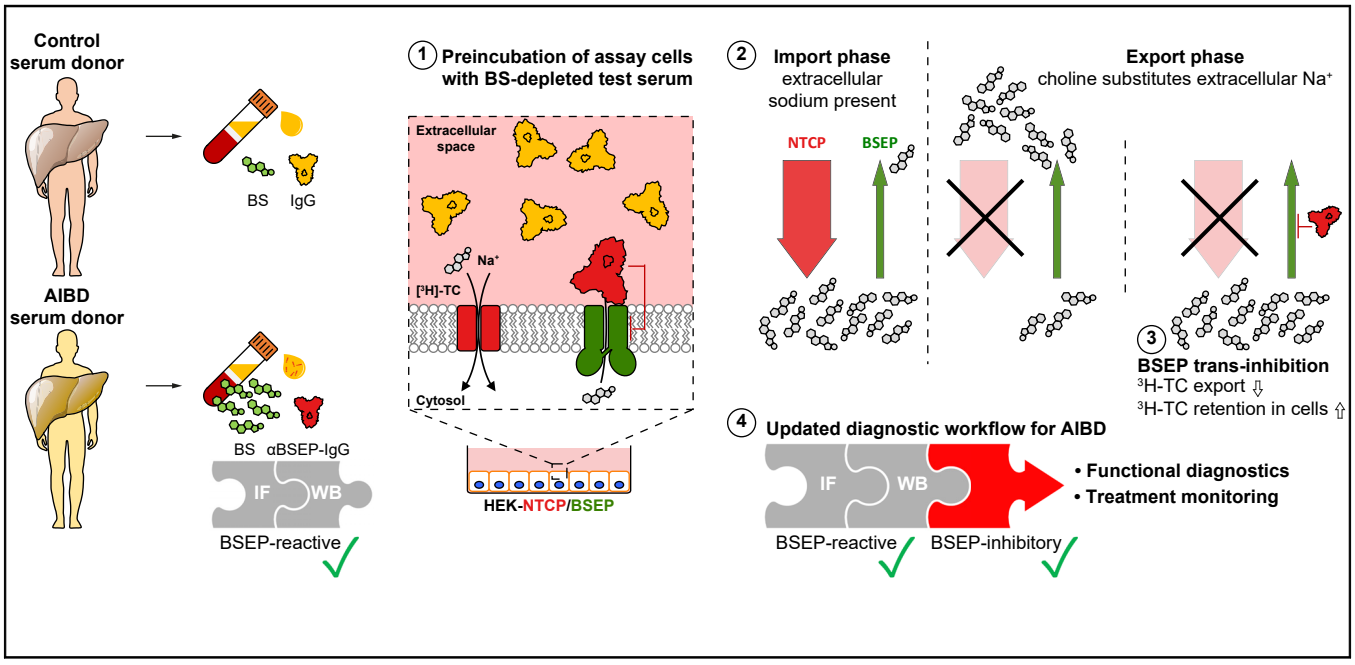
Graphical abstract



Highlights

- Development of a functional diagnostic assay for antibody-induced BSEP deficiency (AIBD).
- Confirmation of BSEP inhibition by serum antibodies corroborates AIBD diagnosis.
- Updated diagnostic workflow for AIBD confirming presence of inhibitory anti-BSEP antibodies.
- Monitoring of onset and treatment response of AIBD using this functional test.

Impact and Implications

Antibody-induced BSEP deficiency (AIBD) is a potentially serious complication that may affect patients with PFIC-2 after liver transplantation. To improve its early diagnosis and thus immediate treatment, we developed a novel functional assay to confirm AIBD diagnosis using a patient's serum and propose an updated diagnostic algorithm for AIBD.

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Research article

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Cell-based BSEP trans-inhibition: A novel, non-invasive test for diagnosis of antibody-induced BSEP deficiency

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Background & Aims: Antibody-induced bile salt export pump deficiency (AIBD) is an acquired form of intrahepatic cholestasis, which may develop following orthotopic liver transplantation (OLT) for progressive familial intrahepatic cholestasis type 2 (PFIC-2). Approximately 8–33% of patients with PFIC-2 who underwent a transplant develop bile salt export pump (BSEP) antibodies, which trans-inhibit this bile salt transporter from the extracellular, biliary side. AIBD is diagnosed by demonstration of BSEP-reactive and BSEP-inhibitory antibodies in patient serum. We developed a cell-based test directly measuring BSEP trans-inhibition by antibodies in serum samples to confirm AIBD diagnosis.
Methods: Sera from healthy controls and cholestatic non-AIBD or AIBD cases were tested (1) for anticanalicular reactivity by immunofluorescence staining of human liver cryosections, (2) for anti-BSEP reactivity by immunofluorescence staining of human embryonic kidney 293 (HEK293) cells expressing BSEP-enhanced yellow fluorescent protein (EYFP) and immunodetection of BSEP-EYFP on Western blot, and (3) for BSEP trans-inhibition using HEK293 cells stably expressing Na^+/taurocholate cotransporting polypeptide (NTCP)-mCherry and BSEP-EYFP. The trans-inhibition test uses [^{3}H]-taurocholate as substrate and is divided into an uptake phase dominated by NTCP followed by BSEP-mediated export. For functional analysis, sera were bile salt depleted.
Results: We found BSEP trans-inhibition by seven sera containing anti-BSEP antibodies, but not by five cholestatic or nine control sera, all lacking BSEP reactivity. Prospective screening of a patient with PFIC-2 post OLT showed seroconversion to AIBD, and the novel test method allowed monitoring of treatment response. Notably, we identified a patient with PFIC-2 post OLT with anti-BSEP antibodies yet without BSEP trans-inhibition activity, in line with asymptomatic presentation at serum sampling.
Conclusions: Our cell-based assay is the first direct functional test for AIBD and allows confirmation of diagnosis as well as monitoring under therapy. We propose an updated workflow for AIBD diagnosis including this functional assay.
Impact and Implications: Antibody-induced BSEP deficiency (AIBD) is a potentially serious complication that may affect patients with PFIC-2 after liver transplantation. To improve its early diagnosis and thus immediate treatment, we developed a

Keywords: AIBD; Progressive familial intrahepatic cholestasis type 2; Non-invasive diagnostic test; Anti-BSEP antibody; Liver transplantation.
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novel functional assay to confirm AIBD diagnosis using a patient's serum and propose an updated diagnostic algorithm for AIBD.

Introduction

Severe bile salt export pump (BSEP) deficiency, also termed progressive familial intrahepatic cholestasis type 2 (PFIC-2), is caused by inherited pathogenic variants in the *ABCB11* (ATP binding cassette transporter superfamily, subfamily B, member 11) gene encoding the canalicular BSEP.[1,2] BSEP is an ATP binding cassette (ABC) transporter exclusively expressed in hepatocytes, which uses the energy obtained from ATP hydrolysis to export bile salts (BS) against their concentration gradient from hepatocytes into the canalicular lumen.[3] *ABCB11* variants resulting in reduced or absent functional BSEP expression at the canalicular membrane lead to retention of BS in the hepatocyte (Fig. 1A, left panel).[1,2,4–6] Patients with PFIC-2 commonly present within the first 6 months of life with failure to thrive, jaundice, and pruritus.[2,4,7] Serum BS levels are drastically increased, and the overall clinical presentation is that of a low gamma-glutamyl transferase (gGT) intrahepatic cholestasis.[1,6,8] Left untreated, PFIC-2 may be associated by severe pruritus and progress to liver cirrhosis and hepatocellular carcinoma, necessitating orthotopic liver transplantation (OLT) of a BSEP-competent donor organ, which restores hepatic BS excretion and enterohepatic BS circulation.[6,9,10]

In 2009, we and others described a novel, acquired form of intrahepatic cholestasis, which results from development of an immunoglobulin gamma (IgG) anti-BSEP response after successful OLT for PFIC-2 and was named antibody-induced BSEP deficiency (AIBD) (Fig. 1A, right panel).[11–13] Patients with PFIC-2 without any residual BSEP expression in their native liver are at increased risk of developing AIBD, as they congenitally lack immunological BSEP tolerance.[13] The clinical presentation of AIBD mimics PFIC-2 and is caused by antibody-mediated BSEP inhibition from the extracellular side in the canalicular lumen (trans-inhibition; Fig. 1A).[11–13] In the majority of reported cases, this phenotypic disease recurrence was at least temporarily manageable by intensified immunosuppressive therapy, antibody removal via immunoadsorption or plasmapheresis, and/or depletion of antibody-producing B cells by rituximab treatment.[14–16] Notably, a patient with AIBD refractory to these therapeutic approaches was cured by allogeneic haematopoietic stem cell transplantation as an individualised therapeutic salvage approach.[17]

Current diagnostic workup of AIBD is a multistep process[16] based on (1) post-OLT recurrence of a low-gGT intrahepatic cholestasis phenotype in patients with PFIC-2 accompanied by (2) the presence of anticanalicular antibodies in the patient's serum, (3) which are directed against BSEP.[13,15,16] Although canalicular antibody deposits in affected transplants are highly characteristic of AIBD,[12,13] their detection requires a liver biopsy for immunostaining and is thus not regularly used for diagnosis. We could previously demonstrate that preincubation of BSEP-containing inside-out vesicles (IOVs) with purified IgG from the sera of patients with AIBD resulted in BSEP transport inhibition.[13,15] Because the intravesicular side (corresponding to the biliary side) of the IOV membrane is not directly accessible to the antibodies, IgG need to be included into the IOVs in a time-consuming freeze–thaw procedure[18] before assaying BS transport, which in turn requires special vacuum filtration equipment.[3,16,19] To facilitate a more direct functional readout, we developed a simplified, cell-based assay that permits direct measurement of BSEP trans-inhibition by serum antibodies, the pathophysiological cause of AIBD.[11,13]

Materials and methods

Additional method descriptions can be found in the Supplementary information.

Patient material

This study was performed according to the guidelines of the Declaration of Helsinki. All serum donors gave written consent for the investigation (approved by the local ethics committee, study number 5350). AIBD sera were obtained from patients for diagnostic workup, of which seven were included in this study (one female and six male). Sera of nine healthy individuals (five female and four male) and of five patients presenting with cholestasis and increased serum BS but lacking anti-BSEP antibodies (non-AIBD; three female and two male) were used as reference samples throughout this study. All sera were stored at -80 °C. The non-tumorous liver metastasis resection margin of a patient with colorectal cancer was used as control liver tissue for detecting anticanalicular reactivity in serum samples.

Generation of stable HEK293 cell lines expressing human NTCP and BSEP

Preparation of lentiviral particles and subsequent transduction were carried out as described.[13] Briefly, lentiviral particles were generated in human embryonic kidney 293 (HEK293) T cells using either puc2CL6EGIP-Na^+/taurocholate cotransporting polypeptide (NTCP)-mCherry or -BSEP-enhanced yellow fluorescent protein (EYFP) vector plasmid and separately used to transduce HEK293 cells (Fig. S1A). After expansion, cells were sorted by EYFP-(BSEP) or mCherry-(NTCP) fluorescence on a FACSAria III cell sorter (Becton Dickinson, Heidelberg, Germany), followed by clonal separation by dilution into hybridoma dishes. Suitable clones for both NTCP-mCherry and BSEP-EYFP were picked, and activity of both transporters was confirmed by cellular [^{3}H]-taurocholate (TC) uptake for NTCP-mCherry (see Results) and a vesicular transport assay for BSEP-EYFP (Fig. S1B and C). Finally, the monoclonal HEK293-BSEP-EYFP cell line was transduced with NTCP-mCherry before sorting and subsequent clonal isolation in hybridoma dishes.

BS depletion of serum samples and BS analysis

All steps were carried out at room temperature. In total, 200 µl serum was diluted 10-fold with PBS before concentration back to 200 µl in a spin concentrator with a molecular weight cut-off of 10 kDa (Amicon Ultra-2 ml, Merck, Darmstadt, Germany). This dilution/concentration was repeated four times. After the last concentration, the volume was adjusted to exactly 200 µl with PBS. Extraction and analysis of BS levels in original and BS-depleted samples was carried out by liquid chromatography-tandem mass spectrometry as described.[20]

Purification of IgG from serum samples

Total IgG was purified from serum as previously described.[13] Peak fractions were pooled and buffer-exchanged to PBS to the volume of the original serum sample using 10-kDa cut-off spin concentrators. NTCP/BSEP cells were preincubated with comparable amounts of total IgG as described below (~325 μg per well).

Cell-based BSEP trans-inhibition test

NTCP/BSEP cells were kept under puromycin selection and were passaged every 4–5 days in high-glucose DMEM (Biochrom, Berlin, Germany). At near confluence, cells were trypsinised and resuspended in 10 ml medium (per 75 cm^2 culture surface), diluted twofold in medium, and seeded onto poly-L-lysine (PLL; Sigma-Aldrich, Heidelberg, Germany)-coated 12-well plates at 1 ml per well. After overnight cultivation, the medium on the confluent monolayer was changed to 470 μl of 25 mM HEPES/TRIS (pH 7.4)-buffered low-glucose DMEM. Thirty minutes later, 25 μl of BS-depleted sera, purified IgG, or PBS was added per well, and plates were incubated for 1 h. For complement inactivation, BS-depleted sera were incubated for 30 min at 56 °C before being assayed. All samples were assayed in triplicates. Transport was started by addition of 5 μl [^{3}H]-TC (freshly diluted into ddH_2O; final concentration, 20 nM per well). Plates were kept at 37 °C for 3 min, after which supernatants (supernatant 1 [S1]) were transferred to reaction tubes. Cells were washed three times with 500 μl ice-cold PBS. After addition of 500 μl prewarmed export buffer (20 mM HEPES/TRIS pH 7.4, 1.8 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM KCl, and 144 mM choline chloride), plates were kept at 37 °C for 3 min and put on ice, and supernatants (supernatant 2 [S2]) were transferred to fresh reaction tubes. Cells were washed as above and lysed in 500 μl PBS with 0.5% (w/v) SDS and 1 μl/ml Benzonase (Merck, Darmstadt, Germany) to reduce sample viscosity. Each well yields three samples: S1 (import phase), S2 (export phase), and cell lysate L (intracellular BS retention). Afterwards, 400 μl of each sample was mixed with 4 ml of liquid scintillation cocktail (Ultima Gold™, BD Biosciences, Heidelberg, Germany) by vigorous vortexing for 20 s and then measured on a liquid scintillation counter (Packard Instruments, Frankfurt, Germany). The sum of averaged counts per minute (cpm) for S1, S2, and L (triplicates) was set to 100% for the HEK293 cell line in Fig. 1E to visualise the distribution of total radioactivity between S1, S2, and L during the test. All other cpm data for transduced cell lines was transformed into percent of HEK293 cells. All serum-based BSEP trans-inhibition test values are expressed in percent of cpm (S1 + S2 + L) of the respective no-serum measurements. All graphs were prepared using GraphPad Prism 5.0a (GraphPad Software, San Diego, CA, USA). S2 and L data from sera AIBD7-7.3 and from the no-serum controls were used to calculate the thresholds (L/S2 as mean ± SD) of maximum (3.28 ± 0.34) and no inhibition (0.36 ± 0.12), respectively, which are shown in Fig. 2C and E.

Immunofluorescence staining of liver cryosections

Immunostaining of liver cryosections was carried out as previously described.[13] Briefly, methanol-fixed cryosections of non-cholestatic human liver were immunostained with human serum samples at 1:100 for anticanalicular reactivity and a murine monoclonal antibody against multidrug resistance-associated protein 2 (MRP2; M2I-4; Alexis, Grünberg, Germany) as a canalicular marker (1:25). Goat antihuman-IgG-Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and goat antimouse-IgG-Alexa Fluor 488 (Invitrogen, Karlsruhe, Germany) served as secondary antibodies (both at 1:500). All images were acquired via the ZEN software (black edition, Ver.2.3 SP1) on an LSM 880 confocal laser scanning microscope (Zeiss, Jena, Germany) using Immersol 518 F (Zeiss) oil on a Zeiss Plan-APOCHROMAT 63 × Oil/DIC objective at room temperature.

Immunofluorescence staining of HEK293 cells

For staining with sera, empty HEK293 and BSEP cells were trypsinised and mixed at a ratio of 1:5 before seeding onto coverslips in 12-well plates and overnight cultivation. Cells on coverslips were washed with PBS, fixed with ice-cold methanol for 30 s, and blocked for 30 min (UltraVision Block, Thermo, Germany). Cells were stained with patient sera (1:50) for 1 h followed by goat antihuman-IgG-Cy3 (1:500) and DAPI (1:20,000) for 1 h. Coverslips were mounted on microscopic slides using Dako fluorescence mounting medium (Dako, Waldbronn, Germany).

Live cell immunofluorescence staining

NTCP/BSEP cells were seeded into PLL-coated IBIDI dishes (ibiTreat μ-dish 35 mm, high, ibidi, Gräfelfing, Germany) and incubated overnight to reach confluency. The medium was changed to 25 mM HEPES/TRIS-buffered low-glucose DMEM without puromycin. After 30 min, the medium was aspirated, and the edge around the observation area in the dish was carefully dried with a cleaning tissue. The cell layer was then covered with 25 μl of BS-depleted serum or PBS in 500 μl buffered medium to conserve serum (leaving out the dried edge), and dishes were incubated for 1 h. Dishes were put on ice, the medium was aspirated, and the cell layer was gently washed three times with ice-cold PBS containing Ca^{2+} and Mg^{2+} before fixation for 30 s with ice-cold methanol. Cells were washed twice with PBS, blocked for 30 min (UltraVision Block), and stained for 1 h either for human IgG (1:500; goat antihuman-IgG (H + L) Alexa Fluor 647 Fab fragment, Jackson ImmunoResearch Laboratories) or for Na^+/K^+-ATPase (1:200; clone M7-PB-E9, Sigma-Aldrich) followed by goat antimouse-IgG1 Alexa Fluor 647 IgG for 1 h (1:500; Jackson ImmunoResearch Laboratories). All secondary antibody dilutions contained DAPI at 1:20,000. Cells were gently washed three times with PBS and once with H_2O before mounted with an 18-mm round coverslip using Dako mounting medium.

Western blot analysis of patient sera

Membrane vesicle preparations (10 μg protein per lane) from HEK293 and BSEP-EYFP cells were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Blot membranes were cut into segments and blocked for 1 h in TBS-T containing 10% (w/v) non-fat dried milk. Segments were probed with human serum samples (1:1,000 in block) for 1 h followed by goat antihuman IgG-HRP conjugate (1:10,000 in TBS-T) for 1 h before detection using Western Lightning Plus chemiluminescent substrate (PerkinElmer, Rodgau, Germany) in a ChemiDoc MP Imaging System (Biorad, Feldkirchen, Germany).

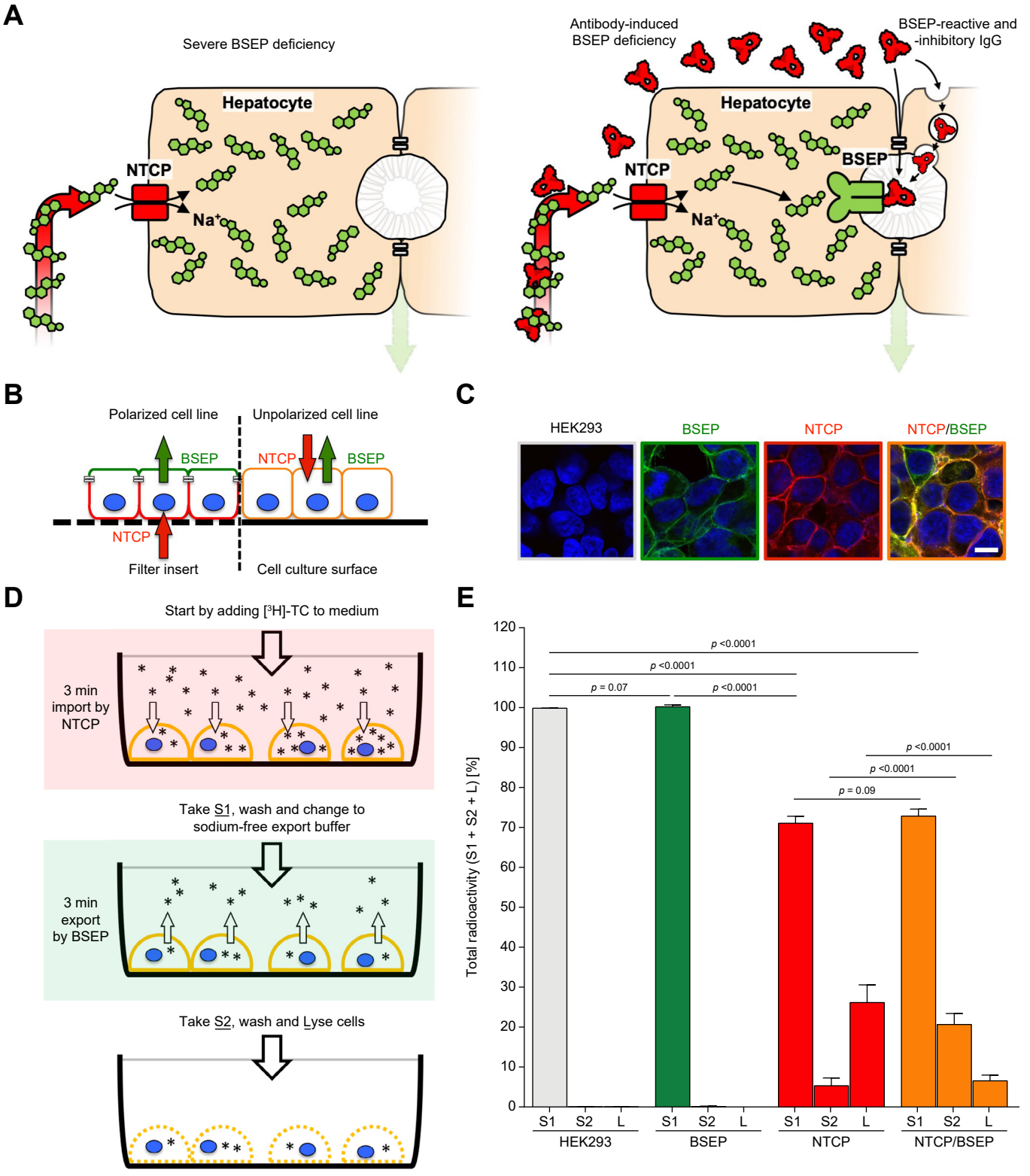

Fig. 1. Development of a cell-based BSEP trans-inhibition assay for AIBD diagnosis. (A) (left) Maintaining the enterohepatic circulation of BS is an essential function of the liver. Severe BSEP deficiency (PFIC-2) is caused by reduced or absent BSEP expression and results in disruption of the enterohepatic BS circulation and accumulation of BS within hepatocytes and the circulation (right) Functional BSEP expression in the transplant restores enterohepatic BS circulation. Some patients, however, show a recurrence of symptoms caused by development of BSEP-inhibitory antibodies, termed 'antibody-induced BSEP deficiency' (AIBD. After entering the canalicular space either by a paracellular route or by transcytosis, they may bind and can trans-inhibit BSEP. (B) As vectorial BS transport is not necessary to recapitulate BSEP trans-inhibition, unpolarised cells may be used as long as BSEP activity can be separated from NTCP activity. (C) HEK293 cells were transduced with recombinant lentiviral vectors for constitutive expression of either human NTCP-mCherry, BSEP-EYFP, or both transporters (also see Fig. S1A). Confocal microscopy images of the established cell lines. Nuclei were stained with DAPI. Bar = 10 μm. (D) Assay principle. Cells are incubated with [^{3}H]-TC during

Results

Development of a cell-based BSEP trans-inhibition assay for AIBD diagnosis

To confirm AIBD diagnosis, a suitable transport inhibition assay needs to recapitulate BSEP trans-inhibition. This could be achieved either by use of a polarised cell line allowing spatial separation of NTCP-mediated uptake and BSEP-mediated export (Fig. 1B) or by use of an assay strategy that allows temporal separation of NTCP from BSEP activity. The latter was achieved by transduction of HEK293 cells with recombinant lentiviral vectors, allowing constitutive expression of both human NTCP-mCherry and BSEP-EYFP (Fig. 1C and Fig. S1A). Cells transduced with either transporter alone served as references [^{3}H]-TC served as a model BS substrate for both transporters. First, cells were incubated with 20 nM [^{3}H]-TC for 3 min (Fig. 1D). After incubation, remaining radioactivity in supernatant S1 was measured by liquid scintillation counting. Lacking any endogenous BS uptake system, both HEK293 (grey in Fig. 1E) and BSEP cells (green) failed to show any [^{3}H]-TC import from the supernatant into the cytosol during the first incubation step. Thus, all [^{3}H]-TC remained in S1 (Fig. 1E). In contrast, NTCP cells (red) showed significant [^{3}H]-TC import as indicated by reduced radioactivity in S1. In the double-transduced NTCP/BSEP cell line (orange) [^{3}H]-TC was detectable in S1 to similar levels as observed for the NTCP cells (Fig. 1E), demonstrating a higher capacity for NTCP import than for BSEP export. This both indicates successful [^{3}H]-TC loading of cells via NTCP and necessitates subsequent NTCP inactivation to measure BSEP export. Accordingly, after the first incubation phase cells were washed and incubated in export buffer containing choline instead of sodium. As TC uptake by NTCP depends on sodium symport, removal of extracellular sodium renders NTCP inactive. After 3-min export, the second supernatant (S2) was obtained, and cells were washed and lysed (L). Without [^{3}H]-TC import into HEK293 and BSEP cells, there was no detectable radioactivity in the supernatant after the BSEP export phase (S2) or in their cell lysate (L) (Fig. 1E). In contrast, NTCP cells showed only minimal amounts in S2, indicating lack of endogenous [^{3}H]-TC export and thus substantial intracellular [^{3}H]-TC accumulation (L). In NTCP/BSEP cells, the amount of [^{3}H]-TC was significantly higher in S2 and significantly lower in L than that in NTCP cells. This demonstrates a significant, BSEP-mediated [^{3}H]-TC export in the NTCP/BSEP cells. Taken together, redistribution of [^{3}H]-TC between S1, S2, und L during both incubation (*i.e.* import and export) phases clearly demonstrated NTCP-mediated import followed by BSEP-mediated export in the NTCP/BSEP cells.

Validation of the BSEP trans-inhibition assay using serum samples from different cohorts

To validate the assay, we used serum samples from healthy controls without cholestasis (n = 9), from patients with cholestasis but without the presence of anti-BSEP antibodies (non-AIBD, n = 5), and from patients diagnosed with AIBD (n = 7) (Fig. 3). AIBD cases were identified by (1) detection of anticanalicular antibodies in the patient's serum by immunofluorescence (IF) staining on cryosections of normal human liver tissue (Fig. S2A); (2) demonstration of anti-BSEP reactivity by IF staining of BSEP-EYFP-expressing HEK293 cells (Fig. S2B); and (III) Western blot detection of BSEP in plasma membrane preparations from BSEP-EYFP cells using the patient's serum (Fig. 3A and Fig. S2C). Complete diagnostic datasets of each cohort member including individual BSEP trans-inhibition data can be found in Fig. S2 (AIBD), Fig. S3 (control), and Fig. S4 (non-AIBD). Of all patients included in this study, only AIBD6 and non-AIBD3 have previously been published.[13,21,22] All identified BSEP variants underlying PFIC-2 in this cohort were either predicted or confirmed to cause absent BSEP expression in native liver (corresponding to type 3 in the NAPPED classification) in all but one case (AIBD1: c.2944G>A = p(Gly982Arg) homozygous).[6,23]

Serum samples were first depleted of excess BS, which may compete with the radioactive reporter substrate for transport by both NTCP and BSEP during the assay, thus effectively mimicking BSEP inhibition (false-positive assay result). To characterise the extent of this substrate competition in NTCP/BSEP cells, increasing amounts of unlabelled TC were added at assay start (Fig. S5A). NTCP-driven [^{3}H]-TC import remained unaffected up to a concentration of 500 μM unlabelled TC (S1). In contrast, reduction of [^{3}H]-TC export by BSEP was already observed between 50 and 100 μM TC (S2), leading to gradually increasing intracellular retention of the radioactive reporter substrate (L). This indicated a higher import than export capacity of this cell line for BS, which corresponds to the physiological situation where BSEP mediates the rate-limiting step of serum-to-bile transport of BS.[24,25] Consequently, high serum BS concentrations characteristic of both PFIC-2 and AIBD may lead to misinterpretation of BSEP transport assays (false-positive result). Total serum BS (TBS) levels in our AIBD cohort ranged from 153.7 to 512.5 μM with an average concentration of 268.9 ± 119.7 μM (n = 5; two samples were too small for TBS measurement). In the non-AIBD cohort, TBS levels ranged from 52.4 to 467.8 μM (203.6 ± 158.7 μM; n = 5). Accordingly, BS were depleted from all serum samples by repeated dilution and concentration steps using spin concentrators retaining anti-BSEP antibodies. BS, which are largely bound to serum (lipo)proteins,[26] were sufficiently decreased after five rounds of dilution/concentration to 28.9 ± 20.4 μM in AIBD sera (n = 5) and to 7.5 ± 5.1 μM in non-AIBD cholestatic sera (n = 5), as determined by mass spectrometric analysis (Fig. S5B). BS in control sera (n = 9) were decreased from 8.0 ± 5.6 μM (range 2.4 to 19.6 μM) to 0.9 ± 0.5 μM after depletion. Following BS depletion, BS concentrations in all three cohorts were below the inhibitory concentration of BSEP activity.

Preincubation of live NTCP/BSEP cells with BS-depleted AIBD sera, but not with non-AIBD or control sera, led to staining of the cell surface with human IgG (Fig. 3B and Fig. S6A). This staining was caused by BSEP-reactive antibodies, as all AIBD sera only stained BSEP-EYFP cells but not empty HEK293 cells (Fig. S2B). To establish whether this antibody decoration triggered BSEP internalisation, which would reduce BS export and thus could be misinterpreted as BSEP inhibition (false-positive result), Na^+/K^+-

an import phase dominated by NTCP (also see Fig. S2A), which is followed by an export phase mediated by BSEP. During export, re-uptake of exported [^{3}H]-TC by sodium-dependent NTCP is prevented by replacing extracellular sodium with choline. Radioactivity contained in S1, S2, and cell L is measured by liquid scintillation counting. (E) Proof of assay principle using empty control (HEK293; n = 7), BSEP (n = 6), NTCP (n = 7), and NTCP/BSEP (n = 7) cells. Data are shown as mean ± SD. Significances were calculated using the two-sided, unpaired Student's *t* test. AIBD, antibody-induced BSEP deficiency; BS, bile salts; BSEP, bile salt export pump; EYFP, enhanced yellow fluorescent protein; IgG, immunoglobulin gamma; L, lysate; NTCP, Na^+/taurocholate cotransporting polypeptide; PFIC-2, progressive familial intrahepatic cholestasis type 2; S1, supernatant 1; S2, supernatant 2; TC, taurocholate.

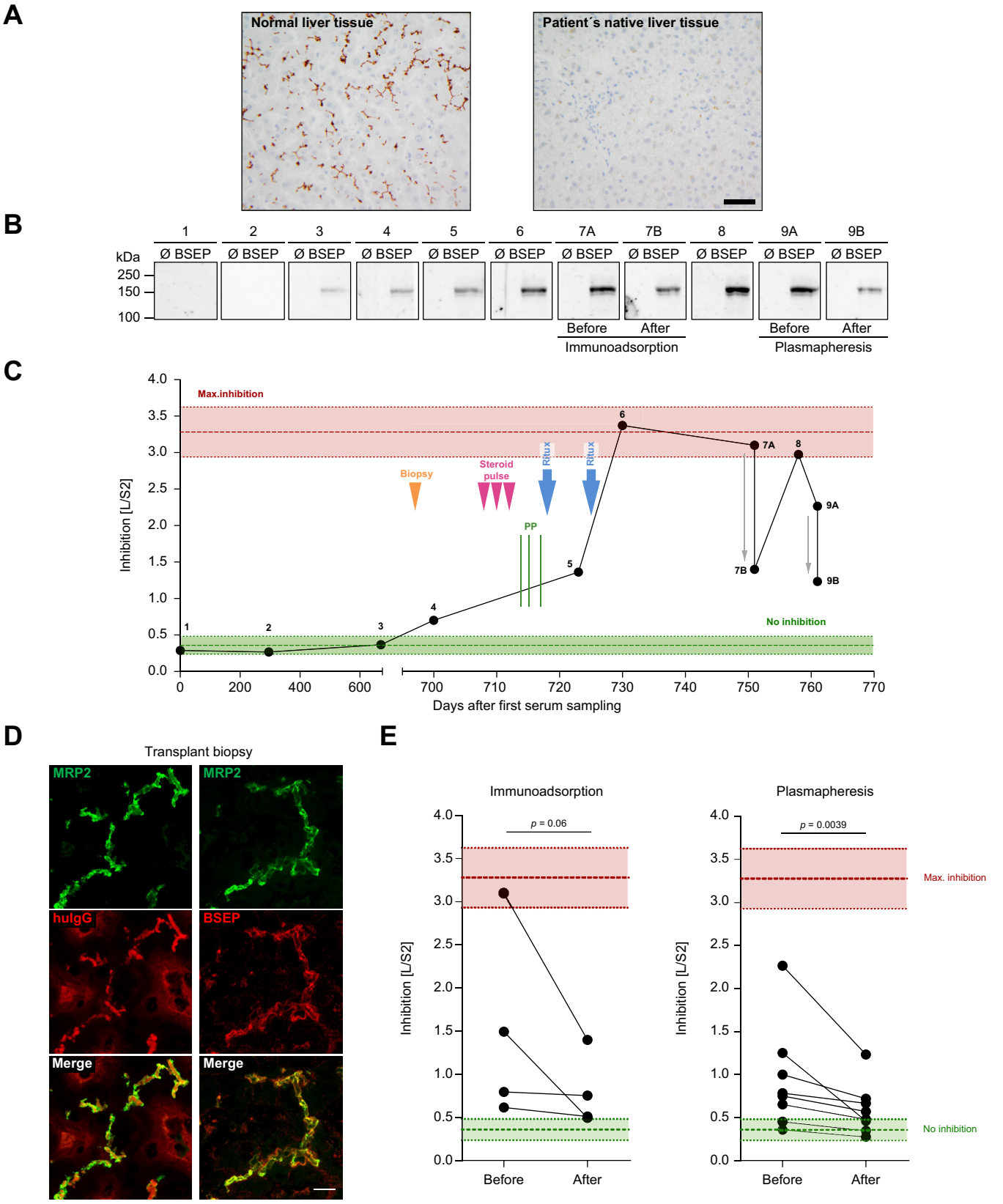

Fig. 2. Disease onset and response to treatment in patient AIBD7. (A) Immunohistochemistry staining of a normal liver (left) and the patient's liver (right) for BSEP. Bar = 50 μm. (B) BSEP reactivity of serum samples on Western blots using plasma membrane preparations from HEK293 cells (∅) and BSEP-EYFP-expressing cells (BSEP). (C) BSEP trans-inhibition by serum antibodies was plotted as L/S2 (both averages from triplicate measurements). Thresholds for maximum (AIBD7-7.3) and no inhibition (no serum) are indicated in red and green, respectively. (D) Detection of canalicular IgG deposits (left, red) alongside normal BSEP

ATPase was stained as a plasma membrane marker (Fig. 3C). Subsequent colocalisation analysis demonstrated that extracellular antibody decoration of BSEP-EYFP did not change its colocalisation with Na^+/K^+-ATPase (Fig. S6B and C), indicating that induction of BSEP internalisation upon antibody binding was negligible.

Next, BSEP trans-inhibition was tested using BS-depleted serum samples from all cohorts. Neither control nor non-AIBD sera showed any reduction in BSEP-mediated transport in comparison with the no-serum reference (Fig. 3D), preserving the substrate distribution S2 >L. In contrast, preincubation with all seven AIBD sera resulted in decreased export (S2) and increased cellular retention (L) of [^{3}H]-TC (arrows in Fig. 3D and Fig. S2D), shifting the substrate distribution to L >S2. Notably, this distribution resembled that of cells expressing only NTCP (Fig. 3D, inset). Preincubation of NTCP/BSEP cells with total purified IgG from AIBD but not control serum resulted in BSEP trans-inhibition, demonstrating that BSEP trans-inhibition was antibody-mediated (Fig. 3E). As antibody decoration of cells is the first step in the classical complement pathway, it may lead to cell lysis triggered by incubation with fresh AIBD sera and may compromise the test. Fresh samples of a BS-depleted AIBD and control serum, however, did not show any difference in BSEP trans-inhibition between native and heat-inactivated states (Fig. S7). Thus, we could rule out that measured BSEP inhibition in this assay was an artefact of BSEP internalisation from the plasma membrane, of excess serum BS competing with the reporter substrate, or of cell lysis caused by complement activation. Therefore, the BSEP trans-inhibition measured in our test with AIBD sera was antibody-mediated and caused by direct inhibition of BSEP-driven BS transport.

Functional testing substantiates diagnosis of AIBD and provides a readout of treatment response: example of an affected patient

The functional readout of BSEP trans-inhibition not only corroborates AIBD diagnosis but can be used to monitor antibody-depleting therapeutic approaches and thus help guide subsequent interventions. A boy of 11 years (AIBD7) initially presented with pruritus and strongly elevated serum BS (204.5 μM). A biopsy of his native liver showed fibrosis stage III and evidence of chronic intrahepatic cholestasis. Immunohistochemistry staining for BSEP was negative, suggesting severe BSEP deficiency (Fig. 2A), which was confirmed by detection of a pathogenic homozygous splice-site variant in the *BSEP* (*ABCB11*) gene. Partial external biliary diversion was performed 6 months after diagnosis and temporally alleviated symptoms. Twelve months later (at 12.5 years of age), the patient received the full organ of an unrelated deceased donor, after which his clinical condition improved and serum BS normalised (from 133.5 to 8.1 μM). Because this patient was at high risk of developing AIBD as a result of congenital absence of BSEP,[13] he was screened for emergence of serum anti-BSEP antibodies. Two serum samples taken within the first year after OLT showed no BSEP-reactivity on Western blots (Fig. 2B, sera 1 and 2). A transplant biopsy was performed for suspected acute rejection 1 month after OLT and found portal inflammation, endotheliitis, and ductulitis. Eleven months after OLT, an elevated alanine aminotransferase (163 U/L) level prompted a second biopsy to rule out rejection and showed liver fibrosis stage I and uncharacteristic portal inflammation. A third biopsy prompted by transaminase elevation 6 months later (17 months after OLT) showed progression of fibrosis to stage II–III, and thus, immunosuppression was increased (tacrolimus, mycophenolate mofetil, and prednisolone). One month later (18 months after OLT), donor-specific antibodies were detected. Then, 23 months after OLT, anti-BSEP antibodies were detected for the first time in the patient's serum (Fig. 2B, serum 3). Pruritus recurred, and alanine aminotransferase (160 U/L) was persistently elevated despite normal gGT (11 U/L). Serum TBS increased from normal levels (4.3 μM) to 230.7 μM over the course of 1.5 months. BSEP trans-inhibition was first detected from serum drawn 2 years after OLT (Fig. 2C, serum 4). The fourth liver biopsy was performed to rule out other differential diagnoses besides AIBD and showed normal canalicular BSEP expression alongside canalicular IgG deposits (Fig. 2D), which are highly characteristic of AIBD.[12,13] Based on absence of other causes of liver disease, presence of canalicular IgG deposits, and BSEP trans-inhibition, he was diagnosed with AIBD, and treatment was initiated (Fig. 2C). The patient received a first round of three steroid pulses (10–20 mg/kg/day prednisolone) followed by three plasmapheresis treatments and two doses of rituximab (375 mg/m^2 each). This was followed by another treatment with immunoadsorption and plasmapheresis, both of which reduced serum anti-BSEP antibody levels (Fig. 2B) and BSEP trans-inhibition (Fig. 2C). To further reduce systemic anti-BSEP antibody load, a series of immunoadsorption and plasmapheresis treatments (12 instances over 3.5 months) was initiated (Fig. 2E). Each treatment instance resulted in a reduction of BSEP trans-inhibition *in vitro*, coinciding with reduced serum anti-BSEP reactivity on Western blot (Fig. S8). Although stable, the patient continues to require intensified immunosuppression and regular plasmapheresis treatment alongside i.v. IgG supplementation to manage his AIBD.

An updated diagnostic workflow for AIBD

To illustrate the additional diagnostic value of a functional assay, we compiled data regarding anti-BSEP antibody detection[16] from three patients with PFIC-2 post OLT (Fig. 4A–C). In contrast to control serum, sera from all three patients showed anti-canalicular reactivity on human liver tissue (Fig. 4A). In AIBD7 and patient 1, but not patient 2, this reactivity was directed against BSEP as shown by IF and Western blot based on BSEP cells (Fig. 4B and C). Accordingly, AIBD7 and patient 1 would have been diagnosed with AIBD by demonstration of anti-BSEP antibodies in their sera. Applying the BSEP trans-inhibition assay, we demonstrated that BS-depleted serum from AIBD7, but not from patients 1 and 2, trans-inhibited BSEP (Fig. 4D). Patient 1, the sibling of another patient with AIBD from a previous study,[13] lacked congenital BSEP expression and also had detectable levels of serum anti-BSEP antibodies (Fig. 4B and C), which could bind to extracellular BSEP epitopes (Fig. S9A).

expression (right, red) in transplant biopsy by IF staining. MRP2 (green) served as canalicular marker. Bar = 10 μm. (E) Changes in BSEP trans-inhibition in response to multiple sessions of immunoadsorption (n = 4) and plasmapheresis (n = 8). Significances were calculated using Wilcoxon's matched-pairs test (one-sided). AIBD, antibody-induced BSEP deficiency; BSEP, bile salt export pump; EYFP, enhanced yellow fluorescent protein; IF, immunofluorescence; IgG, immunoglobulin gamma; L, lysate; multidrug resistance-associated protein 2; Ritux, rituximab; PP, plasmapheresis; S2, supernatant 2.

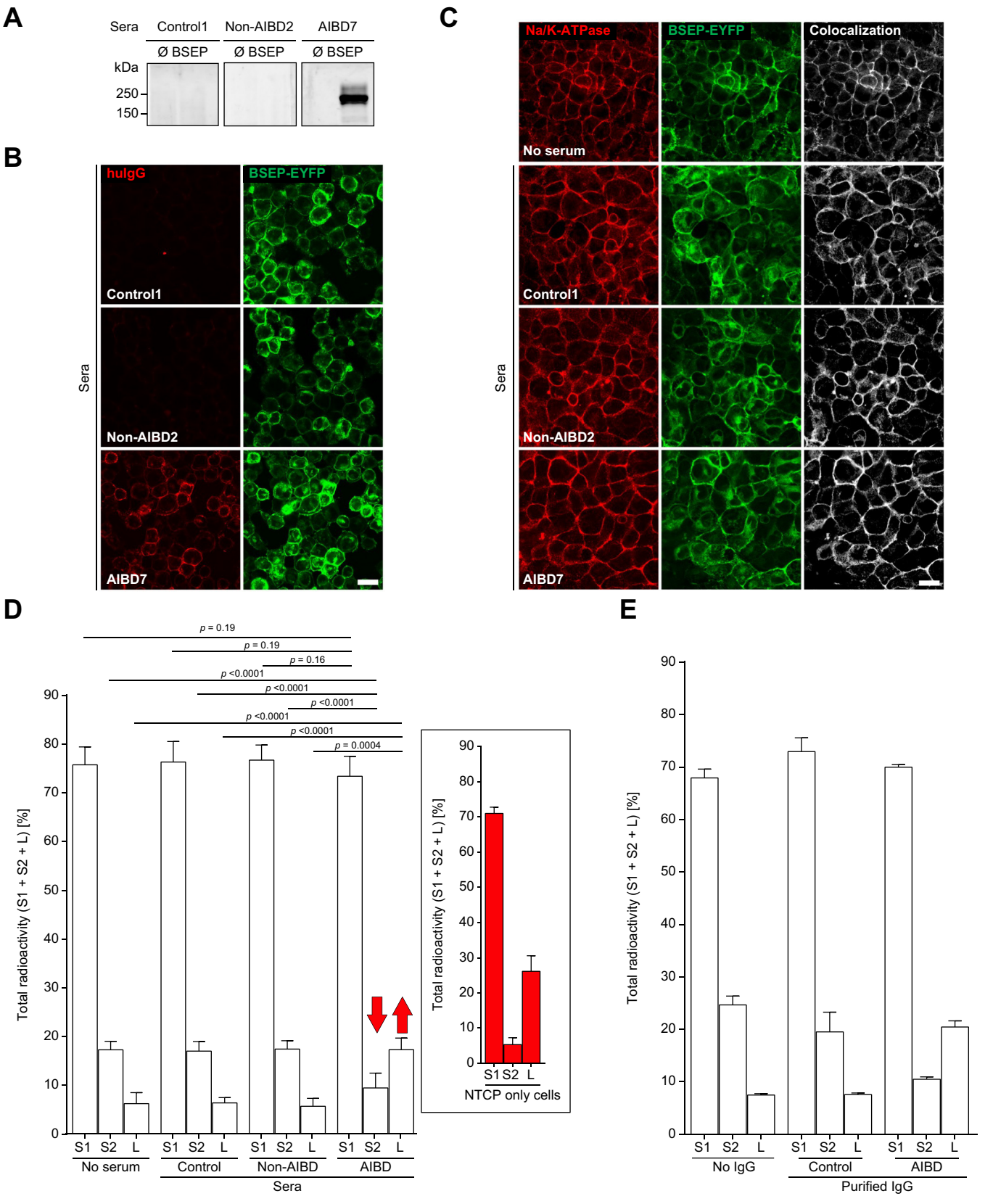

Fig. 3. Validation of the BSEP trans-inhibition assay using serum samples from different cohorts. (A) Western blot data of representative sera used in the test. Crude plasma membranes were prepared from HEK293 cells (∅) and BSEP-EYFP-expressing cells (BSEP), separated via SDS-PAGE and transferred onto nitrocellulose. Membranes were then probed with indicated sera as primary antibody. (B) Anti-BSEP antibodies (huIgG) are present in BSEP-reactive (AIBD) but absent from non-AIBD and control sera and bind to BSEP-EYFP on the surface of live HEK293 cells. NTCP/BSEP cells seeded onto PLL-treated dishes were

However, at the time of analysis, the anti-BSEP antibodies detected in the serum of patient 1 did not significantly inhibit BSEP transport activity (Fig. 4D), even after doubling the serum amount during preincubation (Fig. S9B). In line with this, TBS levels in his serum were normal (4.8 μM), and patient 1 was symptom-free at the time of serum sampling. In contrast, the serum of patient 2 contained anticanalicular antibodies, which were not reactive towards BSEP (Fig. 4B and C), accordingly showing no BSEP trans-inhibition (Fig. 4D). Thus, we could exclude AIBD in patients 1 and 2 at the time of serum sampling. However, as shown for AIBD7 (see Fig. 2), inhibitory antibodies may develop in patient 1 over time. Incorporation of the BSEP trans-inhibition assay into the AIBD diagnostic algorithm allows functional confirmation of AIBD in patients with PFIC-2 post OLT (Fig. 5).

Discussion

In the present study, we developed and validated a cell-based assay that directly detects antibody-mediated BSEP inhibition from the extracellular side (trans-inhibition) using a BS-depleted serum sample, recapitulating the central causative factor of AIBD pathogenesis.[11–15,17,21,27–31] Similar to IgA, the major biliary immunoglobulin IgG is a natural component of bile[32] and thus can access extracellular BSEP epitopes. Mostly derived from the circulation, it may play a role in biliary immunosurveillance.[32] Although the transport mechanism of IgG from serum to bile is still unknown, a paracellular or transcytotic pathway seems a plausible route.[16,33] It is less likely that newly synthesised BSEP is decorated by IgG at the sinusoidal membrane, as it seems to be directly targeted from the Golgi apparatus to the canalicular membrane.[34] At the same time, IgG is unable to reach the cytosol of intact cells outside of an immune complex.[35] This, together with the demonstration that AIBD serum stained BSEP in unpermeabilised freshly isolated hepatocytes, in which the canalicular membrane is accessible,[11] strongly suggests trans-inhibition of BSEP as the pathophysiological cause of AIBD.

Given that an estimated 8–33% of patients with PFIC-2 develop AIBD post OLT,[2] diagnostic testing for this potentially serious complication should be considered upon symptom recurrence in patients with PFIC-2 who underwent a transplant.[11,13–15,17,27–29,31] Five of the six previously unreported AIBD cases in this study carry variants either predicted or confirmed to cause complete loss of BSEP expression, which further corroborates that congenital absence of BSEP expression poses a risk factor for AIBD development.[13]

Many pharmacological studies use polarised cell lines in which BS undergo vectorial transcellular transport from the basal to the apical side of the cell monolayer.[36,37] These tests require a confluent cell layer and handling of cells on cell culture inserts. However, for measuring BSEP trans-inhibition, vectorial BS transport is not required. Therefore, our novel approach makes use of unpolarised cells (Fig. 1B and D). In this assay, the only function of NTCP is to preload the cells with BS during the import phase. This is possible because NTCP import, by far, supersedes BSEP export in the assay cell line (Fig. S5A), resulting in a substantial net uptake of [^{3}H]-TC in the first assay phase as depicted by comparable S1 levels in NTCP and NTCP/BSEP cells (Fig. 1E). After preloading the cells, NTCP activity is diminished by removal of extracellular sodium to exclusively measure BSEP transport (and its inhibition) in the export phase. Here, the bulk of accumulated [^{3}H]-TC is exported from the cells into S2, resulting in the substrate distribution S2 > L. Intracellular BS retention caused by BSEP inhibition, a characteristic of AIBD, was exclusively observed in all tested AIBD sera, causing a shift in substrate distribution to L > S2. Accordingly, a shift from S2 to L indicates BSEP trans-inhibition and AIBD. Moreover, substrate distribution and thus trans-inhibition can readily be expressed as L/S2 (Fig. 2C and E).

As AIBD leads to cholestasis accompanied by high serum BS levels, BS depletion is a prerequisite for robust test performance and prevention of false-positive results. A simple approach using spin concentrators sufficed to reduce BS levels below inhibitory values (Fig. S5B). Furthermore, complement inactivation by heat treatment was not necessary for proper test function (Fig. S7). Cell surface decoration with anti-BSEP antibodies could be expected to trigger the classical complement cascade pathway, leading to membrane attack complex formation and cell lysis during preincubation. Previous studies have not found any impact of human complement on human cell lines, which has been attributed to the general presence of cell surface complement regulatory factors such as decay accelerating factor (CD55) CD59 and MCP (CD46), all of which are expressed on the surface of HEK293 cells,[38–41] to protect cells and tissues from homologous complement activation.

Patients who undergo transplantation are treated with immunosuppressants and a variety of other medications. Although small molecular weight compounds should be removed along with the excess BS during spin column depletion, a substantial modulation of NTCP transport activity during import should raise a red flag of concern, as some compounds that modulate NTCP may also modulate BSEP activity.[42] This problem could be overcome by purification of total IgG from the serum sample (Fig. 3E). We do not routinely purify serum antibodies, as this requires more hands-on time and introduces more variation in terms of recovery as a result of the small serum volumes routinely obtained from paediatric patients. Antibody-mediated BSEP internalisation during preincubation is another potential confounder of the assay that could be excluded by colocalisation analysis (Fig. S6B and C). This lack of BSEP internalisation *in vitro* is in line with observations of normal canalicular BSEP localisation in biopsy material from AIBD livers (also see Fig. 2D),

incubated with sera, fixed, and immunostained for human IgG with an Alexa Fluor 647-coupled secondary antibody (shown in red). Complete sets of original image channels can be found in Fig. S6A. Bar = 20 μm. (C) Binding of anti-BSEP antibodies to their target on the surface of live cells does not trigger BSEP internalisation. NTCP/BSEP cells seeded onto PLL-treated IBIDI dishes were incubated with indicated sera, fixed, and immunostained for Na^+/K^+-ATPase as a plasma membrane marker (using Alexa Fluor 647, channel shown in red; see Fig. S6B). Bar = 20 μm. (D) Validation of cell-based BSEP trans-inhibition test using BS-depleted control (n = 9), non-AIBD (n = 5), and AIBD (n = 7) sera and untreated cells (no serum, n = 15). Data are shown as mean ± SD. Significances were calculated using the two-sided, unpaired Student's *t* test. Radioactivity measurements in S1, S2, and L after AIBD preincubation resemble cells expressing only NTCP (inset). (E) Cell-based BSEP trans-inhibition test using serum IgG. Total IgG was purified from serum control 1 and AIBD6. NTCP/BSEP cells were preincubated with the IgG amount present in the serum volume normally assayed. Data are shown as mean ± SD of triplicate measurements. AIBD, antibody-induced BSEP deficiency; BS, bile salts; BSEP, bile salt export pump; EYFP, enhanced yellow fluorescent protein; IgG, immunoglobulin gamma; L, lysate; NTCP, Na^+/taurocholate cotransporting polypeptide; PLL, poly-L-lysine; S1, supernatant 1; S2, supernatant 2.

supporting direct functional BSEP inhibition at the canaliculus *in vivo*.[11,13]

By prospective screening of a patient who underwent a transplant for PFIC-2, who was at high risk of developing AIBD as a result of congenital absence of BSEP expression, we were able to functionally monitor seroconversion and thus prompt immediate treatment while evaluating its outcome by assaying BSEP trans-inhibition activity (Fig. 2). Ultimately, the ongoing treatment regime led to stabilisation of the patient. Notably, another patient with PFIC-2 (patient 1; Fig. 4) developed anti-BSEP antibodies after OLT, which recognised extracellular BSEP epitopes (Fig. S9A) yet failed to show any BSEP trans-inhibition (Fig. 4D), even after doubling the amount of BS-depleted serum during preincubation (Fig. S9B). Although we could previously demonstrate that both intracellular and extracellular BSEP epitopes are recognised by AIBD-derived anti-BSEP antibodies,[13] information on the complexity of the anti-BSEP allo-immune response with respect to polyclonality is elusive. Based on the recent BSEP cryo-electron microscopy structure,[43] only 8% of its amino acids (~110 of 1321) are accessible from the extracellular side and therefore are pathophysiological targets of binding and trans-inhibition. Thus, only a fraction of the various anti-BSEP antibody specificities contained in a given humoral response can bind to their target from the canalicular lumen. Antibodies that do not confer trans-inhibition upon BSEP binding could leave its carrier essentially asymptomatic, as canalicular antibody decoration

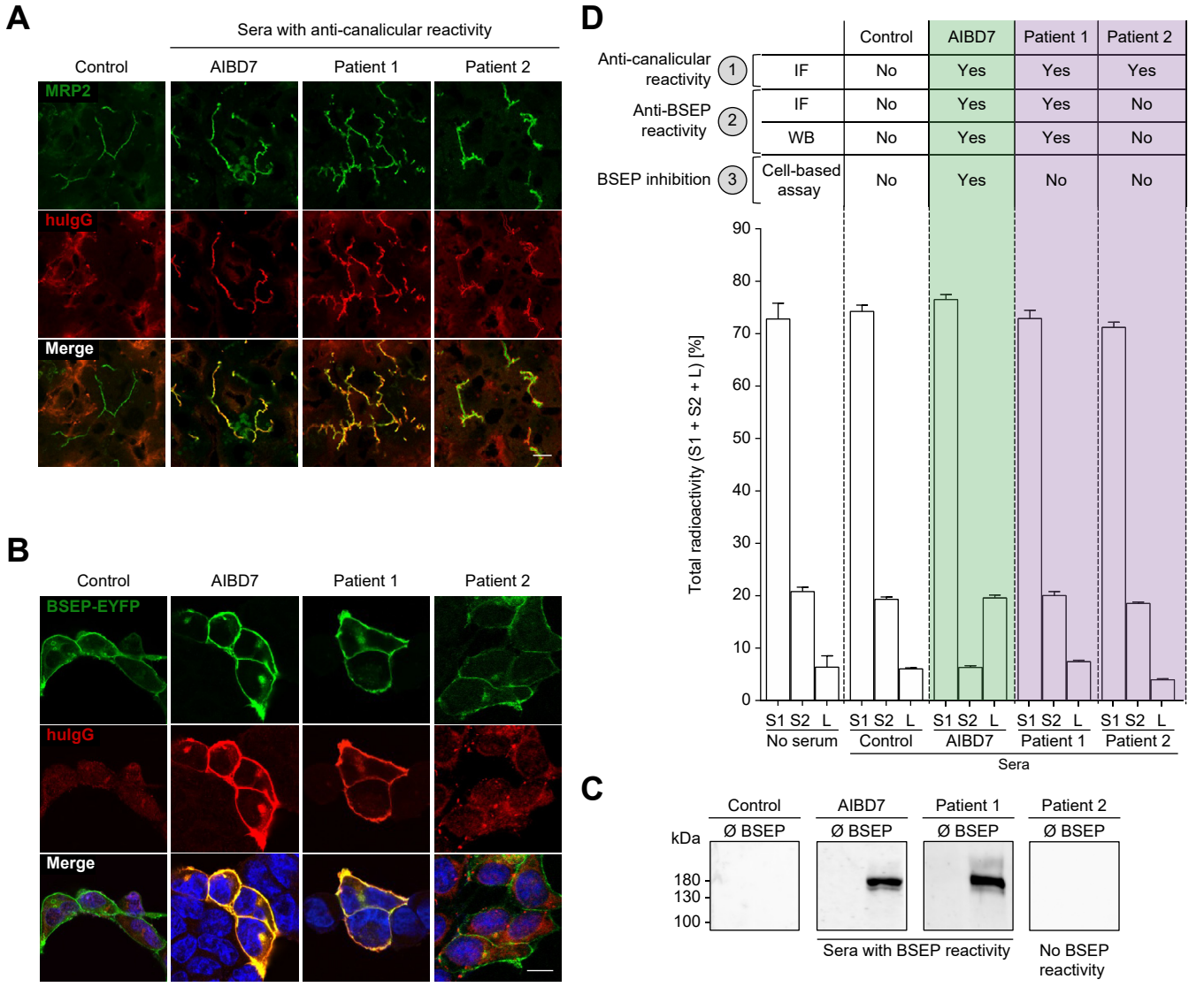

Fig. 4. Current diagnostic workup of AIBD is based on the detection of BSEP-reactive antibodies in patient serum samples. (A) Cryosections of normal human liver tissue were immunostained with patient sera alongside normal control serum for detection of anticanalicular antibodies (human IgG, red). MRP2 (green) stained as canalicular marker. Bar = 10 μm. (B) Immunostaining of HEK293 cells expressing BSEP-EYFP (green) with control or patient sera (red). Nuclei were stained with DAPI. Bar = 10 μm. (C) BSEP reactivity of patient sera was tested by WB using plasma membrane preparations from HEK293 cells (∅) and BSEP-EYFP-expressing cells (BSEP). (D) Summary of diagnostic workup for (1) IF testing for anticanalicular reactivity as depicted in (A), (2) IF and WB data for BSEP specificity as shown in (B) and (C), and (3) BSEP trans-inhibition test data. Data are shown as mean ± SD of triplicate measurements. AIBD, antibody-induced BSEP deficiency; BSEP, bile salt export pump; EYFP, enhanced yellow fluorescent protein; IF, immunofluorescence; L, lysate; MRP2, multidrug resistance-associated protein 2; S1, supernatant 1; S2, supernatant 2; WB, Western blot.

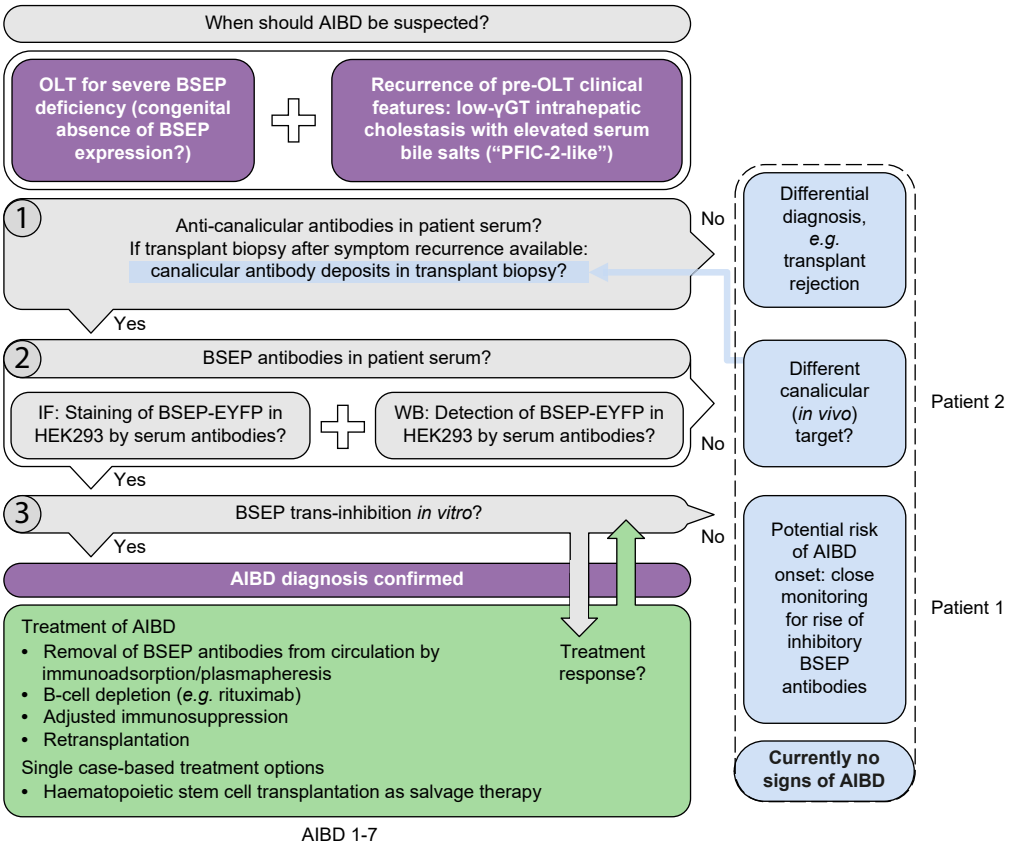

Fig. 5. Updated proposed diagnostic workup for AIBD incorporating the cell-based BSEP trans-inhibition test (also see Fig. 4D). According to this procedure, patients AIBD1–7 were conclusively diagnosed with AIBD, whereas AIBD was excluded for patients 1 and 2 at the time of serum sampling. AIBD, antibody-induced BSEP deficiency; BSEP, bile salt export pump; EYFP, enhanced yellow fluorescent protein; IF, immunofluorescence; OLT, orthotopic liver transplantation; PFIC-2, progressive familial intrahepatic cholestasis type 2; WB, Western blot; γGT, gamma-glutamyl transferase.

does not trigger any changes in canalicular BSEP localisation in affected livers (see above).

In summary, patients with PFIC-2 who underwent a transplant may develop AIBD. We strongly encourage immediate diagnostic testing whenever phenotypic symptom recurrence post OLT is suspected, as this enables a timely therapeutic intervention. The 'classical' AIBD diagnosis is strongly corroborated by functional demonstration of BSEP-reactive antibodies with trans-inhibitory capacity and may help rule out other differential diagnoses of post-OLT cholestasis. Using NTCP/BSEP co-expressing cells and a temporal separation of their respective activity into import and export phases allow testing for BSEP trans-inhibition. The AIBD diagnostic workflow proposed here unites detection of anti-BSEP antibodies with functional testing for BSEP trans-inhibitory antibodies.

Abbreviations

ABCB11, ATP binding cassette transporter superfamily, subfamily B, member 11; AIBD, antibody-induced BSEP deficiency; BS, bile salts; BSEP, bile salt export pump; cpm, counts per minute; EYFP, enhanced yellow fluorescent protein; gGT, gamma-glutamyl transferase; HEK293, human embryonic kidney 293; IF, immunofluorescence; IgG, immunoglobulin gamma; IOV, inside-out vesicle; L, lysate; MRP2, multidrug resistance-associated protein 2 (*ABCC2*); NTCP, Na+/taurocholate cotransporting polypeptide (*SLC10A1*); OLT, orthotopic liver transplantation; PFIC-2, progressive familial intrahepatic cholestasis type 2; PLL, poly-L-lysine; S1, supernatant 1; S2, supernatant 2; TBS, total bile salts; TC, taurocholate.

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Conflicts of interest

EL has received honoraria for lectures from Albireo and Mirum and has served on advisory boards for both companies and on the GPGE board of directors. UB has received grants from Mirum Pharma, Albireo Pharma, and Alexion Pharma and has received consulting fees and honoraria from Mirum, Albireo, Alexion, Nestle, and Vivet. CK has received travel grant from Falk. TB has received grants, consulting fees, and/or honoraria from Abbvie, Alexion, Bayer, BMS, Gilead, GSK, Eisai, Enyo Pharma, Falk Foundation, HepaRegeniX GmbH, Humedics, Intercept, Ipsen, Janssen, Medupdate GmbH, MSD/Merck, Merz, Norgine, Novartis, Orphalan, Roche, Sequana Medical, SIRTEX, SOBI, and Shionogi. VK has received honoraria for speaker's bureau from Falk, Albireo, CSL, Sanofi, Gilead, Abbvie, and Intercept and has served on one advisory board for Astra Zeneca. All other authors declare that there is no potential conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Conceptualisation: JS, VK. Treatment of patients: EL, SK, EDP, UB, EG, MAS, YMG, JH, TB, ASC, VK. Diagnostic workup: JS, CD, AS, CK, PP, VK. Data generation: JS, CD, MW, PP, DH, CW, HH. Data analysis and interpretation: JS, CD, EL, RL, DH, EM, TL, VK. Manuscript preparation: JS, CD, VK. Critical review and editing: all authors.

Data availability statement

The NTCP-, BSEP-, and NTCP-/BSEP-expressing HEK293 cell lines and all datasets generated during the current study are available from the corresponding authors upon request.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2023.100690.

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Author names in bold designate shared co-first authorship

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