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ABCB4 Gene Variants as Potential Modifiers of Biliary Atresia Outcomes

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ABCB4 Gene Variants as Potential Modifiers of Biliary Atresia Outcomes

By

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B.A., University of Pennsylvania, 2009

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ABSTRACT

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Biliary atresia (BA), a progressive neonatal cholestatic liver disease, is the leading indication for pediatric liver transplantation in the United States. The Kasai hepatoportoenterostomy is successful in restoring bile drainage and delaying liver transplantation in a subset of patients. Early age at operation is generally associated with improved clinical outcomes, although the optimal age at surgery is contested. African Americans with BA may undergo the Kasai procedure at a later age than white patients. Mutations in the *ABCB4* gene, a phospholipid floppase, are associated with severe cholestatic liver disease in children and adults. We performed a case-control study comparing *ABCB4* variants between subjects from the Childhood Liver Disease Research and Education Network (ChiLDREN) database who required a liver transplant by age 2 (Early Transplant, ET, n=98) with those who survived with their native liver 4 years after the Kasai with a normal platelet count (Survive with Native Liver, SNL, n=97). Controlling for race, each 1-day delay in age at Kasai increased the odds of ET outcome by 3% ($p<0.001$). The A934T polymorphism was significantly associated with ET outcome (Trend Test, $p=0.044$) and was only found in African American subjects. A linked study examining an independent cohort of African American ChiLDREN subjects (n=104) identified 2 heterozygotes for p.A934T. One subject received a liver transplant less than 1 year after Kasai, and 1 survived with his native liver until age 9. A significant difference in survival with native liver at 2 years post-Kasai was not detected until after 120 days of age ($p=0.015$). A total serum bilirubin $\geq$ 2 mg/dl at 3 months post-Kasai was a stronger predictor of death or transplant by 2 years after Kasai than age at surgery (OR=12.89, 95% CI: 1.31, 127.26). Heterozygosity for p.A934T may expedite the progression of biliary cirrhosis among African Americans with BA who undergo Kasai. Kasai at an early age may not be an important prognostic determinant of survival with native liver among African Americans. More comprehensive analyses of African Americans with BA, inclusive of patients who forego the Kasai, and functional assays of A934T must be performed to confirm these findings.

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INTRODUCTION

Biliary atresia (BA) is a devastating cholestatic liver disease that is uniformly fatal in childhood unless patients receive an orthotopic liver transplant or undergo a surgical operation, the Kasai hepatoportoenterostomy procedure, intended to restore bile flow (1, 2). International population-based studies typically report that approximately 50% of patients who receive the Kasai survive with their native liver until 4 to 5 years after the surgery (3). Despite significant patient heterogeneity in the progression of biliary cirrhosis, the importance of genetic variants in prognosticating outcomes in BA after the Kasai is unclear. A hypothesis-generating whole exome sequencing pilot study discovered a higher frequency of variants in the *ABCB4* gene among BA subjects who died or required a liver transplant in the first year of life compared to those who survived at least 4 years after Kasai with their native liver (4).

To further delineate the impact of *ABCB4* variants on clinical outcomes of BA, a case-control genetic association study and a linked cohort study were performed using subjects enrolled in the Childhood Liver Disease Research and Education Network (ChiLDREN) database. The objective of the case-control study was to determine if the presence of non-synonymous variants in the *ABCB4* gene is a risk factor for early liver transplant in patients with BA who have undergone the Kasai procedure. Genetic variants were compared between children with poor outcomes, consisting of liver transplantation or death by 2 years after Kasai (Early Transplant, ET), and children with favorable outcomes after Kasai, defined as survival with native liver to age 4 and normal platelet count (Survival with native liver, SNL). The identification of the single-nucleotide polymorphism, p.A934T, present in the ET group among African American subjects

motivated a second study to examine outcomes by genotyping a larger cohort of African Americans. Since this constituted the largest population of African American patients with BA studied to-date, we also examined the effect of non-genetic determinants of outcomes, including the age at Kasai, on survival with native liver.

The hypothesis of the cohort study was that African American subjects who underwent the Kasai procedure at a later age had decreased survival with native liver after 2 years compared to subjects who received the Kasai at a younger age. A cohort analysis, stratified by age using cutpoints of 45, 60, 90 and 120 days, was conducted to determine if the cohorts exhibited differential survival at 2 years after Kasai. By extension, we sought to determine if there was a specific age threshold beyond which survival with native liver was compromised among African American subjects. We also examined whether failure to clear jaundice by 3 months after the Kasai, as defined by a total serum bilirubin $\geq$ 2mg/dl, was associated with an increased risk of death or liver transplant by 2 years after the Kasai. Finally, we aimed to assess the impact of the A934T polymorphism on survival with native liver after Kasai, although a survival analysis was not performed due to the rarity of this variant in our sample.

BACKGROUND

Biliary atresia is a neonatal disease that causes progressive fibrosis and inflammatory obstruction of the extrahepatic and intrahepatic bile ducts. Unless surgical intervention is performed in the first two years of life, BA inevitably results in cholestasis and liver failure (1, 5, 6). The incidence of BA is reported to range widely from approximately 1 in 5,000 live births in Taiwan to 1 in 19,000 live births in northern Europe (7, 8). The etiology has not been elucidated, although there is a putative role for environmental and infectious exposures, immune dysregulation, and a genetic predisposition for the disease (6). The majority of infants present with isolated BA and lack associated congenital anomalies (1, 2).

The Kasai hepatoportoenterostomy procedure is a palliative operation effective at restoring bile flow in a subset of infants. Multiple factors affect the success of the Kasai in delaying liver transplantation, including the patient's age, cirrhotic histology at the time of surgery, postoperative clearance of jaundice, experience of the surgical center, anatomic variation of the biliary remnant, and the presence of associated congenital anomalies (1, 9, 10). In the United States, BA is the most common indication for liver transplantation in children (11).

The short-term efficacy of the Kasai is commonly evaluated on the basis of clearance of jaundice within 3 months after the operation. Indeed, patients who achieve a total serum bilirubin less than 2mg/dl at any time point after the Kasai, evidence of repaired biliary drainage, are more likely to survive into adulthood with their native liver intact (10, 12-14). Conversely, patients with unremitting cholestasis after the Kasai often require evaluation for liver transplantation in the first few years of life (8, 15).

Consequently, some authors utilize transplant-free survival at 2 years after the Kasai as an indicator for surgical success (10, 12, 16, 17). This metric is presumed to reflect factors associated with surgical technique and immediate pre- and post-operative management of treated infants, as well as early diagnosis (12).

After receiving the Kasai, patients with BA display a marked stratification of outcomes during childhood, commonly measured as 4 and 5 year transplant-free survival. Studies of European, Asian, and American populations consistently demonstrate that only 30-60% of children survive with their native liver 4 to 5 years after the Kasai procedure (8, 18-22). Despite this steep decline in transplant-free survival early in life, epidemiological studies of long-term outcomes for BA have arrived at mixed conclusions regarding the eventual need for transplantation. Data from a nationwide French cohort study suggests that, among children who do not require a liver transplant by the age of 5, over 70% survive into adulthood with their native liver (21). However, other studies from the Netherlands and France inclusive of all patients that receive the Kasai consistently found that by 20 years after surgery, only 20-30% of patients with BA remain alive with their native liver (23, 24).

Age at the time the Kasai is performed has received considerable scrutiny as a prognostic indicator for clinical outcomes in BA. While it indisputable that sustained cholestasis can lead to irreversible liver damage in an untreated infant, the evidence favoring a specific age cutpoint for the operation has been mixed (25). Multiple studies have demonstrated superior outcomes if the Kasai is performed no later than 30 or 45 days of age (21, 22, 26). However, other studies did not identify a significant effect of age on transplant-free survival until Kasai was performed after 60, 75, or even 90 days,

and some studies failed to find an effect of age altogether (8, 10, 27-29). In 2007, the biliary atresia working group sponsored by the National Institutes of Health acknowledged that Kasai prior to 60 to 90 days of life was associated with improved outcomes in BA (30). Thus, it is generally accepted that the effectiveness of the Kasai in delaying liver transplantation during childhood is dependent on the timing of the procedure, with the greatest benefits seen in younger patients, although the precise age at which survival with native liver is compromised is unclear. Nevertheless, the outcome for an individual patient is difficult to predict, and reports of patients with a favorable prognosis despite receiving a Kasai after 4 months of age support a plausible role for genetic variance in shaping the adaptive response to cholestasis among patients with BA (14, 23).

It has been proposed that visual detection of jaundice is challenging in patients with more darkly pigmented skin, which could mask the degree of hyperbilirubinemia in affected infants, a problem that could be compounded by inexperienced caretakers who may fail to perceive other clinical hallmarks of BA like acholic stools (7, 31). It is conceivable that African American race may be associated with a delay in the recognition of neonatal cholestasis and arrival at the diagnosis of BA, which would be reflected in a later age at Kasai. A retrospective cohort study of all children who underwent the Kasai at a tertiary referral center in the Southeastern US between 1986-1999 demonstrated that African American patients were significantly older at the time of surgery compared to white patients, with a trend toward worse short and long term outcomes, but the study was limited by a sample size of only 30 African American subjects (32).

To date, few consortium-based investigations of outcomes in BA among centers in North America have been conducted which specifically address metrics such as age at the time of Kasai and survival with native liver among racial subpopulations. Shneider et al. did not find that the proportion of subjects belonging to diverse racial and ethnic subgroups differed between clinical outcome groups at 2 years post-Kasai (12). However, this study found that non-Hispanic white subjects were more likely to undergo the Kasai at an earlier age compared with African American subjects and those belonging to other racial and ethnic groups, although only 17 African Americans were included in the analysis. Superina et al. included fewer than 38 African American subjects and did not find that the probability of jaundice clearance at 3 months after Kasai or the hazard ratio for the risk of death or transplant differed according to race (10).

The imperative to investigate the course of BA among African American patients is underscored by epidemiological data demonstrating that incidence of BA is higher among children born to African American mothers than white mothers. One study of the epidemiology of BA using the New York State Congenital Malformations Registry spanning 1983-1998 found the rate of delivering a child with BA in African American mothers was 1.94 times the rate in white mothers (33). Similarly, a population-based cohort study of birth defects in Atlanta found a higher incidence of BA among non-white children relative to white children, with African American infants comprising the majority of cases (34).

The paucity of effective medical interventions that can slow the progression of biliary cirrhosis has generated a tremendous need to identify potentially modifiable factors that may explain the marked heterogeneity in disease course, especially biological

targets that may be amenable to rationally designed therapeutics (30). Despite the disparity in outcomes for children with BA, the importance of individual genetic variation in contributing to phenotypic severity is unknown. To date, genetic modifiers of outcomes after the Kasai procedure have not been identified.

To better understand genetic determinants of BA outcomes, an unbiased, whole exome sequencing study of 20 children with isolated BA was performed to discover genes that may modulate disease severity. Whole exome sequencing has revolutionized the search for disease-causing genes, particularly for rare Mendelian disorders such as Miller Syndrome and Freeman-Sheldon syndrome (35, 36). Examining the protein-coding region of the genome is an efficient approach to identify deleterious variants, since pathogenic mutations are predominantly located within exons, and the functional significance of non-coding variants can be difficult to interpret (36). We selected the *ABCB4* gene for a candidate gene-association study after the whole exome sequencing pilot determined cases harbored disproportionately more non-synonymous variants at this locus compared with controls (4). In light of the pathophysiology of cholestatic liver disease, it is attractive to speculate that missense variants in *ABCB4* could accelerate the progression of liver disease in children with BA.

The *ABCB4* gene encodes for a member of the evolutionary conserved ATP-binding cassette transporter family, also known as multidrug resistance protein 3 (MDR3), which is a phospholipid translocator expressed at the apical membrane of hepatocytes (37). *ABCB4* operates as a "floppase" to excrete a crucial component of bile, phosphatidylcholine, into the canaliculus (38). Missense genetic variants in *ABCB4* reduce the functionality of the transporter and consequently lower the phospholipid

concentration of bile (39). The imbalance in bile composition prevents the formation of mixed micelles necessary to solubilize cholesterol and the unimpeded detergent properties of hydrophobic bile salts cause biliary epithelial injury, as illustrated in Figure 1 (40, 41). As a consequence of this hostile microenvironment, a cascade of inflammation and fibrosis eventually obstructs flow through the bile ducts (42). Mutations in *ABCB4* are implicated in a clinically heterogeneous spectrum of liver disease including progressing familial intrahepatic cholestasis type 3, low phospholipid-associated cholelithiasis, intrahepatic cholestasis of pregnancy and drug-induced cholestasis (40).

The role of *ABCB4* variants as determinants of disease progression in BA has never been explored. We sought to investigate the role of *ABCB4* variants in BA outcomes by examining a sample of racially diverse subjects who exemplified disparate clinical outcomes after the Kasai procedure in order to assess the association of each variant with ET outcome. After identifying the A934T polymorphism in association with ET outcome, we sought to genotype this variant in a larger, independent cohort of African American subjects. Furthermore, we aimed to quantify the effect of age at Kasai on survival with native liver in the African American population and establish a threshold for age beyond which clinical outcomes are compromised.

METHODS

Two linked studies were performed using independent samples of BA subjects. The de-identified DNA and clinical characteristics for all subjects were obtained from the National Institute of Diabetes and Digestive and Kidney Diseases-supported Childhood Liver Disease Research and Education Network. This consortium-based database represents the largest longitudinally collected repository in the world for pediatric liver disease, with 16 centers in North America. All subjects were enrolled between 2004 and 2014. For the case-control study, a multiracial sample of subjects was selected on the basis of dichotomous clinical outcomes in BA, in order to assess if *ABCB4* variants segregated between children who fared well with their native liver and those who required an early liver transplant. After identifying the A934T polymorphism as a potential genetic modifier of the risk of early liver transplant, an unrelated cohort of African American subjects was genotyped to investigate the effect of A934T and other clinical parameters on survival with native liver.

Case-Control Study. The hypothesis of the case-control study was that missense variants in *ABCB4* increase the risk of death or liver transplant by age 2 in BA. Two years of age was chosen as a more stringent endpoint compared with 2 years after Kasai, since timing of the procedure is variable. We performed a case-control study to assess the association between non-synonymous variants and clinical outcomes. The inclusion criteria for cases (Early Transplant, ET) consisted of death or liver transplantation by 2 years of life. Eligible subjects for the control group (Survival with Native Liver, SNL) were alive with their native liver at least 4 years after undergoing the Kasai procedure, without any reports of gastrointestinal bleeding, varices, or ascites, and a platelet count

greater than 150,000/μl, intended to exclude subjects with portal hypertension and liver cirrhosis. Subjects were excluded from the study if they had a syndromic phenotype of BA, including situs inversus, asplenia or polysplenia, the presence of a choledochal cyst, cause of death unrelated to liver disease, and if the Kasai procedure was not performed. A diagram depicting subject selection is shown in Figure 2. The Emory University Institutional Review Board determined that this study was exempt from IRB review because it did not constitute human subjects research.

Genotyping was performed using the Sanger capillary electrophoresis method for all 27 coding exons of the *ABCB4* gene, across splice junctions, and the promoter region. Variants were called by comparison to reference NM_018849.2. After two rounds of Sanger sequencing, we were able to achieve >90% coverage of exons across all subjects, and had 100% coverage of exon 23 spanning the A934T polymorphism.

Clinical data obtained for all subjects included self-reported race, age at Kasai, and age at the time of death or liver transplant. Genetic data regarding the minor allele frequency in ancestral populations was derived from the National Heart, Lung, and Blood Institute Exome Sequencing Project and subsequently verified using the Exome Aggregation Consortium Data Set (43, 44). The PolyPhen-2 software tool was used to generate prediction scores for the functional effects of amino acid substitutions (45). Sample-size considerations for a rare variant association study were undertaken based on the work of Zuk *et al*., 2014 (46). We estimated that 100 cases would be required to detect an association for an allele that increased the odds of ET outcome by 10 times and was present in the population at a minor allele frequency of around 1%.

All statistical analyses were performed using SAS 9.4 unless otherwise noted (SAS Institute, Cary NC). A p-value ≤ 0.05 was considered statistically significant. Descriptive statistics were performed to examine the distribution of self-reported race by outcome group and mean and median age at Kasai stratified by race. A chi-square test was used to assess differences between the proportion of racial groups between ET and SNL outcome groups. One-way analysis of variance F-test was performed to compare the mean age at Kasai between racial groups. A box-and-whiskers plot was constructed depicting the mean age at Kasai for each outcome group stratified by race, with whiskers extending to the interquartile range [25%, 75%]. Two-sided *t*-tests were conducted for pairwise comparisons of mean age at Kasai between outcome groups for each race, and between races within outcome groups. A multivariate logistic regression was conducted to determine the effect of each additional day of age until Kasai and racial group on the odds of ET outcome, modeling white race as the reference.

The Cochran-Armitage trend test was performed to determine the association of each variant in the *ABCB4* gene with ET outcome. The trend test uses the genotype as the unit of analysis rather than the allele count and does not assume Hardy-Weinberg Equilibrium. Due to the small number of individuals who were heterozygous for rare *ABCB4* variants, and the absence of homozygotes in our sample, this was considered the most appropriate test for our study. The trend test was executed using PLINK v1.07 (47, 48).

Cohort Study. The hypothesis of the cohort study was that African American subjects who undergo the Kasai at an earlier age have increased transplant-free survival relative to those who undergo the Kasai at a later age. Our objectives were to establish a

threshold age beyond which survival with native liver is decreased among African American subjects with BA and assess whether clearance of jaundice at 3 months, as defined by a total serum bilirubin <2mg/dl, is associated with increased transplant-free survival. In addition, we sought to determine if heterozygous status for the A934T polymorphism is associated with reduced transplant-free survival after the Kasai. We performed a retrospective case-control study inclusive of all African American subjects with isolated BA in the ChiLDREN database with DNA available in the repository, for which the age at Kasai was known, and the age at death or liver transplantation was known for subjects who did not survive with their native liver.

Genotyping was performed using the Sanger capillary electrophoresis method for exon 23 of the *ABCB4* gene. Variants were called by comparison to reference NM_018849.2. Clinical data obtained for all subjects included self-reported race, age at Kasai, age at death or liver transplant, total serum bilirubin 3 months after the Kasai, and the timing of the last recorded laboratory data, a proxy for the length of follow-up for those subjects that did not experience an adverse event. The majority of subjects were missing laboratory data. Consequently, the Kaplan-Meier curve stratifying subjects on the basis of jaundice clearance at 3 months and the univariate logistic regressions examining jaundice clearance were restricted to patients for which data was available. The ChiLDREN Data Coordinating Center was queried for all subjects with implausible clinical data and these data were subsequently corrected. *A priori* power calculations were not performed as this cohort was intended to represent all African American BA subjects in the ChiLDREN database.

Descriptive statistics were performed for all subjects examining gender, age at Kasai, mean age at death or liver transplantation, and median age at last follow-up for subjects who did not experience an adverse outcome. In addition, descriptive statistics were performed examining the mean and median age at Kasai in our sample, including 95% confidence intervals, and the proportion of subjects alive with their native liver at 2 and 4 years after Kasai, as well as the 95% confidence interval for each binomial proportion. The distribution of age at Kasai among African American subjects was illustrated with a histogram. A Kaplan-Meier survival curve was constructed to depict time to death or liver transplant. We obtained point estimates for quartiles, calculated as the mean time at which a percentage of subjects experienced the event, and 95% confidence intervals.

We conducted survival analysis to examine the effects of covariates on survival with native liver at 2 years after Kasai. The outcome was defined as death or liver transplant and all observations were censored at 2 years after the time of Kasai. Subjects were divided into 5 cohorts based on age at Kasai using cutpoints of 45, 60, 90, and 120 days. For each cohort, summary statistics were calculated to examine the mean age at the time of death or liver transplant and the median length of follow-up for subjects who did not experience an adverse event. Kaplan-Meier curves were generated for each cohort and the overall difference between the survival curves was analyzed, as well as the difference between each pair of cohorts. To assess the presence of a threshold age beyond which survival with native liver is reduced, we bisected the cohort to compare the Kaplan-Meier curves for subjects who underwent the Kasai up to 45, 60, 90, and 120 days of age, with all those who received the procedure at a later age. Subsequently, we

compared Kaplan-Meier curves for subjects who achieved a total serum bilirubin <2 mg/dl by 3 months after Kasai with subjects who did not experience jaundice clearance at 3 months. The Log-Rank test was used for all comparisons of survival curves, and when appropriate, the Wilcoxon test was also used. The Tukey-Kramer method was implemented to control the familywise error rate when performing multiple pairwise comparisons.

Univariate logistic regressions were performed to determine the odds ratio for death or liver transplant at any time point after Kasai modeling age at Kasai as a continuous variable as well as a dichotomous variable comparing subjects who received the Kasai up to 45, 60, 90, 120 days of age with those who received the Kasai at a later age. We examined the effect of age at Kasai, modeled as a continuous predictor, on the outcomes of jaundice clearance at 3 months post-Kasai and death or liver transplant by 2 years after Kasai for subjects with available laboratory data. Lastly, the effect of bilirubin clearance at 3 months post-Kasai was examined on the odds of death or transplant by 2 years after Kasai.

RESULTS

Case-Control Study. A total of 195 ChiLDREN subjects, 98 ET and 97 SNL, were enrolled. All subjects in the ET group received a liver transplant, and none of the subjects died. We categorized subjects according to self-reported race into white, African American, and other groups. The other category was comprised of subjects who self-reported their race as other, Asian, American Indian or Alaska Native, or refused to indicate their race (Table 1). The distribution of self-reported race was similar between ET and SNL groups. Summary statistics on the age at Kasai for each racial group are shown in Table 2. The mean age at Kasai was similar across all races. A pairwise *t*-test comparing the mean age at Kasai between ET and SNL outcome groups when aggregating all races demonstrated the ET group was significantly older (Figure 3a). Stratifying by race, white subjects in the ET group were significantly older compared to white subjects in the SNL group; the mean age at Kasai was comparable for African American and other subjects (Figure 3b).

The results of a multivariate logistic regression examining the effect of race and age at Kasai on outcome are shown in Table 3. African American race, relative to white race, did not significantly increase the odds of ET outcome when controlling for age at Kasai. The odds for ET outcome were 62.6% lower for subjects of other race, controlling for age at Kasai. Each 1-day increase in age until Kasai increased the odds for ET outcome by 3% when controlling for race ($p < 0.001$) (Table 3).

Genotyping of *ABCB4* identified 1 splice site, 9 synonymous and 9 non-synonymous variants. The approximate location of all amino acid substitutions in the ABCB4 transporter identified in more than 1 subject is depicted in Figure 4. At least 1 variant was

identified in 90% and 92% of subjects in the ET and SNL groups, respectively. 29% of subjects in the ET group and 25% of subjects in the SNL group were heterozygous for at least 1 missense variant. The distribution of each variant present in more than 1 subject, separated by heterozygous and homozygous status, between ET and SNL groups for each racial category is shown in Table 4. Omitted polymorphisms present in 1 subject each in the SNL group include T34M, A232A, T438T, V474L, and E1058K, present in 1 subject each in the SNL group, as well as E528D and R590Q, present in 1 subject each in the ET group. The Cochran-Armitage trend test demonstrated that the polymorphism A934T was significantly associated with ET outcome when examining all races, as well as the silent polymorphisms S49S and F153F ($p=0.044$ for all) (Table 5). These variants were exclusively found in 4 African American subjects in the ET group and not present in any subjects in the SNL group. The PolyPhen-2 prediction algorithm estimated that p.A934T is "probably damaging" to protein function with a score of 0.99 (Table 5).

Cohort Study. A total of 104 African American subjects with BA in the ChiLDREN database met the inclusion criteria. Fifty-seven subjects underwent a liver transplant and 1 subject died as a direct complication of end-stage liver disease without undergoing transplantation. Summary measures of age at Kasai and outcomes after 2 and 4 years are summarized in Table 6. The mean age at Kasai was 72.1 ± 37.6 days. The distribution of age at Kasai is depicted in a histogram of count data (Figure 5).

Genotyping of the A934T polymorphism identified 2 heterozygous subjects. One subject underwent the Kasai at 111 days and received a liver transplant at 381 days of age. The other subject underwent the Kasai at 67 days and developed splenomegaly, thrombocytopenia, ascites, and a variceal bleed during age 6 of life. He had not yet

received a transplant by the time of his last follow-up at age 9. We were unable to compare Kaplan-Meier survival curves between subjects heterozygous for p.A934T and the wild type amino acid due to the low frequency of this polymorphism and the small number of heterozygotes in our dataset. However, since we had hypothesized that subjects heterozygous for p.A934T would have decreased transplant-free survival compared to the rest of the cohort, these 2 subjects were subsequently eliminated from any further survival analysis.

The Kaplan-Meier survival curve demonstrating survival with native liver after the Kasai procedure for the remaining 102 subjects is shown in Figure 6. A sharp decline in survival with native liver is evident for the first 1000 days after the operation, after which the curve begins to assume a plateau configuration. Table 7 displays point estimates of time-to-event for the 50^{th} and 25^{th} percentiles of subjects who died or received a liver transplant. We were unable to obtain an estimate for the 75^{th} percentile due to the large number of censored observations in our sample. The median survival time for this cohort was 641 days.

Next, we divided subjects into 5 cohorts according to age at Kasai. For each cohort, the mean age at death or transplant and the median age at the time of last follow-up for subjects who survived with their native liver is depicted in Table 8. Due to the wide variability in age at the time of last follow-up, we elected to focus on 2-year survival with native liver as the primary outcome for the age cohort analysis. The data for all subjects was censored at 2 years after the Kasai, and 6 subjects who were censored ultimately experienced an event later in life. Kaplan-Meier curves illustrating 2-year survival with native liver post-Kasai according to the age at surgery are shown in Figure

7, and demonstrated a significant overall difference as determined by the Log-Rank (p=0.044) and Wilcoxon (p=0.030) tests. After adjusting for multiple pairwise comparisons, the cohort of subjects who underwent the Kasai between 91-120 days had significantly increased transplant-free survival compared to subjects who underwent the Kasai after 120 days (corrected Log-Rank test p=0.05, corrected Wilcoxon test p=0.022) (Table 9). To determine if there was a threshold effect for age at Kasai, we compared Kaplan-Meier curves for subjects who underwent the Kasai up to 120 days with subjects who received surgery after 120 days. Subjects who underwent the Kasai after 120 days had significantly decreased transplant-free survival at 2 years (Log-Rank p=0.015, Wilcoxon p=0.006) (Figure 8). Survival curves comparing subjects who underwent the Kasai up to ages 45, 60, and 90 days with subjects who underwent the procedure at an older age were not significantly different (data not shown).

Univariate logistic regressions were performed to examine the effect of age at Kasai on the odds of death or transplant at any time point after surgery. Age was analyzed as a continuous variable as well as a binary variable comparing subjects who received the Kasai after 120 days of life with those who underwent surgery at an earlier age. The odds for death or transplant did not increase with each additional day until Kasai or if the Kasai was performed after 120 days of life (Table 10).

Laboratory values for total serum bilirubin at 3 months post-Kasai were available for 28 subjects. Subjects who successfully cleared their jaundice at 3 months (n=10), as defined by a total serum bilirubin <2mg/dl, had significantly increased 2-year transplant-free survival compared to those with a higher serum bilirubin level (Figure 9). Univariate logistic regressions examining the effect of age at Kasai on death or transplant at 2 years

post-Kasai and on successful clearance of jaundice, as defined by a total serum bilirubin <2mg/dl at 3 months post-Kasai, showed no significant associations (Table 11). The risk of death or transplant by 2 years post-Kasai was 12.89 times higher for subjects who exhibited a total serum bilirubin of 2mg/dl or higher at 3 months post-Kasai compared with subjects who had lower bilirubin levels ($p=0.03$). Since none of the 28 subjects received a liver transplant more than 2 years after the Kasai, these odds ratios are equivalent when analyzing the entire length of follow-up.

DISCUSSION

We describe the first genetic study of *ABCB4* variants as potential modifiers of clinical outcomes in BA. After a hypothesis-generating whole exome sequencing pilot study identified the *ABCB4* gene as a priority locus, we performed a genetic association study and a linked cohort study to evaluate the impact of *ABCB4* missense variants on the risk of undergoing liver transplantation in childhood.

A case-control study examining a racially and ethnically diverse sample of BA subjects from the ChiLDREN database identified the A934T polymorphism as the only non-synonymous variant significantly associated with the outcome of death or liver transplantation by age 2. We found that p.A934T separated completely between African American subjects who underwent early transplant and those who survived with their native liver after the Kasai procedure. In a large US population database, p.A934T is disproportionately represented among African Americans, with a minor allele frequency of 1.23%, compared to less than 0.001% among European Americans (43). The alanine at position 934 is the first amino acid of the 11th transmembrane domain of the ABCB4 protein (49). The threonine substitution is expected to exert a damaging effect on protein function according to the PolyPhen-2 *in silico* prediction tool (50). As a consequence of the predicted functional ramifications and low minor allele frequency, we decided to prioritize p.A934T for further validation genotyping in an independent cohort of African American subjects. This decision was made prior to the Exome Aggregation Consortium (ExAC) public data release, characterizing genetic variation in over 60,000 presumably healthy individuals, wherein the A934T polymorphism is listed at a similar minor allele frequency of around 1.5% in individuals with African ancestry (44). Since it is expected

that deleterious variants undergo negative selective pressure in the population, proposing that a moderately rare variant is maladaptive is consistent with evolutionary theory (51, 52). Strikingly, there are no homozygotes for p.A934T listed in the ExAC dataset, which further suggests selection against this variant in the general population (44).

The pathogenic potential of p.A934T is underscored by descriptions of heterozygous adult patients who presented with low phospholipid-associated cholelithiasis (LPAC) or unexplained fibrosing cholestatic liver disease and did not possess other mutations in *ABCB4* (53, 54). One of the first case reports describing oral contraceptive pill-induced cholestasis identified p.A934T in a compound heterozygote female patient who also possessed a splicing mutation (55). Intriguingly, the 4 African American patients with p.A934T harbored a unique constellation of silent variants including p.S49S and p.F153F. Although it is difficult to ascertain the impact of silent polymorphisms using bioinformatics tools, synonymous mutations may affect gene expression and protein function by interfering with mRNA and protein stability and influencing transcriptional regulation (56). It is possible that these three polymorphisms represent a haplotype predictive of poor prognosis in BA; however, parental DNA sequencing is necessary to confirm that these alleles were inherited on the same chromosome before a haplotype effect can be asserted.

The cohort study only identified 2 patients out of 104 who were heterozygous for the A934T variant. The first subject underwent the Kasai at 67 days and developed signs of advanced liver disease at age 6 years. This subject retained his native liver unexpectedly late, until his last follow-up at age 9, but the severity of his cirrhotic manifestations renders it unlikely that he would survive into his teenage years without a

liver transplant. Conversely, the second subject underwent the Kasai at 111 days and received a liver transplant less than 1 year after the Kasai procedure. It is interesting to consider the synergistic deleterious effects of a relatively late Kasai procedure in the context of increased hydrophobicity of bile, and whether *ABCB4* missense variants potentiate the progression of fibrosis in patients with late Kasai procedures.

In the case-control study, we identified missense variants in around 25% of subjects in the ET and SNL groups, and found that 20% of African Americans who did poorly after the Kasai possessed the A934T polymorphism. These results initially generated speculation that *ABCB4* variants may be involved in the etiopathogenesis of BA. However, since the cohort study identified p.A934T at a frequency that approximates the African American population as a whole, the high prevalence of the variant in the ET group in the case-control study is likely attributable to random error.

The presence of other non-synonymous variants, besides A934T, was not predictive of outcomes in BA, including the R652G polymorphism, which is common in healthy individuals across racial groups (57). However, many of the missense changes identified in our subjects have been reported in patients with LPAC, including p.R788Q, p.T175A, p.E528D, and p.R590Q (58, 59). Although none of these variants differed significantly between cases and controls, the R788Q polymorphism was slightly more prevalent in the SNL group, an unexpected finding since it is predicted to adversely affect protein function according to the PolyPhen-2 prediction score (50). However, p.R788Q is common to both African American and European American populations, with a minor allele frequency exceeding 7%, reducing the likelihood that this variant imparts a strong predilection for worsening liver disease. The polymorphism T175A was found in one ET

and one SNL subject, making it difficult to draw inferences from our sample, however, this is a known loss-of-function substitution with a minor allele frequency of 1.2%, consistent with a harmful mutation (43, 59).

Due to the life-threatening complications of rapid progression to liver failure after the Kasai**,** there is a critical need to identify potentially modifiable factors that could delay the onset of liver cirrhosis, especially parameters that can be leveraged for a public health intervention (30, 60). We examined age at the time of the Kasai procedure as the principal non-genetic determinant of outcomes in both the case-control and cohort study, with discordant results. In the case-control study, we found an unexpectedly large decrease in the risk of ET outcome among subjects that we classified in the other racial category, which included predominantly Asian and self-identified other subjects. Since we could not ascertain the racial or ethnic background of these subjects using genetic methods, it is unclear why a robust protective effect for this group was found. However, this finding may implicate either genetic or modifiable factors that merit further investigation in a well-phenotyped and comprehensively genotyped cohort of subjects.

The case-control study determined that later age at the time of Kasai was significantly associated with poor outcomes in BA, with each additional day conferring a 3% increase in the risk of early liver transplant. However, a significant difference in age between case and control groups was only observed for white subjects, which constituted the largest racial group in this sample. This disparity in age among white subjects may have accounted for the finding that the ET group was older, on average, than the SNL group when aggregating all races. Nevertheless, we found the average age for African American subjects at the time of Kasai was comparable to white and other subjects.

Controlling for age, African American race was not a significant predictor of clinical outcomes. This lessens the possibility that a race-specific effect for p.A934T was the result of a bias caused by delayed timing of Kasai among African American subjects.

In contrast, the cohort study did not find that each 1-day delay in the timing of Kasai increased the risk of death or liver transplant. To better evaluate the impact of age on survival with native liver after the Kasai, we performed an age cohort analysis, using cutpoints of 45, 60, and 90 days, since these ages have been reported in the literature to be associated with differential survival (8, 26, 27). We chose 120 days to represent the extreme of age, since it is unusual for a patient to receive the Kasai after 4 months. We elected to examine outcomes at 2 years after Kasai, since the median length of follow-up for subjects was highly variable, with some followed for just a few months after birth, whereas others were evaluated into the late teenage years.

Surprisingly, African American subjects who underwent the Kasai between 91-120 days appeared to have the highest survival with native liver 2 years after the surgery. Since these subjects comprised the second-oldest cohort, our finding differs from other studies that observed the highest short-term survival rates among patients operated on early in life, by age 30 or 45 days (21, 22, 26). We did not detect a decrease in the survival probability until subjects underwent the Kasai after 120 days. This threshold is later compared with results from other published studies, but confirms that a prolonged period of biliary obstruction during infancy will result in greater cumulative liver damage and worse clinical outcome. Ultimately, we found that effective restoration of bile flow 3 months after Kasai, as measured by a total serum bilirubin < 2mg/dl, was a stronger

predictor of survival with native liver at 2 years compared to early age at surgery, which is in agreement with a previously published study using the ChiLDREN database (10).

Taken together, our studies suggest that early age at Kasai may improve survival with native liver among white subjects, however, this advantage is eliminated among African Americans with BA, raising a provocative question into the underlying causes of this potential racial disparity. It is plausible that factors not considered in our analysis, such as socioeconomic status, could negatively impact survival with native liver among African American patients, but a more comprehensive study is needed to address this issue. The apparent lack of a benefit of early Kasai surgery among African Americans also seems to argue against the utility of a comprehensive national screening program for BA, which has been adopted by nations such as Taiwan, but has not been implemented in the US (7).

The available international data regarding the mean and median age at Kasai stratified by country is depicted in Table 12 (7, 8, 10, 12, 20-22, 26, 28, 61). Since the study conducted by Superina et al., also took advantage of the ChiLDREN dataset, it is possible that some of the subjects in our study have been published as part of that analysis (10). The mean age of 72 days for the African American subjects in our study is consistent with a smaller North American cohort from an earlier time period (12). The median age of 63 days is similar to the median age in a multiracial cohort from Canada and a Swiss national cohort, but is slightly later than ages from other Western and Northern European countries (8, 20-22, 26, 28).

The chief strength of these studies is rooted in our innovative approach. This is the first investigation of *ABCB4* variants in children with BA. Many genetic studies aim

for a racially homogenous sample since variant interpretation depends on knowledge of an individual's ancestral background. However, our case-control study deliberately included a large, racially and ethnically diverse patient population for genotyping. For the cohort study, our inclusion of over 100 African American subjects with BA is noteworthy since previously published North American-based studies have comprised fewer than 40 African American subjects (10, 12). Finally, we chose to examine a potential modifier gene, in contrast to the preponderance of research that focuses on causative genes by comparing variants in children with BA with non-BA control subjects.

We recognize that both studies have important limitations. The reliance on self-reported race and ethnicity precluded the ability to control for genetic admixture and misclassification of race. Since we could not verify ancestry though genetic methods, this could render our interpretation of rare variants less reliable. In addition, subjects who identified as Hispanic were categorized according to their self-reported race, although this could obscure the effect of darker skin color on clinical detection of jaundice and alter the timing of Kasai.

The case-control study was underpowered to detect extremely rare variants with a minor allele frequency <1% among racial subgroups. For subjects in the cohort study, we only acquired permission to genotype the A934T polymorphism, and it is possible that we missed other rare and *de novo* variants in the *ABCB4* gene that are unique to African Americans and could impair transporter function. As a consequence of the candidate gene approach, we did not examine mutations in other canalicular hepatobiliary transporters responsible for severe cholestatic disease, including the bile salt export pump,

introducing another layer of genetic variability that may conflate the clinical picture in our subjects (62).

Candidate gene studies are susceptible to false positive findings, which we attempted to minimize in the case-control study by focusing on subjects that exhibited the extreme ends of the phenotypic spectrum in BA. The case-control study excluded patients with intermediate clinical outcomes, therefore, this approach may understate the phenotypic burden imposed by *ABCB4* mutations in BA. Both studies utilized a hard endpoint of death or liver transplant as the primary outcome. However, there are regional differences in the availability of organs, and the time at which a subject is listed for transplant could serve as a more accurate surrogate measure of the severity of liver disease. Another limitation common to both studies is the omission of subjects who proceeded directly to liver transplantation and did not receive the Kasai, which represents a population with highly advanced liver disease that may possess pathogenic *ABCB4* mutations.

For both studies, we possessed limited or no clinical information on non-genetic factors that are important for survival with native liver, including clearance of jaundice after the Kasai, the anatomic subtype of BA, and the experience of the surgical center (10, 18, 19). Laboratory data, including 3 month bilirubin levels post-Kasai, were not available for the majority of subjects in the cohort study, so we were unable to assess other biomarkers that could have prognostic significance for early liver transplant. Ideally, a multivariate analysis incorporating these factors as covariates would have further delineated the impact of age at Kasai on survival with native liver in the cohort study.

The objective of the cohort study was to enroll as many African American subjects in the ChiLDREN database as possible, but due to missing clinical data, we were forced to exclude over 100 subjects that were otherwise eligible. Due to the variability in the length of follow-up for subjects in the cohort, we did not have 2-year outcome data for all subjects, which undermined our survival analysis.

We postulate that BA is a form of biological stress that leads to decompensation of the heterozygous state in patients with *ABCB4* missense mutations and promotes accelerated liver disease (63). This view parallels other models of *ABCB4*-linked disease, such as intrahepatic cholestasis of pregnancy and oral-contraceptive induced cholestasis, wherein the presence of heterozygous mutations in *ABCB4* is unmasked by a hormonal challenge (40, 64).

We suggest that genetic profiling of *ABCB4* may have direct therapeutic relevance for the treatment of BA and has the potential to improve risk stratification by identifying aggressive clinical phenotypes. Ideally, a patient's genomic profile will be used to prognosticate outcomes and personalize medical care with drugs such as ursodeoxycholic acid, especially since missense mutations in the *ABCB4* gene can serve as predictive biomarkers of the response to treatment (65, 66). This finding also creates opportunities to investigate new drugs, such as molecular chaperones, to rescue defective transporters, and use targeted agents that augment ABCB4 function. Moreover, ascertaining a patient's genotype may inform transplant decisions, since individuals with *ABCB4* mutations may not experience long-term benefits from the Kasai. Patients who receive an urgent transplant due to a failed Kasai may be exposed to iatrogenic risks from

the first operation and experience a higher rate of post-operative complications such as sepsis after undergoing transplant (67).

Future steps include investigating the full length of the *ABCB4* gene in a larger cohort of African American subjects, including those who proceed directly to liver transplant, which will be a more representative population to study the effects of damaging variants. Comprehensive functional validation must be performed for p.A934T to examine the effects on protein expression, trafficking to the apical membrane, and floppase activity. Consideration should also be granted to the silent variants, p.S49S and p.F153F, which accompanied this polymorphism in our 4 African American subjects and may also contribute to clinical outcomes. If functional assays of p.A934T confirm that ABCB4 floppase activity is impaired, this study represents the first description of a polymorphism, in any gene, with a pathophysiological link to accelerated liver disease in BA.

The vast majority of polymorphisms in *ABCB4* have not undergone functional validation to confirm pathogenicity, and isolating the clinical manifestations of a single nucleotide variant is complicated by considerations of allele penetrance and expressivity (68). One gene is incapable of explaining the range of clinical outcomes observed in BA, however, we hope this finding spurs further research into modifier genes with substantial translational potential, including *ABCB4*, and emphasizes the need to integrate genetic data with clinical trial design to better quantify treatment effects in pediatric liver disease. Widespread application of whole exome and whole genome sequencing approaches to racially and ethnically diverse patients is bound to uncover myriad additional modifier

genes in BA. Although BA is a rare disease, focusing on the *ABCB4* transporter can ultimately benefit both children and adults affected by cholestatic liver diseases.

REFERENCES

1. Hartley JL, Davenport M, Kelly DA. Biliary atresia. *Lancet* 2009;374(9702):1704-13.
2. Petersen C, Davenport M. Aetiology of biliary atresia: what is actually known? *Orphanet journal of rare diseases* 2013;8:128.
3. Jimenez-Rivera C, Jolin-Dahel KS, Fortinsky KJ, et al. International incidence and outcomes of biliary atresia. *J Pediatr Gastroenterol Nutr* 2013;56(4):344-54.
4. Mezina A, Gandhi K, Sabo A, et al. 845 Whole Exome Sequencing Identifies ABCB4 Gene Variants As Modifiers of Biliary Atresia Outcomes. *Gastroenterology*;146(5):S-928.
5. Mack CL, Sokol RJ. Unraveling the pathogenesis and etiology of biliary atresia. *Pediatr Res* 2005;57(5 Pt 2):87R-94R.
6. Moreira RK, Cabral R, Cowles RA, et al. Biliary atresia: a multidisciplinary approach to diagnosis and management. *Archives of pathology & laboratory medicine* 2012;136(7):746-60.
7. Hsiao CH, Chang MH, Chen HL, et al. Universal screening for biliary atresia using an infant stool color card in Taiwan. *Hepatology (Baltimore, Md)* 2008;47(4):1233-40.
8. de Vries W, de Langen ZJ, Groen H, et al. Biliary atresia in the Netherlands: outcome of patients diagnosed between 1987 and 2008. *The Journal of pediatrics* 2012;160(4):638-44 e2.
9. Wildhaber BE. Biliary atresia: 50 years after the first kasai. *ISRN surgery* 2012;2012:132089.
10. Superina R, Magee JC, Brandt ML, et al. The anatomic pattern of biliary atresia identified at time of Kasai hepatoportoenterostomy and early postoperative clearance of jaundice are significant predictors of transplant-free survival. *Annals of surgery* 2011;254(4):577-85.
11. Barshes NR, Lee TC, Balkrishnan R, et al. Orthotopic liver transplantation for biliary atresia: the U.S. experience. *Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society* 2005;11(10):1193-200.
12. Shneider BL, Brown MB, Haber B, et al. A multicenter study of the outcome of biliary atresia in the United States, 1997 to 2000. *The Journal of pediatrics* 2006;148(4):467-74.
13. McKiernan PJ, Baker AJ, Lloyd C, et al. British paediatric surveillance unit study of biliary atresia: outcome at 13 years. *J Pediatr Gastroenterol Nutr* 2009;48(1):78-81.
14. Nio M, Sasaki H, Wada M, et al. Impact of age at Kasai operation on short- and long-term outcomes of type III biliary atresia at a single institution. *Journal of pediatric surgery* 2010;45(12):2361-3.
15. Hung PY, Chen CC, Chen WJ, et al. Long-term prognosis of patients with biliary atresia: a 25 year summary. *J Pediatr Gastroenterol Nutr* 2006;42(2):190-5.
16. Grosfeld JL, Fitzgerald JF, Predaina R, et al. The efficacy of hepatoportoenterostomy in biliary atresia. *Surgery* 1989;106(4):692-700; discussion -1.

17. Lilly JR, Karrer FM, Hall RJ, et al. The surgery of biliary atresia. *Annals of surgery* 1989;210(3):289-94; discussion 94-6.
18. Davenport M, De Ville de Goyet J, Stringer MD, et al. Seamless management of biliary atresia in England and Wales (1999-2002). *Lancet* 2004;363(9418):1354-7.
19. Serinet MO, Broue P, Jacquemin E, et al. Management of patients with biliary atresia in France: results of a decentralized policy 1986-2002. *Hepatology (Baltimore, Md)* 2006;44(1):75-84.
20. Wildhaber BE, Majno P, Mayr J, et al. Biliary atresia: Swiss national study, 1994-2004. *J Pediatr Gastroenterol Nutr* 2008;46(3):299-307.
21. Chardot C, Buet C, Serinet MO, et al. Improving outcomes of biliary atresia: French national series 1986-2009. *J Hepatol* 2013;58(6):1209-17.
22. Schreiber RA, Barker CC, Roberts EA, et al. Biliary atresia: the Canadian experience. *The Journal of pediatrics* 2007;151(6):659-65, 65 e1.
23. Lykavieris P, Chardot C, Sokhn M, et al. Outcome in adulthood of biliary atresia: a study of 63 patients who survived for over 20 years with their native liver. *Hepatology (Baltimore, Md)* 2005;41(2):366-71.
24. de Vries W, Homan-Van der Veen J, Hulscher JB, et al. Twenty-year transplant-free survival rate among patients with biliary atresia. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association* 2011;9(12):1086-91.
25. Davenport M. Biliary atresia: clinical aspects. *Seminars in pediatric surgery* 2012;21(3):175-84.
26. Serinet MO, Wildhaber BE, Broue P, et al. Impact of age at Kasai operation on its results in late childhood and adolescence: a rational basis for biliary atresia screening. *Pediatrics* 2009;123(5):1280-6.
27. Davenport M, Caponcelli E, Livesey E, et al. Surgical outcome in biliary atresia: etiology affects the influence of age at surgery. *Annals of surgery* 2008;247(4):694-8.
28. Davenport M, Ong E, Sharif K, et al. Biliary atresia in England and Wales: results of centralization and new benchmark. *Journal of pediatric surgery* 2011;46(9):1689-94.
29. Carvalho E, Santos JL, Silveira TR, et al. Biliary atresia: the Brazilian experience. *Jornal de pediatria* 2010;86(6):473-9.
30. Sokol RJ, Shepherd RW, Superina R, et al. Screening and outcomes in biliary atresia: summary of a National Institutes of Health workshop. *Hepatology (Baltimore, Md)* 2007;46(2):566-81.
31. Watchko JF. Hyperbilirubinemia in African American neonates: clinical issues and current challenges. *Seminars in fetal & neonatal medicine* 2010;15(3):176-82.
32. Lee H, Lewis J, Schoen BT, et al. Kasai portoenterostomy: differences related to race. *Journal of pediatric surgery* 2001;36(8):1196-8.
33. Caton AR, Druschel CM, McNutt LA. The epidemiology of extrahepatic biliary atresia in New York State, 1983-98. *Paediatric and perinatal epidemiology* 2004;18(2):97-105.
34. Yoon PW, Bresee JS, Olney RS, et al. Epidemiology of biliary atresia: a population-based study. *Pediatrics* 1997;99(3):376-82.

35. Ng SB, Buckingham KJ, Lee C, et al. Exome sequencing identifies the cause of a mendelian disorder. *Nature genetics* 2010;42(1):30-5.
36. Ng SB, Turner EH, Robertson PD, et al. Targeted capture and massively parallel sequencing of 12 human exomes. *Nature* 2009;461(7261):272-6.
37. Nicolaou M, Andress EJ, Zolnerciks JK, et al. Canalicular ABC transporters and liver disease. *The Journal of pathology* 2012;226(2):300-15.
38. Smith AJ, Timmermans-Hereijgers JL, Roelofsen B, et al. The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. *FEBS letters* 1994;354(3):263-6.
39. Oude Elferink RPJ, Paulusma CC. Function and pathophysiological importance of ABCB4 (MDR3 P-glycoprotein). *Pflugers Arch* 2007;453(5):601-10.
40. Davit-Spraul A, Gonzales E, Baussan C, et al. The spectrum of liver diseases related to ABCB4 gene mutations: pathophysiology and clinical aspects. *Semin Liver Dis* 2010;30(2):134-46.
41. Donovan JM, Timofeyeva N, Carey MC. Influence of total lipid concentration, bile salt:lecithin ratio, and cholesterol content on inter-mixed micellar/vesicular (non-lecithin-associated) bile salt concentrations in model bile. *J Lipid Res* 1991;32(9):1501-12.
42. Trauner M, Fickert P, Halilbasic E, et al. Lessons from the toxic bile concept for the pathogenesis and treatment of cholestatic liver diseases. *Wiener medizinische Wochenschrift* 2008;158(19-20):542-8.
43. Exome Variant Server. Seattle, WA; 2015. (http://evs.gs.washington.edu/EVS/). (Accessed May 5 2014).
44. Exome Aggregation Consortium (ExAC). Cambridge, MA; 2015. (http://exac.broadinstitute.org/). (Accessed February 15 2015).
45. Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. *Nature methods* 2010;7(4):248-9.
46. Zuk O, Schaffner SF, Samocha K, et al. Searching for missing heritability: designing rare variant association studies. *Proc Natl Acad Sci U S A* 2014;111(4):E455-64.
47. Purcell S. PLINK v.1.07: Whole genome data analysis toolset. 2013. (http://pngu.mgh.harvard.edu/~purcell/plink/). (Accessed November 1 2013).
48. Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *American journal of human genetics* 2007;81(3):559-75.
49. Ncbi. Homo sapiens (ABCB4), transcript variant B, mRNA. 2014. (http://www.ncbi.nlm.nih.gov/pubmed/). (Accessed May 5 2014).
50. Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. *Current protocols in human genetics / editorial board, Jonathan L Haines [et al]* 2013;Chapter 7:Unit7 20.
51. Olson-Manning CF, Wagner MR, Mitchell-Olds T. Adaptive evolution: evaluating empirical support for theoretical predictions. *Nature reviews Genetics* 2012;13(12):867-77.
52. Pritchard JK, Cox NJ. The allelic architecture of human disease genes: common disease-common variant...or not? *Hum Mol Genet* 2002;11(20):2417-23.

53. Ziol M, Barbu V, Rosmorduc O, et al. ABCB4 heterozygous gene mutations associated with fibrosing cholestatic liver disease in adults. *Gastroenterology* 2008;135(1):131-41.
54. Poupon R, Rosmorduc O, Boelle PY, et al. Genotype-phenotype relationships in the low-phospholipid-associated cholelithiasis syndrome: a study of 156 consecutive patients. *Hepatology (Baltimore, Md)* 2013;58(3):1105-10.
55. Ganne-Carrié N, Baussan C, Grando V, et al. Progressive familial intrahepatic cholestasis type 3 revealed by oral contraceptive pills. *Journal of Hepatology* 2003;38(5):693-4.
56. Sauna ZE, Kimchi-Sarfaty C. Understanding the contribution of synonymous mutations to human disease. *Nature reviews Genetics* 2011;12(10):683-91.
57. Lang T, Haberl M, Jung D, et al. Genetic variability, haplotype structures, and ethnic diversity of hepatic transporters MDR3 (ABCB4) and bile salt export pump (ABCB11). *Drug metabolism and disposition: the biological fate of chemicals* 2006;34(9):1582-99.
58. Poupon R, Rosmorduc O, Boelle P-Y, et al. Genotype-phenotype relationships in the low-phospholipid-associated cholelithiasis syndrome: a study of 156 consecutive patients. *Hepatology (Baltimore, Md)* 2013;58(3):1105-10.
59. Rosmorduc O, Hermelin B, Boelle P-Y, et al. ABCB4 gene mutation-associated cholelithiasis in adults. *Gastroenterology* 2003;125(2):452-9.
60. Shneider BL, Mazariegos GV. Biliary atresia: a transplant perspective. *Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society* 2007;13(11):1482-95.
61. Leonhardt J, Kuebler JF, Leute PJ, et al. Biliary atresia: lessons learned from the voluntary German registry. *European journal of pediatric surgery : official journal of Austrian Association of Pediatric Surgery [et al] = Zeitschrift fur Kinderchirurgie* 2011;21(2):82-7.
62. Hirschfield GM, Chapman RW, Karlsen TH, et al. The genetics of complex cholestatic disorders. *Gastroenterology* 2013;144(7):1357-74.
63. Trauner M, Fickert P, Wagner M. MDR3 (ABCB4) defects: a paradigm for the genetics of adult cholestatic syndromes. *Semin Liver Dis* 2007;27(1):77-98.
64. Lang C, Meier Y, Stieger B, et al. Mutations and polymorphisms in the bile salt export pump and the multidrug resistance protein 3 associated with drug-induced liver injury. *Pharmacogenet Genomics* 2007;17(1):47-60.
65. Rosmorduc O, Poupon R. Low phospholipid associated cholelithiasis: association with mutation in the MDR3/ABCB4 gene. *Orphanet journal of rare diseases* 2007;2:29.
66. Gordo-Gilart R, Andueza S, Hierro L, et al. Functional analysis of ABCB4 mutations relates clinical outcomes of progressive familial intrahepatic cholestasis type 3 to the degree of MDR3 floppase activity. *Gut* 2015;64(1):147-55.
67. Alexopoulos SP, Merrill M, Kin C, et al. The impact of hepatic portoenterostomy on liver transplantation for the treatment of biliary atresia: early failure adversely affects outcome. *Pediatric transplantation* 2012;16(4):373-8.

68. Katsonis P, Koire A, Wilson SJ, et al. Single nucleotide variations: biological impact and theoretical interpretation. *Protein science : a publication of the Protein Society* 2014;23(12):1650-66.

TABLES AND FIGURES

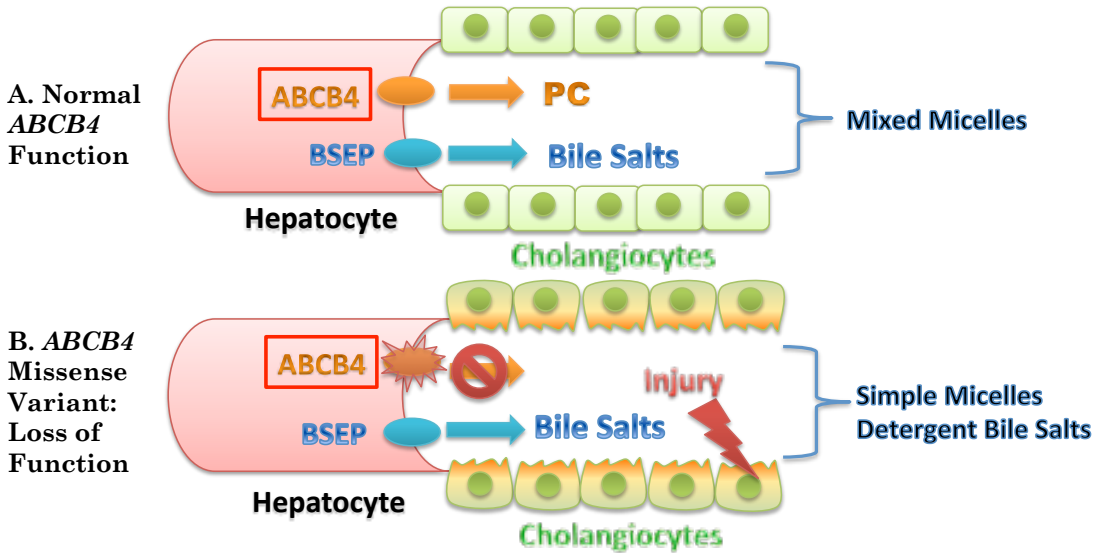


Figure 1. Pathophysiology of *ABCB4*-linked Disease. A. The ABCB4 transporter, located on the apical membrane of hepatocytes, functions as a "floppase" to translocate phosphatidylcholine (PC) from the inner to the outer leaflet of the canalicular membrane. In individuals with intact *ABCB4* function, PC complexes with bile salts and cholesterol to form mixed micelles, protecting the cholangiocytes from the detergent properties of bile salts. B. If an *ABCB4* missense variant impairs transporter function, lowering the phospholipid concentration of bile, simple micelles are formed resulting in damage to the bile duct epithelium.

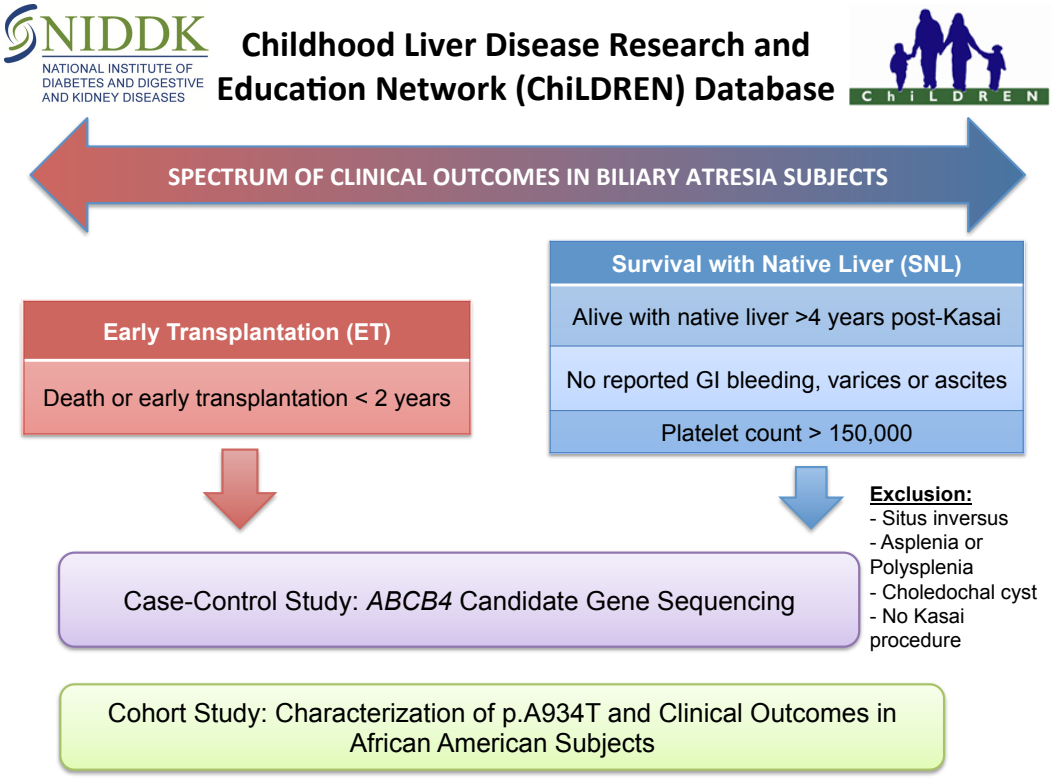


Figure 2. Diagram of Subject Selection. The DNA and clinical characteristics of all subjects with biliary atresia were obtained through the National Institute of Diabetes and Digestive and Kidney Diseases-funded Childhood Liver Disease Research and Education (ChiLDREN) Database. Subjects meeting ET and SNL definitions were selected for the case-control study based on the inclusion and exclusion criteria in the diagram. An independent cohort of African American subjects was selected for the cohort study on the basis of similar exclusion criteria.

Table 1. Univariate Analysis of Race in Biliary Atresia Subjects Stratified by Outcome

Race		ET (n=98) n (%)	SNL (n=97) n (%)	X^2 p-value
White		67 (68.4)	63 (64.9)	
African American		20 (20.4)	13 (13.4)	0.09
Other		11 (11.2)	21 (21.7)	
	Self-Reported as Other	8	8	
	Asian	2	10	
	American Indian or Alaska Native	1	2	
	Refused	0	1	

ET, Early Transplant; SNL, Survive with Native Liver

Table 2. Mean Age at Kasai (days) in Biliary Atresia Subjects According to Race

Race	n	Mean ± SD	Median (Range)	F-test p-value
White	130	54.2 ± 19.5	55 (7-90)	0.14
African American	33	59.0 ± 18.7	58 (26-95)	
Other	32	61.4 ± 24.4	63.5 (15-119)	

ANOVA F-test p-value compares mean age at Kasai across racial subgroups.
Other subgroup includes subjects who self-identified as other (n=16), Asian (n=12), American Indian or Alaska Native (n=3), and refused (n=1).

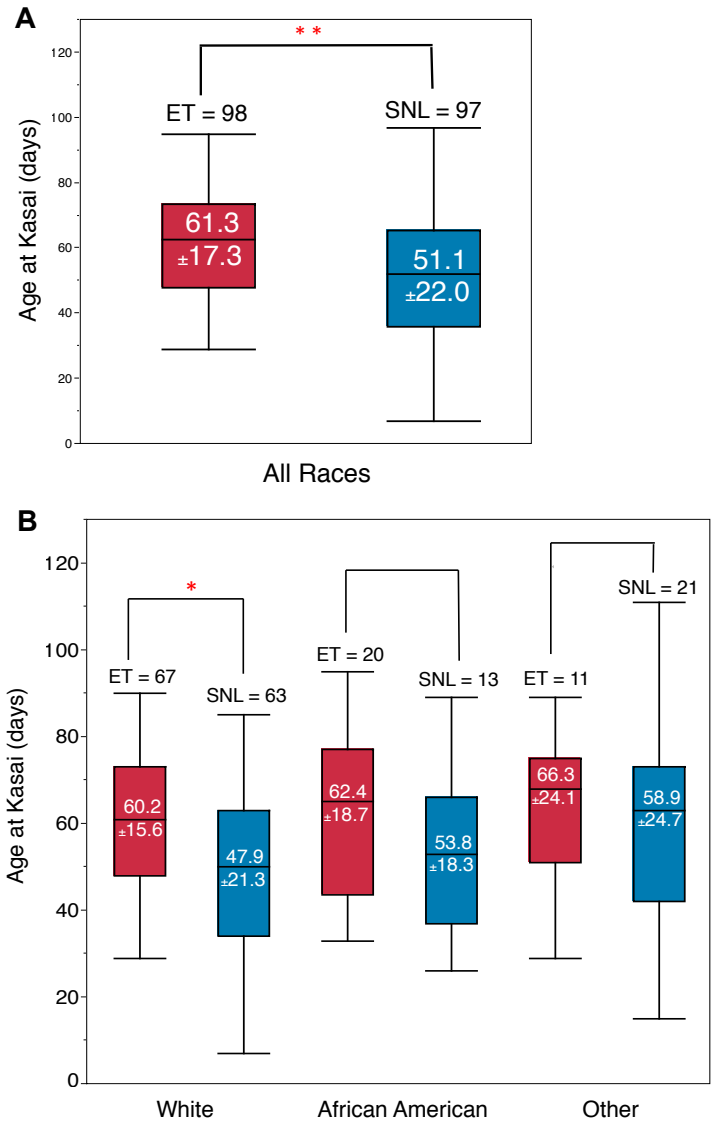


Figure 3. Age at Kasai: Stratified by Race and Outcome. Box and whiskers plots of age at Kasai procedure (average age in days ± standard deviation) for biliary atresia subjects (n=195). Whiskers drawn to the furthest point within 1.5 x the interquartile range (25%-75%) from the box. A. When aggregating subjects of all races, the SNL group received the Kasai procedure at an earlier age than the ET group (**p-value < 0.001). B. Pairwise t-tests comparing age at Kasai between ET and SNL outcome groups demonstrated that white subjects in the SNL group received the Kasai procedure at an earlier age (*p-value < 0.01).
Other subgroup includes subjects who self-identified as other (n=16), Asian (n=12), American Indian or Alaska Native (n=3), and refused (n=1).
ET, Early Transplant; SNL, Survive with Native Liver

Table 3. Multivariate Analysis of Race and Age at Kasai as Risk Factors for ET Outcome in Biliary Atresia Subjects (n=195)

Variable	Odds Ratio	95% CI	Wald X^2 p-value
Kasai (per 1 day – continuous)	1.030	(1.013, 1.046)	<0.001
Race (Reference = White)			
African American	1.300	(0.581, 2.910)	0.5
Other	0.374	(0.158, 0.886)	0.025

Other subgroup includes subjects who self-identified as other (n=16), Asian (n=12), American Indian or Alaska Native (n=3), and refused (n=1).
ET, Early Transplant

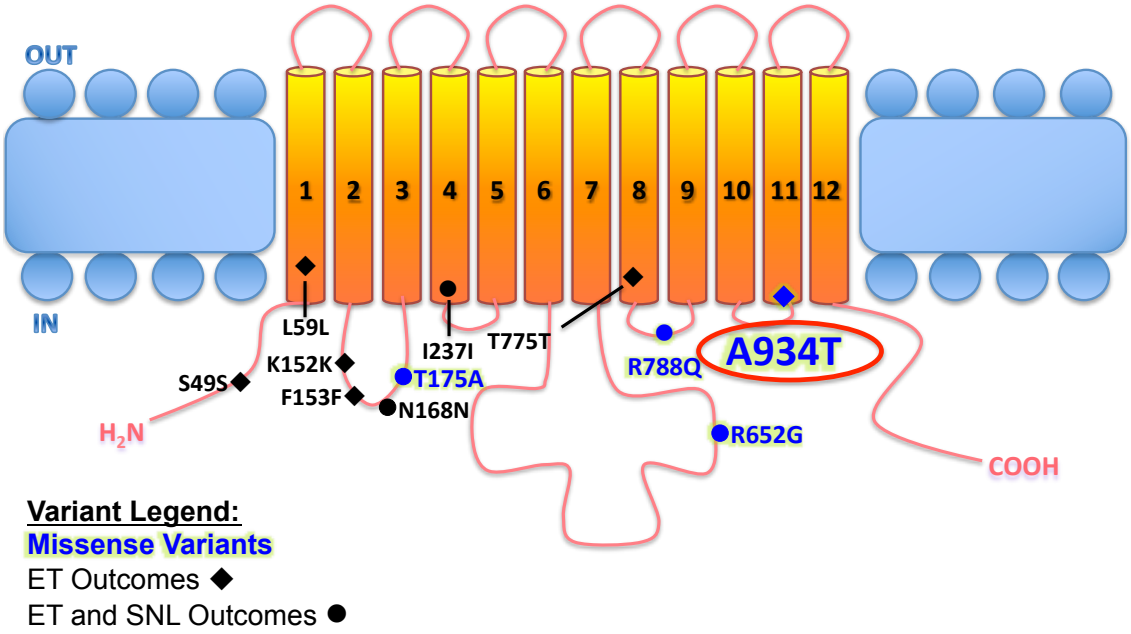


Figure 4. Schematic of *ABCB4* Gene Variants in Biliary Atresia Subjects. The predicted location of all exonic synonymous and missense variants identified in at least 2 subjects with biliary atresia is featured (n=195). Emphasis is added to the A934T polymorphism, which produces a substitution of the first amino acid of the 11th transmembrane domain.
ET, Early Transplant; SNL, Survive with Native Liver

Table 4. *ABCB4* Genotyping Results in Biliary Atresia Subjects Stratified by Race

	White		African American		Other	
	ET (n=67)	SNL (n=63)	ET (n=20)	SNL (n=13)	ET (n=11)	SNL (n=21)
Variant	Het/Homo	Het/Homo	Het/Homo	Het/Homo	Het/Homo	Het/Homo
S49S	-	-	4/0	-	-	-
L59L	13/2	19/2	7/4	10/1	5/1	8/0
K152K	1/0	-	1/0	-	-	-
F153F	-	-	4/0	-	-	-
N168N	23/24	29/19	6/0	3/0	2/3	9/1
I237I	15/4	22/2	5/0	10/0	5/1	7/3
T775T	-	-	1/0	-	1/0	-
Splice Site (Exon 1)	-	-	1/0	2/0	-	1/0
T175A	1/0	1/0	-	-	-	-
R652G	7/1	5/1	13/1	7/0	3/0	6/0
R788Q	1/0	-	-	3/0	-	1/0
A934T	-	-	4/0	-	-	-

Omitted polymorphisms include T34M, A232A, T438T, V474L, and E1058K, present in 1 subject each in the SNL group, and E528D and R590Q, present in 1 subject each in the ET group.
Other subgroup includes subjects who self-identified as Other (n=16), Asian (n=12), American Indian or Alaska Native (n=3), and refused (n=1).
ET, Early Transplant; SNL, Survive with Native Liver

Table 5. Statistical Results for Missense Variants in *ABCB4* in Biliary Atresia

Nucleotide Change	Polymorphism	ET (n=98)		SNL (n=97)		Trend Test p-value	PolyPhen-2 Score
		Het	Homo	Het	Homo		
c.101C>T	T34M	0	0	1	0	-	0.01
c.523A>G	T175A	1	0	1	0	0.99	0.87
c.1423 G>C	V474L	0	0	1	0	-	0.98
c.1584G>C	E528D	1	0	0	0	-	0.00
c.1769G>A	R590Q	1	0	0	0	-	0.99
c.1954A>G	R652G	23	0	18	1	0.26	0.00
c.2363G>A	R788Q	1	0	4	0	0.17	0.99
c.2800G>A	A934T	4	0	0	0	0.044	0.99
c.3172G>A	E1058K	0	0	1	0	-	0.00

Cochran-Armitage Trend Test indicates association with ET outcome for all missense variants identified in the case-control study.
PolyPhen-2 prediction score ranges from 0 (benign) to 1 (probably damaging); pink shading designates amino acid substitutions predicted to have damaging effect on protein function.
ET, Early Transplant; SNL, Survive with Native Liver

Table 6. Characteristics and Outcome Measures for African American Subjects with Biliary Atresia

Characteristics		n	95% CI
Number of patients		104	-
Sex	Males: Females	45:59	-
Age at Kasai (days)			
	Mean ± SD	72.1 ± 37.6	(64.8, 79.4)
	Median (range)	63 (7–210)	(60, 71)
Outcome			
	Transplant	57	-
	Death	1	-
	2-Year Survival Post-Kasai (%)	53 (51.0)	(0.41, 0.60)
	4-Year Survival Post-Kasai (%)	47 (45.2)	(0.36, 0.55)

Figure 5. Distribution of Age at Kasai for African American Subjects with Biliary Atresia

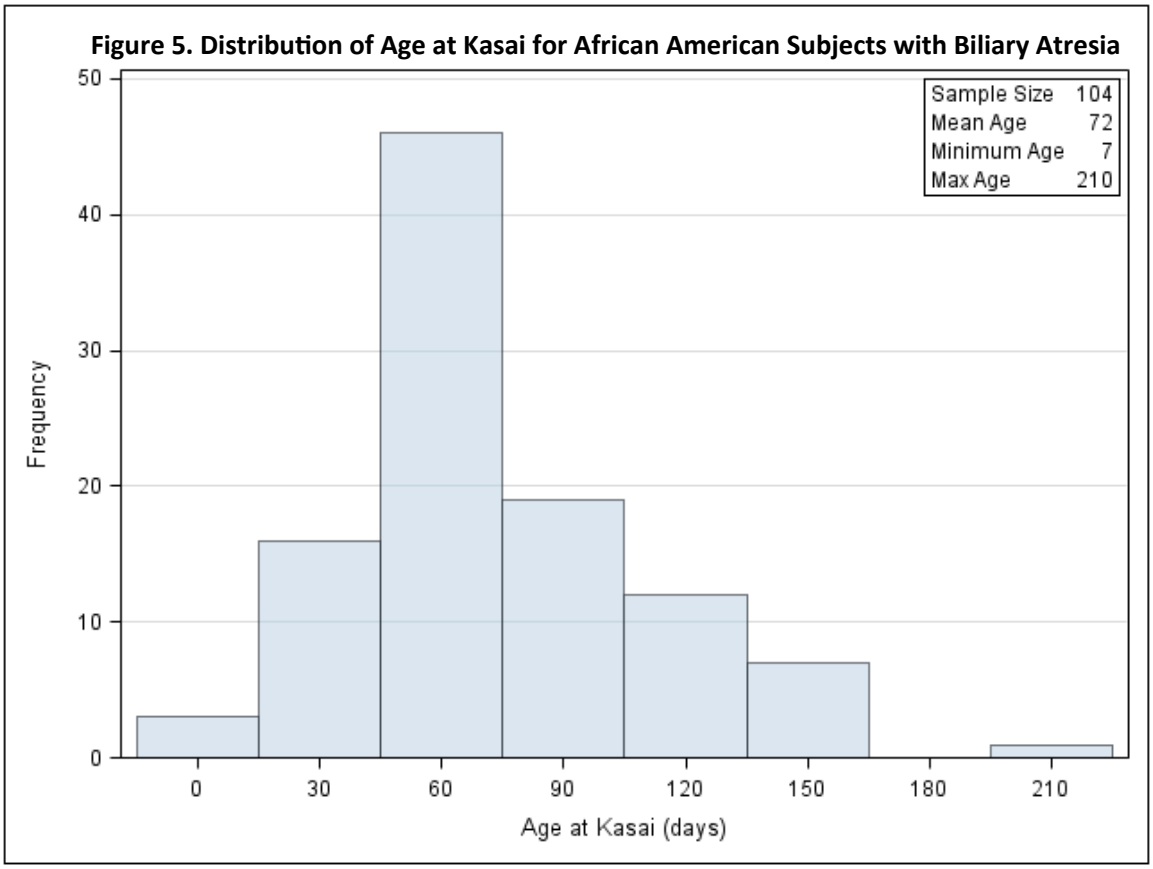
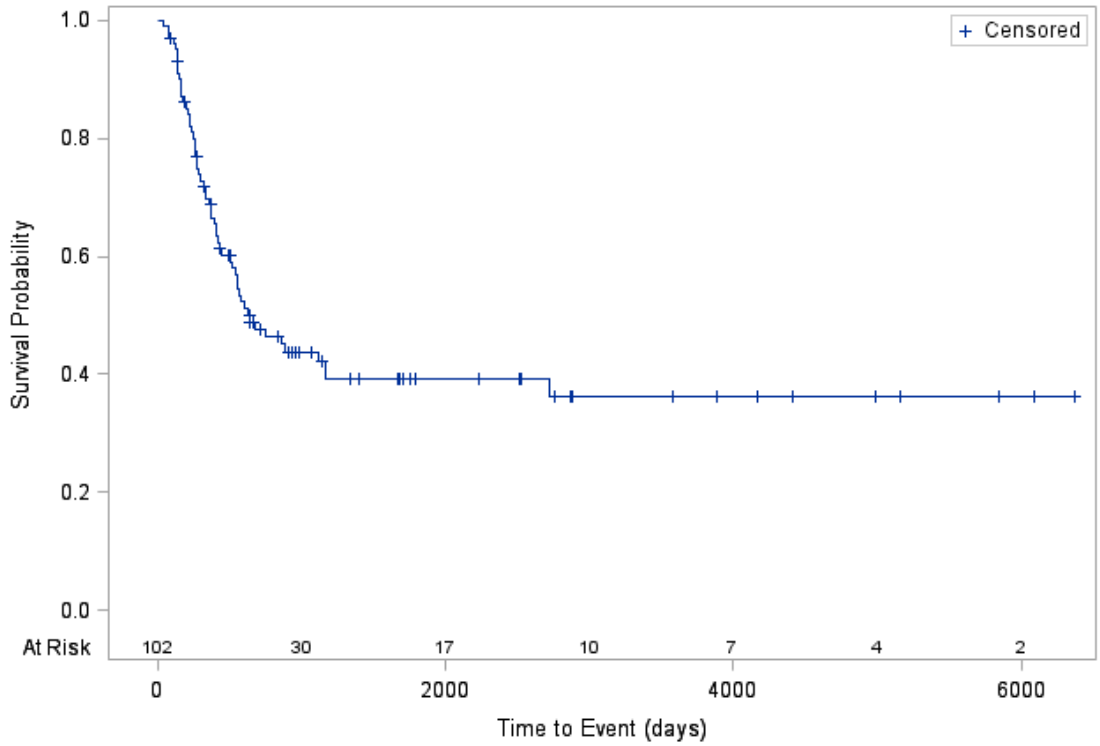


Figure 6. Kaplan-Meier Survival Curve for African American Subjects with Biliary Atresia



Kaplan-Meier survival curve depicts transplant-free survival after the Kasai procedure.
Number of subjects at risk per 1000-day interval is plotted along the x-axis.
Biliary atresia subjects with the A934T polymorphism (n=2) are excluded.

Table 7. Summary Statistics for Estimated Survival for African American Subjects (n=102)

Percentile	Point Estimate (days)	95% CI
50	641.0	(437.0, 1166.0)
25	277.0	(219.0, 375.0)

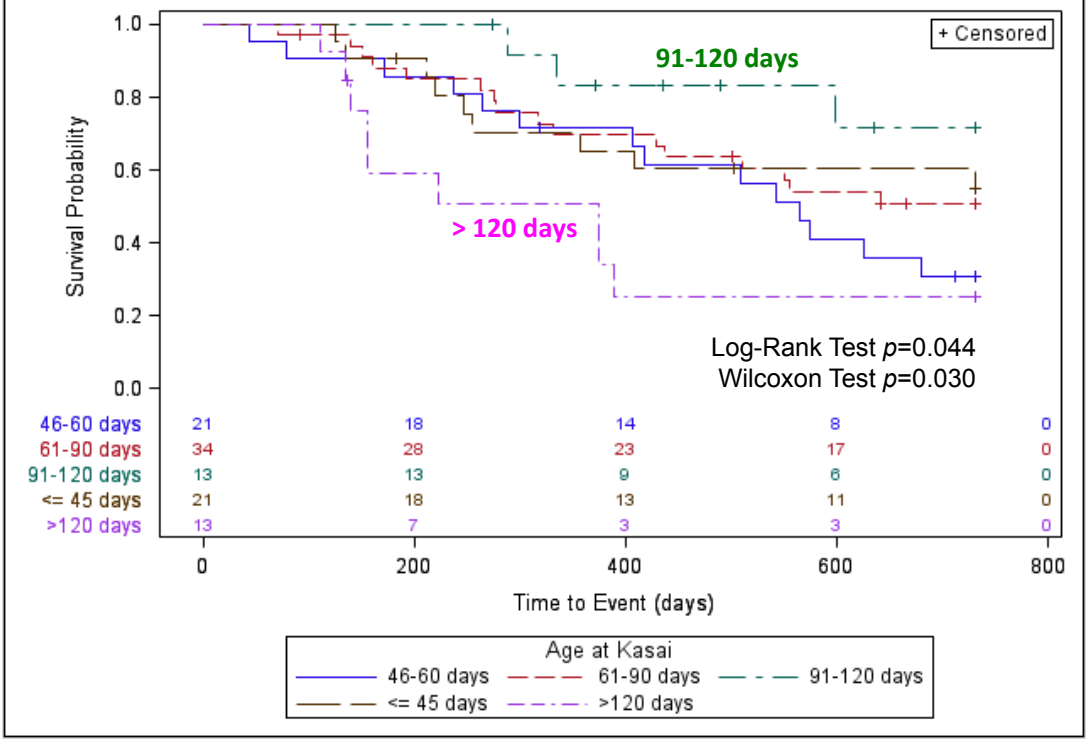
Point estimates for time-to-event, defined as death or liver transplantation, after the Kasai procedure. The 75% could not be calculated due to censored observations.

Table 8. Characteristics of African American Subjects with Biliary Atresia Stratified by Age at Kasai (n=102)

Age at Kasai (days)	n (# of Events)	Age at Event (days) Mean ± SD*	Follow-Up Time (years) Median (Range)**
≤45	21 (11)	658.2 ± 784.0	5.4 (0.6-16.7)
46-60	21 (16)	490.5 ± 240.4	7.0 (1.0-12.2)
61-90	34 (18)†	479.1 ± 282.3	4.0 (0.4-17.7)
91-120	13 (3)†	511.3 ± 175.8	2.5 (1.0-14.4)
>120	13 (9)	378.2 ± 121.7	4.0 (0.7-10.1)

*Event is defined as either death (n=1) or liver transplantation occurring at any time during the study.
**Age at last follow-up is determined for subjects who did not experience an event.
†Excluding 1 subject with the A934T polymorphism (total n=2).

Figure 7. Kaplan-Meier 2-Year Survival Curves Stratified by Age at Kasai



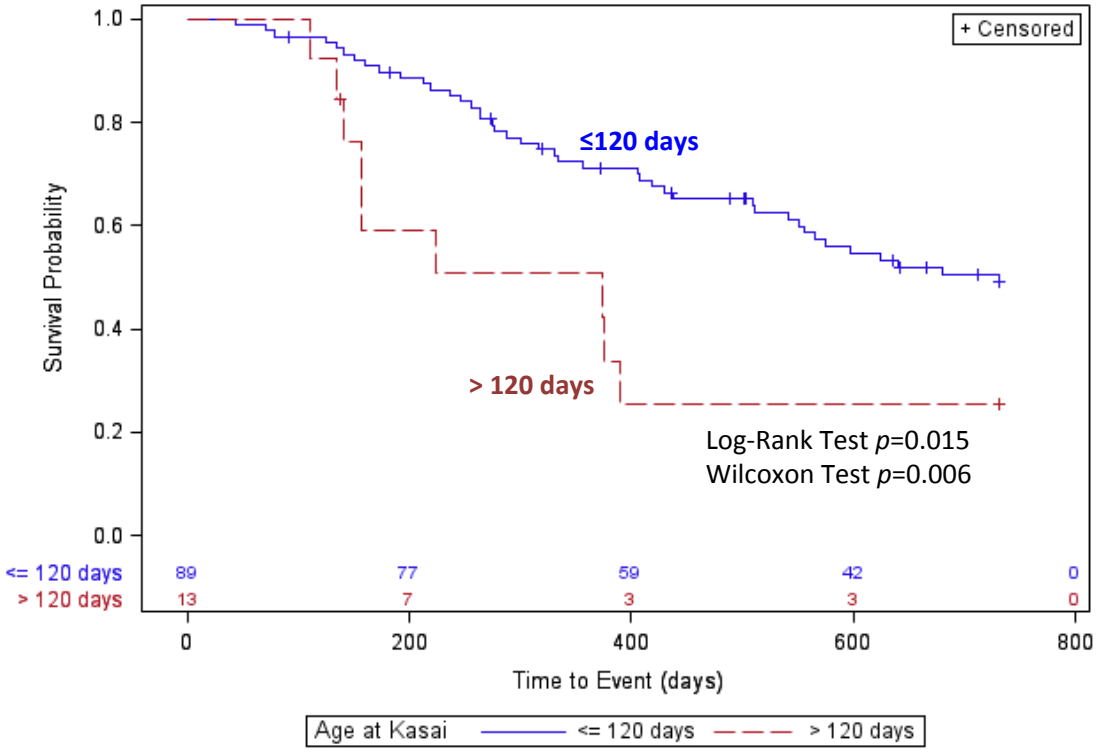
Kaplan-Meier curves demonstrate transplant-free survival after the Kasai for African American subjects with biliary atresia (n=102).
Number of subjects at risk per 200-day interval is plotted along the x-axis.
All observations censored 2 years after the Kasai date.
Six censored subjects experienced an event after 2 years post-Kasai.

Table 9. 2-Year Survival Analysis Stratified by Age at Kasai: Pairwise Comparisons

Comparison of Strata			Log-Rank p value	Log-Rank p value†	Wilcoxon p value	Wilcoxon p value†
91-120 days	**v.**	**> 120 days**	**0.006***	**0.050***	**0.003***	**0.022***
≤ 45 days	v.	> 120 days	0.080	0.403	0.076	0.389
46-60 days	v.	61-90 days	0.292	0.830	0.421	0.929
46-60 days	v.	91-120 days	0.060	0.328	0.103	0.477
46-60 days	v.	≤ 45 days	0.253	0.783	0.475	0.953
46-60 days	v.	> 120 days	0.727	0.997	0.362	0.893
61-90 days	v.	91-120 days	0.617	0.987	0.575	0.981
61-90 days	v.	≤ 45 days	0.963	1.000	0.861	1.000
61-90 days	v.	> 120 days	0.112	0.505	0.078	0.396
91-120 days	v.	≤ 45 days	0.532	0.971	0.392	0.913

Log-Rank and Wilcoxon tests comparing survival curves for transplant-free survival 2 years post-Kasai for African American subjects with biliary atresia (n=102). Subjects stratified by age at Kasai into 5 cohorts: ≤ 45 days (n=21), 46-60 days (n=21), 61-90 days (n=34), 91-120 days (n=13), >120 days (n=13).
Event is defined as death or liver transplantation.
†Tukey-Kramer method used to control the familywise Type I Error rate for multiple comparisons.
*Significant at an alpha = 0.05.

Figure 8. Kaplan-Meier 2-Year Survival Curves Comparing Age at Kasai at 120 days

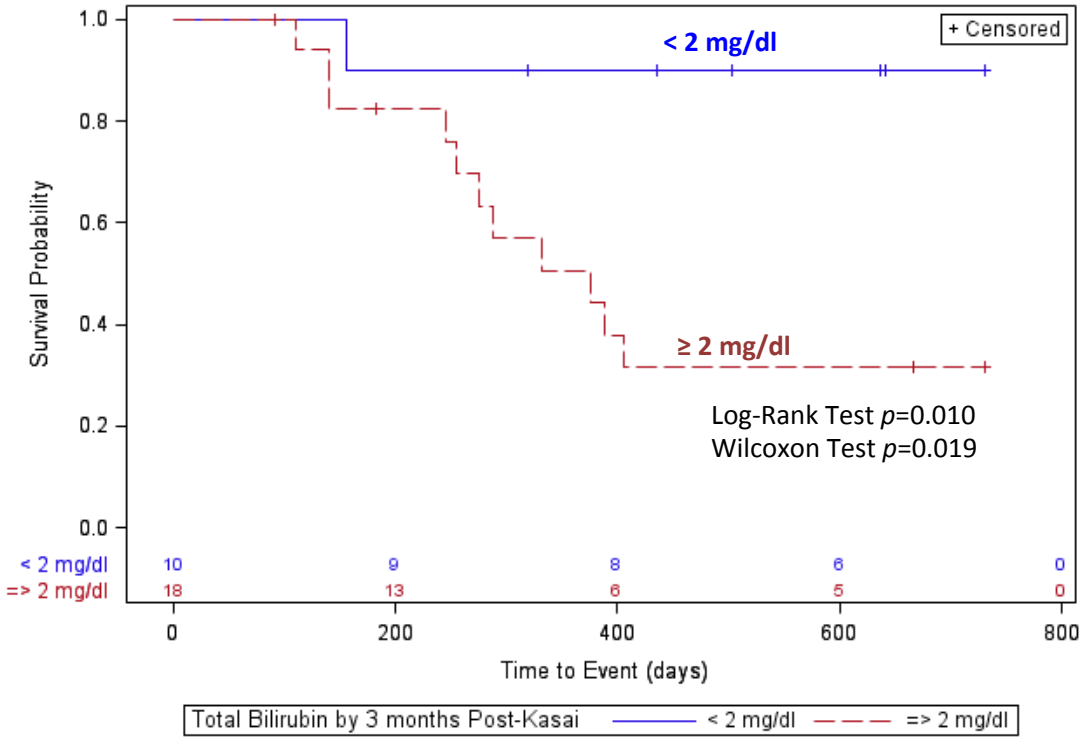


Kaplan-Meier curves demonstrate transplant-free survival after the Kasai for African American subjects with biliary atresia (n=102).
Number of subjects at risk per 200-day interval is plotted along the x-axis.
All observations censored 2 years after the Kasai date.
Six censored subjects experienced an event after 2 years post-Kasai.

Table 10. Univariate Analysis of Age at Kasai as Risk Factor for Death or Transplant (n=102)

Variable	Odds Ratio	95% CI	Wald X^2 p-value
Kasai (per 1 day – continuous)	0.99	(0.99, 1.01)	0.45
Kasai (> 120 days v. ≤ 120 days)	0.52	(0.15, 1.82)	0.3

Figure 9. Kaplan-Meier 2-Year Survival Curves Stratified by Bilirubin at 3 months Post-Kasai



Kaplan-Meier curves demonstrate transplant-free survival after the Kasai for African American subjects with biliary atresia and known total serum bilirubin at 3 months post-Kasai (n=28).
Number of subjects at risk per 200-day interval is plotted along the x-axis.
All observations censored 2 years after the Kasai date.
Six censored subjects experienced an event after 2 years post-Kasai.

Table 11. Univariate Analysis of Jaundice Clearance and Age at Kasai as Risk Factors for 2-Year Transplant Free Survival and Probability of Jaundice Resolution (n=28)

	Risk of Death or Transplant			Probability of Jaundice Clearance at 3M		
Variable	**Odds Ratio**	**95% CI**	**Wald X^2 p-value**	**Odds Ratio**	**95% CI**	**Wald X^2 p-value**
Kasai (per 1 day – continuous)	1.01	(0.99, 1.03)	0.52	0.99	(0.97, 1.01)	0.28
Bilirubin ≥ 2 mg/dl at 3 months	12.89	(1.31, 127.26)	0.03	-	-	-

Table 12. International Data Available on Age at Kasai Hepatoportoenterostomy Procedure

Study	Country	Period	Racial Composition	N	Age at Kasai (days) Mean ± SD	Age at Kasai (days) Median (Range)
Shneider, 2006	North America	1997-2000	Non-Hispanic White	65	55 ± 26	NR
			African American	17	72 ± 29	NR
Schreiber, 2007	Canada	1985-1995	Varied	312	NR	65 (6-200)
Wildhaber, 2008	Switzerland	1994-2004	NR	43	NR	68 (30-126)
Hsiao, 2008	Taiwan	2004-2005	NR	75	55 ± 17	(11-90)
Serinet, 2009	France	1986-2002	NR	695	NR	60 (12-180)
Leonhardt, 2011	Germany	2001-2005	NR	159	57	(7-150)
Superina, 2011	North America*	2004-2010	Varied	244	65 ± 25	NR
Davenport, 2011	England & Wales	1999-2009	NR	424	NR	54 (7-209)
de Vries, 2012	The Netherlands	1987-2008	NR	214	NR	59 (20-210)
Chardot, 2013	France	1986-2009	NR	1044	NR	59 (10-199)
Mezina, 2015	**North America***	**2004-2013**	**African American**	**104**	**72.1 ± 37.6**	**63 (7–210)**

*Data derived from Childhood Liver Disease Research and Education Network database; potential for subject overlap.