Genotype-phenotype relationships of truncating mutations, p.E297G and p.D482G in bile salt export pump deficiency

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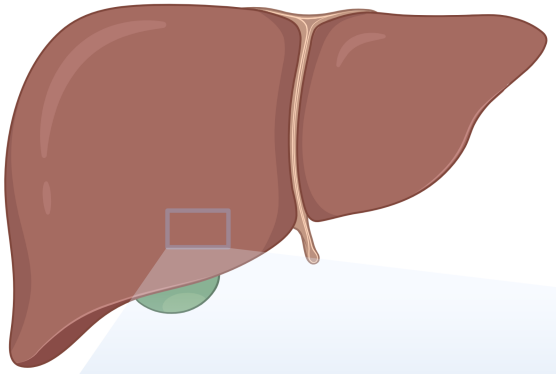
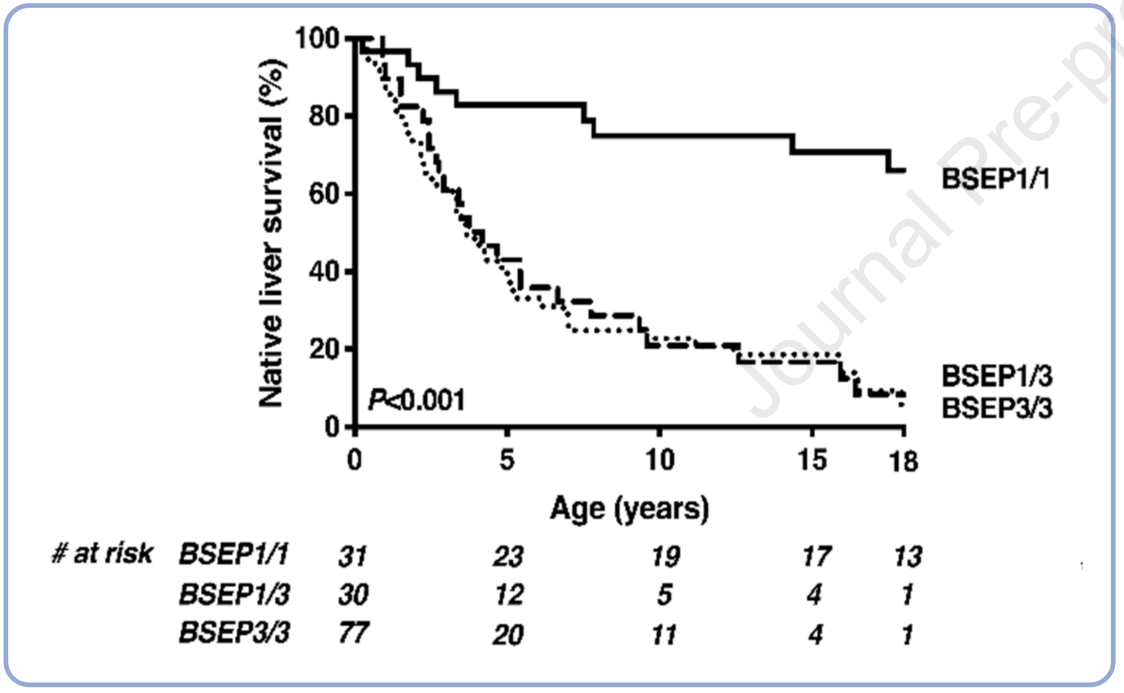


Genotype-phenotype relationships of predicted protein truncating mutations, p.E297G and p.D482G in bile salt export pump deficiency



BSEP1/1	homozygous p.E297G or p.D482G mutations
BSEP1/3	one p.E297G or p.D482G and one truncating mutation
BSEP3/3	two truncating mutations

Patients with BSEP1/3 mutations have very poor native liver survival and do not seem to benefit from (surgical) interruption of the enterohepatic circulation

NAPPED
NAtural course And Prognosis of PFIC and Effect of biliary Diversion

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Abstract

Background & Aims: Bile salt export pump (BSEP) deficiency frequently necessitates liver transplantation in childhood. Homozygous p.D482G or p.E297G mutations are associated with relatively mild phenotypes, responsive to surgical interruption of the enterohepatic circulation (siEHC), in contrast to patients with two predicted protein truncating mutations (PPTM). The phenotype of patients with a compound heterozygous genotype of one p.D482G or p.E297G mutation and one PPTM has remained unclear. We aimed to assess their genotype-phenotype relationship.

Methods: From the NAPPED database, we selected patients with homozygous p.D482G or p.E297G mutations (BSEP1/1; n=31), with one p.D482G or p.E297G, and one PPTM (BSEP1/3; n=30), and with two PPTMs (BSEP3/3; n=77). We compared presentation, native liver survival (NLS), and effect of siEHC on NLS.

Results: The groups had a similar median age at presentation (0.7-1.3 years). Overall NLS at age 10 years was 21% in BSEP1/3 vs. 75% in BSEP1/1 and 23% in BSEP3/3 (P<0.001). Without siEHC in their follow-up, NLS of BSEP1/3 was similar to BSEP3/3 patients, but considerably lower than BSEP1/1 patients (at age 10 years: 38%, 30%, and 71%, resp; P=0.003**).** After siEHC, BSEP1/3 and BSEP3/3 patients had similarly low NLS, while this was much higher in BSEP1/1 patients (10 years after siEHC, 27%, 14%, and 92%, resp.; P<0.001).

Conclusions: BSEP deficiency patients with one p.E297G or p.D482G mutation and one PPTM have a similarly severe disease course and low responsiveness to siEHC as patients with two PPTMs. This identifies a considerable subgroup of patients who are unlikely to benefit from interruption of the enterohepatic circulation by either surgical or ileal bile acid transporter inhibitor treatment.

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Lay summary

This manuscript defines the clinical features and prognosis of BSEP deficiency patients with the combination of one relatively mild and one very severe BSEP deficiency mutation. Until now, it had always been assumed that the mild mutation would be enough to ensure a relatively good prognosis. However, our manuscript shows that the prognosis of these patients is just as poor as that of patients with two severe mutations. They do not respond to biliary diversion surgery and will likely not respond to the new IBAT (ileal bile acid transporter) inhibitors, which have recently been approved for use in BSEP deficiency patients.

Introduction

Deficiency of the bile salt export pump (BSEP), also known as Progressive Familial Intrahepatic Cholestasis type 2 (PFIC2), is a rare disease that results from mutations in the *ABCB11* gene. The BSEP protein transports conjugated bile acids from the hepatocyte into the bile canaliculus across the canalicular membrane.(1, 2) Patients with BSEP deficiency usually present with jaundice, pruritus, high serum bile acid levels (sBA) and transaminases in the first year or two of life.(3-5) Some patients may respond, usually transiently, to medical therapy such as ursodeoxycholic acid (UDCA).(3, 6, 7) Surgical interruption of the enterohepatic circulation (siEHC) can be associated with improved native liver survival (NLS) in some BSEP deficiency patients.(5) The siEHC aims to reduce cholestasis through partially interrupting the enterohepatic circulation and thereby decreasing the amount of bile acids available for reuptake in the terminal ileum. Most patients, however, eventually progress to end-stage liver disease and/or to therapy-resistant pruritus, which usually necessitates liver transplantation (LTx) during childhood. More specifically, only a third of all BSEP deficiency patients reach adulthood with their native liver.(5)
Two frequent *ABCB11* mutations (p.D482G and p.E297G) have been associated with impaired intracellular trafficking to the canalicular membrane, but with residual bile acid transport activity.(8) Patients harboring these mutations usually present with a relatively mild phenotype.(3-5, 9) It has been suggested that carrying at least one of these mutations has a positive impact on the phenotype of the affected patients, possibly irrespective of the mutation on the second allele.(3, 5, 8-10) On the other hand, a genotype with biallelic predicted protein truncating mutations (PPTMs) has been associated with a more severe phenotype (with regard to NLS, response to siEHC and incidence of hepatocellular carcinoma (HCC)).(3, 5, 11, 12) We have

recently established genotype-phenotype relationships in three broad categories of *ABCB11* patients.(3, 5) However, little is known about the combined effects of two different *ABCB11* categories on the phenotype, which may reveal a better understanding of BSEP function and prognosis.(3, 5) By accumulating global data of patients with BSEP deficiency, the NAPPED (Natural course and Prognosis of PFIC and Effect of biliary Diversion) consortium aims to provide data that will allow optimized patient-specific treatment, whilst acknowledging the extensive heterogeneity in the observed genotypes.(5) The unique size of the NAPPED database allows, for the first time, the study of specific mutation combinations. In this study, we addressed the genotype-phenotype relationships in compound heterozygous BSEP deficiency patients with one allele affected by a BSEP1 mutation, i.e. encoding p.D482G or p.E297G, and the second allele by a BSEP3 mutation (PPTM), the latter of which is considered to have absent functional BSEP activity.

Patients and Methods

Data acquisition, patient inclusion and genetic categorization

This cohort study was performed conforming to the 1975 Declaration of Helsinki. At the data extraction for the present analyses (May 19th 2020), the NAPPED consortium included the world-wide collaboration of 68 centers. Retrospective follow-up data were collected in most cases, which was combined with prospective data from North American centers participating in the Childhood Liver Disease Research Network (ChiLDReN, Longitudinal Study of Genetic Causes of Intrahepatic Cholestasis – NCT00571272). Demographic, clinical and outcome data were entered by the participating centers directly into the web-based NAPPED Research Electronic Data Capture (REDCap) environment, using a pre-specified case record form,(13) or prospectively into the central ChiLDReN database. Centers using retrospective data collection identified all patients who had ever been under pediatric care (defined as age 0-18 years) since 1977. The respective *ABCB11* alleles were categorized into either BSEP1, representing a p.D482G or p.E297G mutation, and BSEP3 representing a PPTM. PPTMs were defined as truncating, nonsense or splice site mutation, leading to a predicted non-functional protein. Analyses were performed on patients with a BSEP1/1 (homozygous for p.D482G or p.E297G), BSEP1/3 (compound heterozygous for either p.D482G or p.E297G and one PPTM) and BSEP3/3 (two PPTMs) genotype. Separate analyses were also performed to determine the effect of the individual mutations. Patients were split up into homozygous p.D482G (BSEP1/1), homozygous p.E297G (BSEP1/1) and p.D482G – PPTM (BSEP1/3), p.E297G – PPTM (BSEP1/3) as well as BSEP3/3. Patients with at least one BSEP2 allele (missense mutation other than p.D482G or p.E297G) were excluded from this analysis due to the heterogeneity of mutations and the generally

unpredictable effect on BSEP protein function. Three patients who were compound heterozygous for BSEP1/1 (carrying one p.D482G mutation on one allele and one p.E297G mutation on the other allele) were excluded from the analysis to allow an analysis of only homozygous BSEP1/1 patients. Inclusion of these patients (n=3) in the analyses did not affect the main results (data not shown).

We compared baseline characteristics and liver biochemistry at presentation, overall NLS (time in years between birth and either LTx or death, whichever occurred first), NLS without siEHC, NLS after siEHC as well as liver biochemistry before and after siEHC. siEHC was defined as any internal or external surgical interruption of the enterohepatic circulation. Formally, ileal exclusion (IE) may not have been regarded as a biliary diversion, but we nevertheless included it as siEHC for reasons of consistency in the literature and for the similarities in physiological effects. Other outcomes we compared were occurrence of HCC and pre-transplant mortality. We analyzed clinical and biochemical parameters at the first presentation in the tertiary referral center. Liver biochemistry in relation to siEHC was assessed with the most recent available value before siEHC (pre-siEHC) and the first values available between 2 and 12 months after siEHC (post-siEHC). Parameters were converted to standardized units. End of follow-up was defined as last known visit at the tertiary referral center, LTx or death.

Statistics

Continuous variables were expressed as medians and interquartile range [IQR], unless specified otherwise. Nonparametric tests were used, including Mann-Whitney, Kruskal-Wallis and Wilcoxon signed-rank test, unless stated otherwise. Categorical

data were expressed as number and percentage (n, %) and analyzed using Chi-square, McNemar or Mantel's test for trend. The following variables were included in the statistical analyses: birth year, sex, age at first visit in the tertiary referral center, sBA, total serum bilirubin (TSB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), platelet count, any (current or historical) use of UDCA, rifampicin, phenobarbital, cholestyramine or antihistamine therapy prior to or at first visit, HCC and pre-transplant mortality. Univariate differences in proportion of patients with siEHC and NLS between patient subgroups were assessed by means of age to event analysis using Kaplan-Meier curves that were compared using the log-rank test. Patients without events were censored at last follow-up. A clock-reset approach was used to visualize the association of the time-dependent risk of siEHC with NLS: all patients start without siEHC. Then, patients that underwent siEHC during follow-up are censored at the age of siEHC and restart with a new risk in the siEHC curve. Multiple centers with different caseloads and geographical locations have included their data into the NAPPED database and sensitivity analyses (including geographic region and caseload of the center) were performed to assess for heterogeneity between sites. A two-sided p-value <0.05 was considered statistically significant. All analyses were performed using IBM SPSS Statistics 27.0 (Armonk, NY). Figures were constructed using Prism 8.2.1, GraphPad Software (La Jolla, CA).

Results

Baseline data

From the 759 patients recorded in our REDCap database at the time of data extraction, we selected 354 patients with two disease-causing mutations in *ABCB11*. From these patients, we selected 138 patients with a BSEP1/1 (n=31), BSEP1/3 (n=30) or BSEP3/3 (n=77) genotype (Fig. 1, Supplementary Fig. 1, Supplementary Table 1). Nine of the 85 patients in whom this information was available had originally presented with a BRIC phenotype (i.e., episodic cholestasis and/or transient pruritus and indications for hepatocellular damage), but this had developed into a PFIC phenotype during follow-up (i.e., continuous cholestasis and/or pruritus and continuous hepatocellular damage). The initial BRIC phenotype patients were rather equally distributed across the groups (2/15 (13%) in BSEP1/3, 3/22 (14%) in BSEP 1/1, and 4/48 (8%) in BSEP3/3). Two of the BSEP3/3 patients included were reported to have a copy of the common *ABCB11* risk polymorphism p.V444A in addition to their pathogenic mutations (Supplementary Table 2), however no patients were reported to have an additional mutation in *ABCB4*. No information was available on the potentially protective alleles CIDEB or HSD17B13.

Clinical characteristics and liver biochemistry at baseline

Table 1 depicts the characteristics at first presentation in the tertiary referral hospital for each of the three genotype categories. The median age at first presentation in the tertiary referral center was statistically comparable between the groups (between 0.7 and 1.3 years; P=0.07), as was the sex distribution (P=0.56).

Table 1 shows liver biochemistry at presentation in the tertiary referral center. Median sBA levels were nearly two-fold higher in BSEP1/3 patients (459 μmol/L) compared

to the other two groups (BSEP1/1: 247 μmol/L and BSEP3/3: 209 μmol/L, P=0.004). This difference was also statistically significant upon sub-group statistics, comparing BSEP1/3 to BSEP1/1 and to BSEP3/3 separately (P=0.009 and P<0.001, respectively). ALT levels in BSEP1/3 patients were comparable to BSEP1/1 but nearly half of those in BSEP3/3 (P=0.01). TSB, AST, and platelet levels were not statistically different between the groups. As expected, all patient groups had low to normal GGT levels, with the lowest values in BSEP1/1 patients (P=0.01, Table1).

Association between BSEP1/3 genotype and native liver survival

As far as information was available, the major indication for LTx in the three groups was either intractable pruritus or end-stage liver disease, with HCC and other reasons being the minority (Supplementary Table 3). The overall NLS at 10 years of age was 21% in BSEP1/3 compared to 75% in BSEP1/1 and 23% in BSEP3/3 patients (P<0.001, Fig. 2). In patients that had not (or not yet) undergone siEHC during follow-up, the NLS at age 10 years was only 38% in BSEP1/3 but 71% in BSEP1/1 and 30% in BSEP3/3 (P=0.003, Fig. 2). The percentage of LTx in patients without siEHC at 10 years of age was 92% in BSEP1/3 vs. 29% in BSEP1/1 and 72% in BSEP3/3 (P<0.001, Fig. 3). Sensitivity analyses with respect to center and geographic location yielded comparable results.

Association between BSEP1/3 genotype and response to surgical interruption of the enterohepatic circulation

The information regarding the indication for siEHC was limited but had been pruritus in 16 cases and failure to thrive in combination with portal hypertension in 1 case.

The type of siEHC was a partial external biliary diversion (PEBD) in 33 patients, IE in 8 patients, internal biliary diversion in 2 patients, partial internal biliary diversion in 1 patient and biliostomy in 1 patient. At 10 years after siEHC, the post-siEHC-NLS was 27% in BSEP1/3 compared to 92% in BSEP1/1 and 14% in BSEP3/3 (P<0.001, Fig. 2). The percentage of LTx in patients that had earlier undergone an siEHC at 10 years after siEHC was 73% in BSEP1/3 vs. 8% in BSEP1/1 and 71% in BSEP3/3 (P<0.001, Fig. 4). Sensitivity analyses with respect to center and geographic location yielded comparable results.

Information on paired pre- and post-siEHC liver biochemistry was available in a limited number of patients. siEHC was not associated with an improvement in liver biochemistry in the BSEP1/3 group (Supplementary Fig. 2). Analysis of paired sBA data revealed that siEHC was not associated with a significant decrease in sBA in BSEP1/3 or BSEP3/3 patients, in contrast to BSEP1/1 patients (Fig. 5). The absolute and relative decrease in sBA compared to pre-siEHC values differed between groups (BSEP1/3: from 378 to 343μmol/l (-9%); BSEP1/1: from 314 to 8μmol/l (-97%); BSEP3/3: from 462 to 308μmol/l (-33%)). Cross-sectional analysis of (unpaired) available sBA data before and after siEHC showed similar results (Fig. S3). Median TSB changed from 49 to 44 μmol/L in BSEP1/3 (-10%; P=0.56), from 73 to 8 μmol/L in BSEP1/1 (-90%; P=0.006), and from 44 to 20 μmol/L in BSEP3/3 (-55%; P=0.16; Supplementary Fig. 2). Availability of ALT, AST and GGT values before and after siEHC was rather limited and the observed changes were less remarkable (Supplementary Fig. 2).

A decrease of at least 75% in sBAs has previously been associated with higher 10 year NLS after siEHC.(5) This was also the case in our subgroup of patients (Fig. 6; P = 0.01). None of the 4 BSEP1/3 or of the 3 BSEP3/3 patients from whom data were available reached the 75% cutoff, in contrast to 9 of the 12 BSEP1/1 patients.

Association between genotype, development of hepatocellular carcinoma and pre-transplant mortality

At 10 years of age, the observed incidence of HCC was 0% for BSEP1/3 patients, 4% for BSEP1/1 patients and 20% for BSEP3/3 patients (P=0.006, Fig. 7). One BSEP1/1 patient was diagnosed with cholangiocarcinoma at age 34. The 10-year (pre-transplant) mortality was lowest in the BSEP1/3 group (0%), compared to 14% in the BSEP1/1 and 21% in the BSEP3/3 group (P=0.11, Supplementary Fig. 3). Cause of death was related to liver disease in all cases.

Association between specific type of BSEP1 mutation in BSEP1/3 patients (p.D482G or p.E297G) and native liver survival

The unique size of the NAPPED database previously allowed us to determine differences in the natural history of patients with the p.D482G or the p.E297G mutation.(5) This analysis showed that patients with at least one p.E297G mutation usually had a slightly more severe phenotype. Therefore, we assessed possible differences between the p.D482G and p.E297G within the BSEP1/3 genotype, thus when combined with a PPTM. The NLS of the p.D482G – PPTM patients was consistently lower than that of p.E297G – PPTM patients, but the NLS of each was still comparable to the BSEP3/3 genotype category. The compound heterozygous

combination of p.D482G and PPTM had the worst prognosis without and with siEHC (Supplementary Fig. 4).

N.B. All specific mutations used for this manuscript as well as their categorization into BSEP1 or BSEP3 alleles were listed in Supplementary Table 2.

Discussion

The aim of this study was to assess genotype-phenotype relationships in BSEP deficiency patients with the compound heterozygous combination of one p.D482G or p.E297G (BSEP1) mutation, i.e. encoding a residual function protein, and one PPTM (BSEP3) mutation. Patients in whom a p.D482G or p.E297G mutation was combined with a PPTM (BSEP1/3) had a prognosis that was strikingly similar to that of patients with two PPTMs (BSEP3/3) in terms of limited long-term NLS and poor response to siEHC. Our results indicate that combination of a p.D482G or p.E297G mutation and a PPTM results in a presumed BSEP transport activity below the level needed to mitigate the clinically severe phenotype of the disease.

At initial presentation in the tertiary referral center BSEP1/3 patients had the highest values of sBA, compared to BSEP 1/1 and BSEP3/3 patients. Other liver biochemistry parameters at initial presentation were rather similar between the three groups, except for the previously described high ALT levels in BSEP3 patients.(5) The natural history of the BSEP1/3 patients indicated a more severe phenotype, compared to BSEP1/1 patients, in terms of NLS, with only one fifth of patients alive with native liver at the age of ten years. It had previously been shown that patients with at least one BSEP1 mutation have a relatively good overall prognosis.(3-5, 9)

Strautnieks et al. hypothesized that compound heterozygosity for both a mild (BSEP1) and a severe (BSEP3) mutation results in a milder disease based on the hypothesis that milder missense mutations would allow sufficient residual function.(12) One example in favor of this hypothesis could be derived from a study by Davit-Spraul et al. in which two patients with a combination of missense and truncating mutations in BSEP had a longer NLS than those with biallelic truncating mutations.(3)

Subsequently, this assumption was used to include this category of patients in two clinical trials in BSEP deficiency patients, in contrast to patients with two PPTMs.(14, 15) Our present data on larger number of patients however, show that the initial biochemical presentation and subsequent natural history of the disease of BSEP1/3 patients is more similar to that of patients with two PPTMs (described before as BSEP3, i.e. patients with a BSEP3/3 genotype).(5) Our observations suggest that the residual function conferred by one copy of two BSEP1 variants in this study is not enough to improve disease outcomes compared to those with no residual function.

We analyzed the responsiveness to siEHC in the three groups. siEHC appeared not to be associated with a beneficial effect on long-term NLS in BSEP1/3 patients. The severe course of disease in patients with the BSEP1/3 genotype was observed both in patients that had undergone siEHC and in patients without siEHC. Accordingly, the post-siEHC survival with native liver was poor in BSEP1/3 patients (Fig. 2). We cannot exclude the possibility that those BSEP1/3 patients may have at least some benefit from siEHC, however the NLS at 10 years after siEHC was only 27% in BSEP1/3 patients compared to 38% at age 10 years in patients that did not (or not yet) undergo siEHC during follow-up (Fig. 2; see also clock reset analysis in

Supplementary Fig. 5). Since the former percentage is derived from the time after siEHC the actual age of these patients would be slightly older than 10 years, making the NLS slightly higher. However, the percentage of patients that had undergone liver transplantation at 10 years of age without siEHC (92%) was similar to the 73% of patients who underwent a LTx at 10 years after siEHC. Again, the time after siEHC would make the patients slightly older than 10 years, but not more than by a median of 2-3 years (Fig. 4). Therefore, we conclude that these numbers illustrate a poor prognosis towards NLS and a very limited, if any, benefit of siEHC in BSEP1/3 patients.

Previously, we reported that BSEP3/3 patients have a high risk of HCC (ca. 25% at 10 years of age).(5) Our present data in an expanded group of patients are in line with this observation with a 20% risk of HCC at 10 years in BSEP3/3 patients. It should be noted that the 25% risk in a previous analysis was based on a subfraction of the present larger BSEP3/3 group.(5) However, we did not observe any patient with HCC in the BSEP1/3 group in our present analysis, resulting in an incidence comparable to that of the BSEP1/1 group (4%). This observation could be related to the (single) BSEP1 allele present in the BSEP1/3 group or just to the still relatively low numbers for reliable interpretation. Nevertheless, this outcome stands out of the other phenotypic parameters studied. BSEP1/3 patients performed worse in almost all outcome parameters (i.e., liver function parameters at diagnosis, NLS and decrease in SBAs after siEHC) than the patients with two BSEP1 mutations and they appeared rather similar to the BSEP3 category, except, thus, regarding the high incidence of HCC in the latter. If this observation would be sustained in statistically even more reliable patient numbers, it would be tempting to speculate that the

residual function of BSEP1 in the BSEP1/3 patients may be insufficient for preventing significant cholestasis and liver damage, but nevertheless sufficient to offset the increased risk of HCC development.

In a subset of our patients, we also studied potential differences specific to either the combination of p.D482G with PPTM or that of p.E297G with PPTM. The p.D482G - PPTM patients had a more severe phenotype than p.E297G - PPTM. In homozygosity, the p.D482G mutation interestingly has a less severe natural history than the p.E297G mutation. Our previous study showed that patients with homozygous p.D482G achieved a similar NLS as those with homozygous p.E297G mutations (NLS at 15 years: 73% vs. 69%; p = 0.41) while undergoing siEHC much less often (%siEHC at 15 years: 26% vs. 90%; p = 0.006). (5) In combination with a PPTM, however, this feature seems to be reversed.

To date, a large variety of different BSEP mutations in the *ABCB11* gene have been identified. Several studies have contributed valuable *in vitro* or *in silico* data on the predicted BSEP transport functionality of other *ABCB11* mutants than p.D482G and p.E297G. (8, 16, 17) Nevertheless, knowledge is still lacking for the vast majority of currently known pathological *ABCB11* mutations (ca. 200).(5) It is reasonable to assume that several other mutations result in some residual activity of the BSEP protein. It would therefore be interesting to investigate whether the results of this study are unique to the p.D482G and p.E297G mutations or whether they can be extrapolated for other combinations of a residual function mutation with a PPTM. The large variety of ultra-rare and different BSEP mutations and the lack of insight in protein functionality limits the possibilities to assess whether other missense

mutations are associated with a similarly severe phenotype as p.D482G and p.E297G. This relevant question may become accessible for investigations in the future if information of more patients becomes available and detailed information on functional consequences of mutations are elucidated. A similar remark can be made on the possible influence of (polymorphisms in) other genes, such as CIDEB or HSD17B13, and of the *ABCB11* p.V444A polymorphism, which has been identified with limited BSEP transport function. (18-20) In the NAPPED registry, insufficient genetic information is currently available to determine their possible influence.

The main result of our study is that the severity of the phenotype of patients with one p.D482G or p.E297G mutation and one PPTM seems comparable to those with 2 PPTM. The most likely explanation is the “threshold hypothesis”, such that the *in vivo* BSEP protein transport activity resulting from the two BSEP alleles falls below a certain threshold, needed to prevent a (more) severe phenotype. However, an alternative hypothesis is that the PPTM could have a dominant negative effect over the p.D482G and p.E297G mutations. Although perhaps unlikely, we cannot exclude that some PPTM escape nonsense mediated mRNA degradation (NMD) and lead to translation of a truncated protein that interferes with the translation, folding and/or intracellular transport of the p.D482G or p.E297G BSEP protein originating from the other allele. (21-23) The difference between the natural history in p.D482G-PPTM and p.E297G-PPTM BSEP deficiency patients could also possibly be due to different levels of dominant negative interference. At this moment, however, neither of these two hypotheses can be proven. Future studies in other combinations of residual function mutations and PPTM might provide more clarity, as discussed above.

Our study shows that the phenotype of p.D482G-PPTM and p.E297G-PPTM patients is among the most severe category of BSEP genotypes, i.e., similar to patients with two PPTM mutations. It is generally known that patients with two PPTMs have a poor prognosis.(3, 5) In the NAPPED database these patients currently make up about 22% of all BSEP deficiency patients with two confirmed *ABCB11* mutations (Fig. 1). The category p.D482G-PPTM and p.E297G-PPTM patients add up to about 8% of all patients, resulting in 30% of all NAPPED registered BSEP deficiency patients having a very severe phenotype, poor responsiveness to siEHC and an overall poor prognosis with respect to survival with native liver. Based on this categorization it might be that BSEP1/3 patients may not only be unresponsive to surgical interruption of the enterohepatic circulation but also to medical approaches to interrupt, such as the novel ileal bile acid transporter inhibitors (IBATi).(14, 15, 24) The inclusion of p.D482G - PPTM and p.E297G - PPTM patients could underestimate beneficial effects of (medical) interruption of the enterohepatic circulation in other genetic categories of BSEP patients. We therefore suggest that it is defendable, if not warranted, to (re)assess clinical trial results with and without including data of BSEP1/3 (specifically p.D482G - PPTM and p.E297G – PPTM) patients.

Other therapeutical strategies, such as chaperones or potentiators, could prove to be therapeutically successful for BSEP1/3 patients. Theoretically, chaperones like 4-phenylbutyrate could improve the phenotype of these patients by BSEP retargeting of the BSEP1 mutation with known residual function, as has been shown in patients with other missense mutations.(8, 25, 26) Once retargeted, the residual activity could then possibly be boosted by a potentiator like ivacaftor.(16) However, this is still largely speculative and more studies on the relationship between BSEP deficiency

genotype and response to therapy are needed before clinical application could become reality.

We are aware of limitations to the retrospective component of our study, notwithstanding it being based on the by far largest collection of BSEP deficiency patient data. The absolute number of patients in the BSEP subgroups is still limited and we did encounter missing data, as indicated. Nevertheless, we believe that our numbers were sufficient to draw the present conclusions. We are aware that center bias could play a role: treatment strategies, including the application of siEHC and the indications for LTx, have likely been different among the contributing NAPPED centers. However, sensitivity analyses towards centers or global regions did not impact the main outcomes of our study. Due to its (largely) retrospective nature, our cohort included patients that were treated over an extended period, which could have led to a chronological bias. Finally, some patients had been or still were on UDCA treatment. Unfortunately we do not have more precise data on actual use or dosage of UDCA. We did not see significant differences between patients that had been or still were on UDCA treatment versus those that never had used it (Table 1). Our present data do not allow to assess more detailed possible associations between UDCA use and course of disease.

In conclusion, our global NAPPED database has allowed to further address genotype-phenotype relationships for patients with genetically confirmed BSEP deficiency, or PFIC type 2. We identified a subgroup, representing 8% of all BSEP deficiency patients, with an unexpectedly, severe disease phenotype, both at initial presentation and during follow up, including a poor response to surgical interruption

of the enterohepatic circulation and a low survival rate with native liver. Even beyond the clinical implications, the results of this study will allow for a better prognostication of patients when confronted with a genetic diagnosis of p.D482G -PPTM and p.E297G - PPTM BSEP deficiency. Finally, we feel that the present observations have important consequences for past, current and future therapeutic trials in BSEP patients, aiming to improve their prognosis.

Abbreviations

ABCB11 (ATP-binding cassette, sub-family B member 11), ALT (alanine-aminotransferase), AST(aspartate-aminotransferase), BSEP (bile salt export pump), ChiLDReN (Childhood Liver Disease Research Network), GGT (gamma-glutamyltransferase), HCC (hepatocellular carcinoma), IBATi (ileal bile acid transporter inhibitor), IE (ileal exclusion), IQR (interquartile range), LTx (liver transplantation), NAPPED (NAtural course and Prognosis of PFIC and Effect of biliary Diversion), NLS (native liver survival), NMD (nonsense mediated mRNA degradation), PEBD (partial external biliary diversion), PFIC2 (Progressive Familial Intrahepatic Cholestasis type 2), PPTM (Predicted Protein Truncating Mutation), REDCap (Research Electronic Data Capture), sBA (serum bile acids), siEHC (surgical interruption of the enterohepatic circulation).TSB (total serum bilirubin), UDCA (ursodeoxycholic acid).

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Tables

Table 1. Patient baseline characteristics of the selected BSEP genotypes.

	BSEP1/1 (n=31)		**BSEP1/3 (n=30)**		**BSEP3/3 (n=77)**	
Demographics, median		***P* value BSEP1/1 vs. BSEP1/3*** **		***P* value BSEP1/3 vs. BSEP3/3*** **		***P* value *Overall***
Age first visit, y [IQR] Available n (%)	0.8 [0.3-1.9] 30 (97)	-	1.3 [0.5-4.4] 30 (100)	-	0.7 [0.3-1.9] 77 (100)	0.07
Year of birth, [IQR] Available n (%)	1992 [1987-2009] 31 (100)	0.006*	2001 [1995-2009] 30 (100)	0.008*	2009 [2002-2012] 77 (100)	<0.001****
Females, n (%) Available n (%)	16 (52) 31 (100)	-	19 (63) 30 (100)	-	40 (52) 76 (99)	0.56
Laboratory data at presentation to the tertiary referral center, median [IQR]						
Serum bile acids, (μmol/L) Available n (%)	247 [153-378] 18 (58)	0.009*	459 [354-539] 11 (37)	<0.001*	209 [151-309] 44 (57)	0.002*
Total serum bilirubin, (μmol/L) Available n (%)	95 [44-180] 26 (84)	-	110 [57-150] 20 (67)	-	104 [53-145] 67 (87)	0.99
Alanine-aminotransferase, (IU/L) Available n (%)	126 [63-251] 28 (90)	0.26	148 [92-437] 18 (60)	0.19	293 [138-502] 64 (83)	0.01****
Aspartate-aminotransferase, (IU/L) Available n (%)	246 [102-475] 22 (71)	-	257 [128-648] 18 (60)	-	359 [157-591] 64 (83)	0.22
Gamma-glutamyltransferase,(IU/L) Available n (%)	15 [10-29] 26 (84)	0.07	22 [18-35] 18 (60)	0.37	27 [18-38] 65 (84)	0.01****
Platelet count, (10^9/L) Available n (%)	397 [341-561] 25 (81)	-	321 [192-468] 18 (60)	-	395 [265-539] 54 (70)	0.16
Medication prior to or at moment of first presentation at the tertiary referral center, n (%)						
UDCA	15/31 (48)	-	11/30 (37)	-	30/77 (39)	0.59
Rifampicin	4/31 (13)	-	6/30 (20)	-	19/77 (25)	0.39
Phenobarbital	4/31 (13)	-	2/30 (7)	-	3/77 (4)	0.10
Cholestyramine	7/31 (23)	-	2/30 (7)	-	8/77 (10)	0.18
Antihistamines	4/31 (13)	-	2/30 (7)	-	9/77 (12)	0.94

*A *P* value of <0.05 is considered statistically significant
**Mann-Whitney U, Kruskal-Wallis test, Chi-squared test or Fisher's exact test as appropriate, to test differences between the three groups.
***Sub-group statistics only performed at overall *P* value <0.05
Genotypic categorization clarified in Methods.
BSEP (bile salt export pump); UDCA (ursodeoxycholic acid); IQR (interquartile range); n (number).

Figure Legends

Fig. 1. Flowchart of patient inclusion from NAPPED database. Genotype category explained under methods section. *NAPPED (NAtural course and Prognosis of PFIC and Effect of biliary Diversion); BSEP (bile salt export pump); n (number); created with biorender.com.*

Fig. 2. Observed native liver survival over time in patients with a BSEP1/1, BSEP1/3 and BSEP3/3 genotype. (A) All patients. (B) Patients without siEHC during follow up, patients with siEHC are censored at time of siEHC. (C) Patients after they had siEHC. Genotypic categorization of BSEP1/1, BSEP1/3 and BSEP3/3 groups is defined in the methods section. Log rank tests. *BSEP (bile salt export pump; siEHC (surgical interruption of the enterohepatic circulation).*

Fig. 3. Observed proportion of liver transplants in patients that did not undergo siEHC during follow-up. Genotypic categorization of BSEP1/1, BSEP1/3 and BSEP3/3 groups is defined in the methods section. Log rank tests. *BSEP (bile salt export pump); siEHC (surgical interruption of the enterohepatic circulation).*

Fig. 4. Observed proportion of liver transplants in patients that did undergo siEHC during follow-up. Genotypic categorization of BSEP1/1, BSEP1/3 and BSEP3/3 groups is defined in the methods section. Median age at siEHC was 2.9 years in BSEP1/1, 1.8 years in BSEP1/3 and 2.5 years in BSEP3/3. Log rank tests. *BSEP (bile salt export pump; siEHC (surgical interruption of the enterohepatic circulation).*

Fig. 5. Serum bile acids prior to and after siEHC in patients with a BSEP1/1, BSEP1/3 and BSEP3/3 genotype. Wilcoxon signed-rank test. Bars represent median and IQR. *BSEP (bile salt export pump); siEHC (surgical interruption of the enterohepatic circulation).*

Fig. 6. Observed native liver survival after siEHC, stratified for post-surgical sBA cut-offs. In all available patients (BSEP1/1, BSEP1/3 and BSEP3/3) with a relative decrease in sBAs of < or ≥ 75%. Log rank tests. *sBAs (serum bile acids); siEHC (surgical interruption of the enterohepatic circulation).*

Fig. 7. Observed proportion of patients with hepatocellular carcinoma per genotypic category. Genotypic categorization of BSEP1/1, BSEP1/3 and BSEP3/3 groups is defined in the methods section. Log rank tests. *BSEP (bile salt export pump).*

Fig. Graphical abstract: Created with biorender.com

Figure 1.

Figures

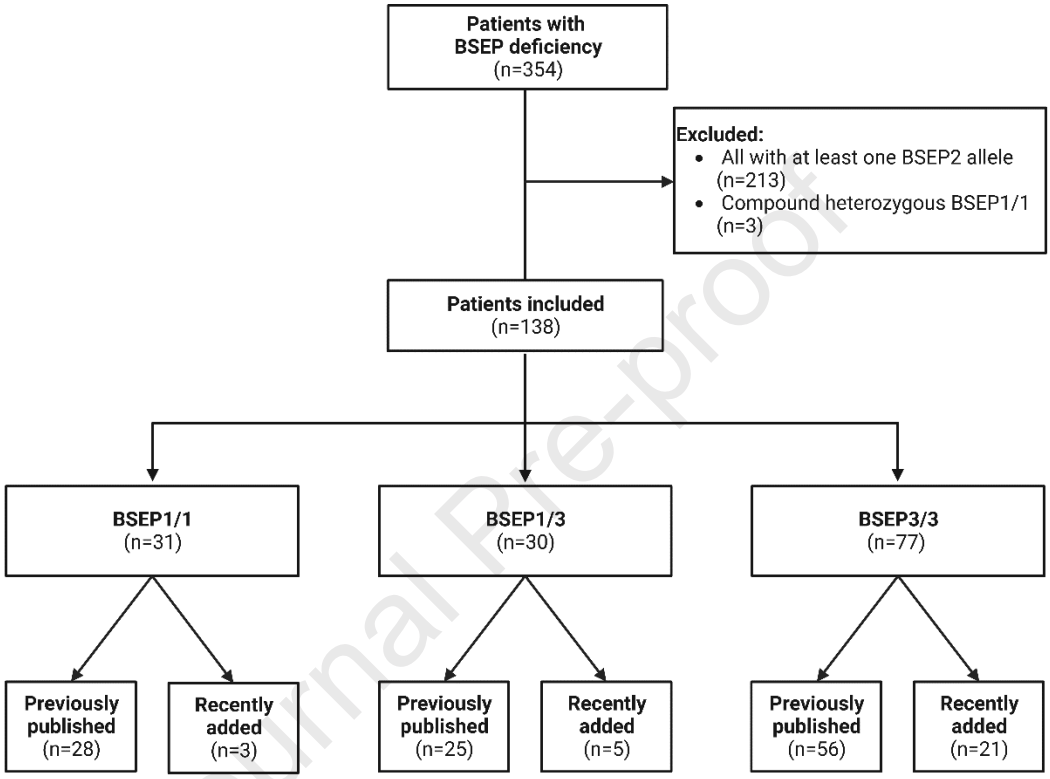


Figure 2.

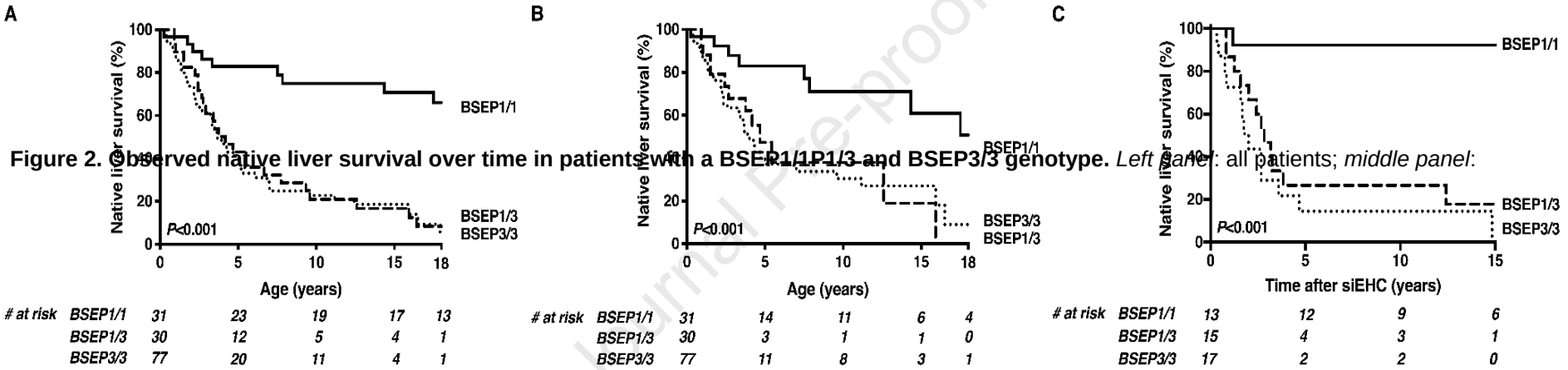
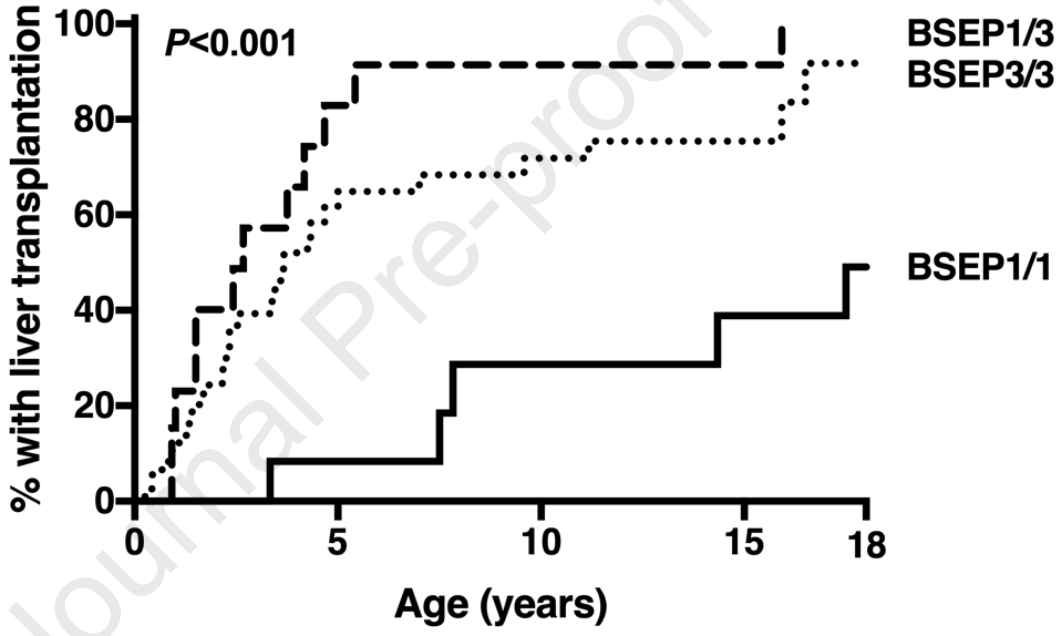


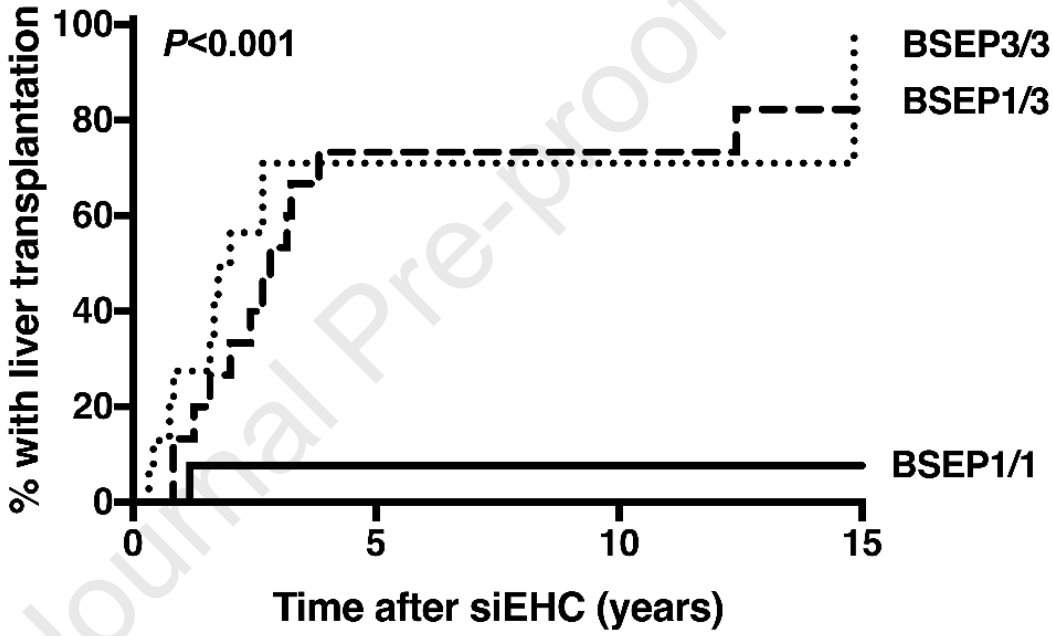
Figure 2. Observed native liver survival over time in patients with a BSEP1/1P1/3 and BSEP3/3 genotype. *Left panel*: all patients; *middle panel*:

Figure 3.



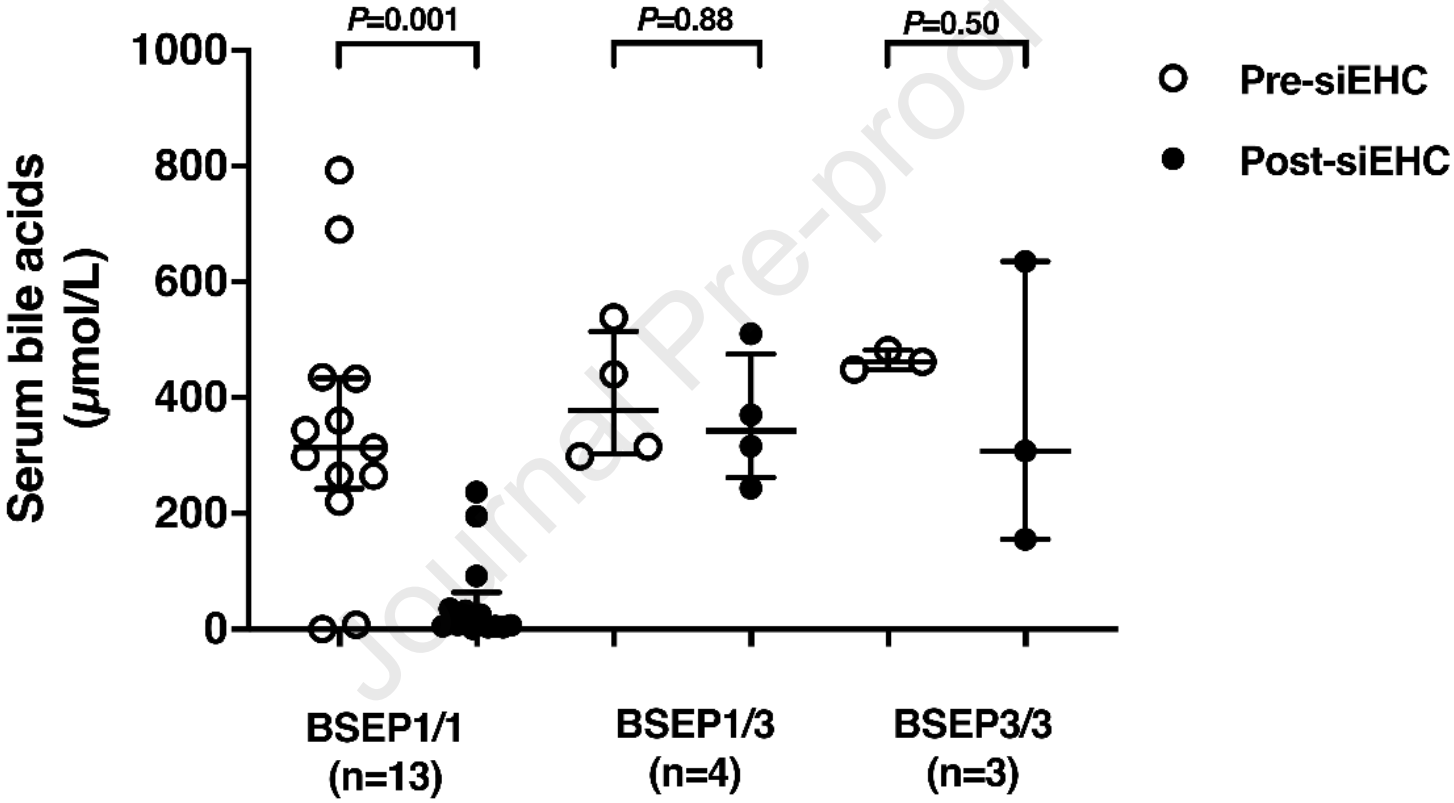
# at risk						
	BSEP1/1	18	10	7	6	4
	BSEP1/3	15	2	1	1	0
	BSEP3/3	60	11	8	3	1

Figure 4.



# at risk					
	BSEP1/1	13	12	9	6
	BSEP1/3	15	4	3	1
	BSEP3/3	17	2	2	0

Figure 5.



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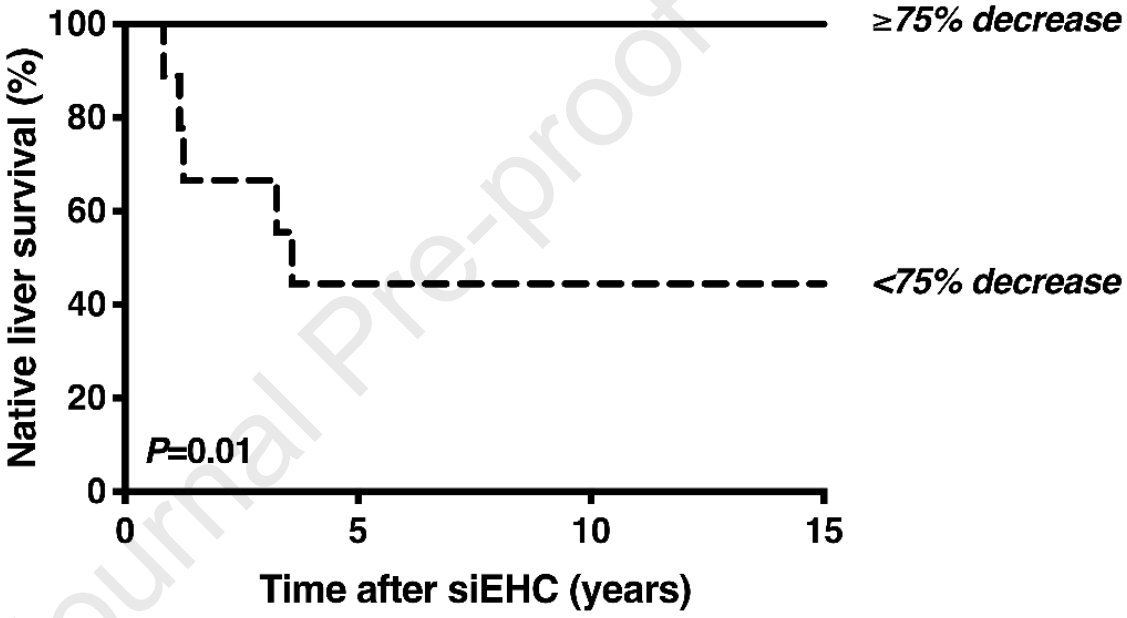
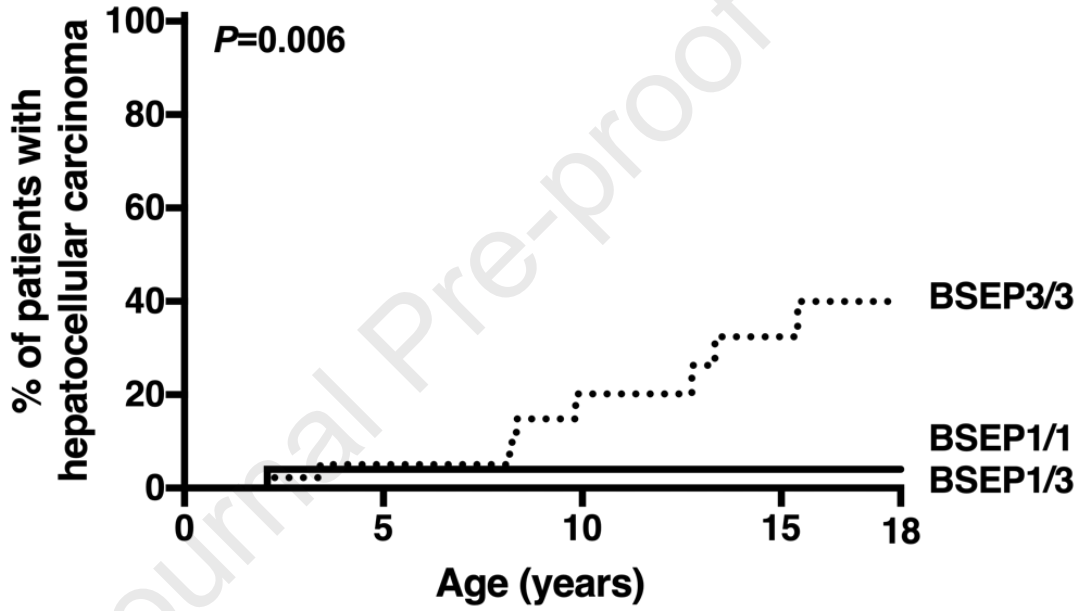


# at risk					
	≥75% decrease	9	9	6	5
	<75% decrease	10	4	3	1

Figure 7.



# at risk						
	BSEP1/1	31	22	19	19	15
	BSEP1/3	30	17	10	9	3
	BSEP3/3	77	26	15	9	5

JHEP Reports Highlights

- Homozygous E297G or D482G mutations in ABCB11 are associated with long-term native liver survival
- Native liver survival in BSEP deficiency patients who carry two truncating mutations is poor
- One protein truncating mutation in ABCB11 overrides the phenotypic effect of a concomitant E297G or D482G mutation
- The indicated heterozygous genotype is also associated with a poor response to surgical biliary diversion
- No hepatocellular carcinoma was observed in patients with a E297G or D482G mutation and a truncating ABCB11 mutation

JHEP Reports Data Availability Statement

The centers participating in the NAPPED Registry keep ownership over the data of their own patients. The data therefore remains confidential.

Data not available / The data that has been used is confidential