

DR. JORGE A BEZERRA (Orcid ID : 0000-0001-9667-7742)

DR. SAUL J KARPEN (Orcid ID : 0000-0002-3379-7592)

Article type : Original

## Identification of *PKD1L1* Gene Variants in Children with the Biliary Atresia Splenic Malformation Syndrome

John-Paul Berauer<sup>1</sup>, Anya I. Mezina<sup>1</sup>, David T. Okou<sup>1</sup>, Aniko Sabo<sup>2</sup>, Donna M. Muzny<sup>2</sup>, Richard A. Gibbs<sup>2</sup>, Madhuri R. Hegde<sup>3</sup>, Pankaj Chopra<sup>3</sup>, David J. Cutler<sup>3</sup>, David H. Perlmutter<sup>4</sup>, Laura N. Bull<sup>5</sup>, Richard J. Thompson<sup>6</sup>, Kathleen M. Loomes<sup>7</sup>, Nancy B. Spinner<sup>8</sup>, Ramakrishnan Rajagopalan<sup>8,9</sup>, Stephen L. Guthery<sup>10</sup>, Barry Moore<sup>11</sup>, Mark Yandell<sup>11</sup>, Sanjiv Harpavat<sup>12</sup>, John C. Magee<sup>13</sup>, Binita M. Kamath<sup>14</sup>, Jean P. Molleston<sup>15</sup>, Jorge A. Bezerra<sup>16</sup>, Karen F. Murray<sup>17</sup>, Estella M. Alonso<sup>18</sup>, Philip Rosenthal<sup>19</sup>, Robert H. Squires<sup>20</sup>, Kasper S. Wang<sup>21</sup>, Milton J. Finegold<sup>22</sup>, Pierre Russo<sup>23</sup>, Averell H. Sherker<sup>24</sup>, Ronald J. Sokol<sup>25</sup>, and Saul J. Karpen<sup>1</sup> for the Childhood Liver Disease Research Network (ChiLDReN)

HEPATOLOGY HEP-18-0915 R1

12.12.2018

**Title:** 92 Characters

**Abstract:** 253 Words

**Text with References:** 5,086 Words

**References:** 50

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1002/HEP.30515](https://doi.org/10.1002/HEP.30515)

This article is protected by copyright. All rights reserved

**Figures:** 4      **Tables:** 2      **Supplement:** 3 Tables and 2 Figures

## **Keywords:**

Neonatal Cholestasis

Whole Exome Sequencing

Cilia

Laterality

Cholangiocyte

## **Article Information:**

<sup>1</sup>Department of Pediatrics; Division of Gastroenterology, Hepatology and Nutrition; Emory University School of Medicine and Children's Healthcare of Atlanta; Atlanta, GA, 30322, USA.

<sup>2</sup>Human Genome Sequencing Center; Baylor College of Medicine; Houston, TX, 77030, USA.

<sup>3</sup>Department of Human Genetics; Emory University School of Medicine; Atlanta, GA, 30322, USA.

<sup>4</sup>Department of Pediatrics; Washington University School of Medicine; St. Louis, MO, 63110, USA.

<sup>5</sup>Institute for Human Genetics, and Liver Center Laboratory, Department of Medicine; University of California San Francisco; San Francisco, CA, 94143, USA.

<sup>6</sup>Institute of Liver Studies; King's College London; London, SE5 9RS, UK.

<sup>7</sup>Department of Pediatrics; Division of Gastroenterology, Hepatology and Nutrition; Perelman School of Medicine at the University of Pennsylvania and Children's Hospital of Philadelphia; Philadelphia, PA 19104, USA.

<sup>8</sup>Department of Pathology and Laboratory Medicine; Division of Genomic Diagnostics; Children's Hospital of Philadelphia; Philadelphia PA, 19104, USA.

<sup>9</sup>Department of Biomedical and Health Informatics; Children's Hospital of Philadelphia; Philadelphia PA, 19104, USA.

<sup>10</sup>Department of Pediatrics; Division of Gastroenterology, Hepatology and Nutrition; University of Utah; and Intermountain Primary Children's Hospital Salt Lake City, UT, 84112, USA.

<sup>11</sup>Department of Human Genetics; University of Utah; Salt Lake City, UT, 84112, USA.

<sup>12</sup>Department of Pediatrics; Division of Gastroenterology, Hepatology and Nutrition; Baylor College of Medicine; Houston, TX, 77030, USA.

<sup>13</sup>University of Michigan Medical School; Ann Arbor, MI, 48103, USA.

<sup>14</sup>Division of Gastroenterology, Hepatology and Nutrition; Hospital for Sick Children and University of Toronto; Toronto, ON, M5G 1X8, Canada.

<sup>15</sup>Department of Pediatrics; Division of Gastroenterology, Hepatology and Nutrition; Indiana University School of Medicine and Riley Hospital for Children; Indianapolis, IN, 46202, USA.

<sup>16</sup>Department of Pediatrics; Division of Gastroenterology, Hepatology and Nutrition; Cincinnati Children's Hospital Medical Center; Cincinnati, OH, 45229, USA.

<sup>17</sup>Department of Pediatrics; Division of Gastroenterology and Hepatology; University of Washington School of Medicine and Seattle Children's Hospital; Seattle, WA, 98105, USA.

<sup>18</sup>Department of Pediatrics; Division of Gastroenterology, Hepatology and Nutrition; Ann and Robert H. Lurie Children's Hospital of Chicago; Chicago, IL, 60611, USA.

<sup>19</sup>Department of Pediatrics; Division of Gastroenterology, Hepatology and Nutrition; University of California San Francisco; San Francisco, CA, 94143, USA.

<sup>20</sup>Department of Pediatrics; Division of Gastroenterology, Hepatology and Nutrition; Children's Hospital of Pittsburgh of UPMC; Pittsburgh, PA, 15224, USA.

<sup>21</sup>Department of Surgery; Division of Pediatric Surgery; Children's Hospital of Los Angeles; University of Southern California; Los Angeles, CO, 90027, USA.

<sup>22</sup>Department of Pediatrics; Department of Molecular and Cellular Biology; Baylor College of Medicine; Houston, TX, 77030, USA.

<sup>23</sup>Department of Pathology and Laboratory Medicine; Children's Hospital of Philadelphia; Philadelphia PA, 19104, USA.

<sup>24</sup>Liver Diseases Research Branch; National Institute of Diabetes and Digestive and Kidney Diseases; National Institutes of Health; Bethesda, MD, 20892, USA.

<sup>25</sup>Department of Pediatrics; Section of Gastroenterology, Hepatology and Nutrition; Children's Hospital Colorado and University of Colorado School of Medicine; Aurora, CO, 80045, USA.

## **Address Correspondence and Reprint Requests To:**

Saul J. Karpen, M.D., Ph.D.

Raymond F. Schinazi Distinguished Biomedical Chair

Division of Pediatric Gastroenterology, Hepatology and Nutrition

Department of Pediatrics

Emory University School of Medicine/Children's Healthcare of Atlanta

1760 Haygood Dr. NE

HSRB E204

Atlanta, GA 30322-1015

E-mail: [skarpen@emory.edu](mailto:skarpen@emory.edu)

Tel: +1-404-727-1463

Fax: +1-404-727-4069

## **Abbreviations:**

*BA, biliary atresia*

*BASM, biliary atresia splenic malformation*

*WES, whole exome sequencing*

*ChiLDReN, Childhood Liver Disease Research Network*

*L-R, left-right*

*PROBE, A Prospective Database of Infants with Cholestasis*

*BASIC, Biliary Atresia Study in Infants and Children*

*SNV, single nucleotide variant*

*INDELS, insertions/deletions*

*dbSNP, Single Nucleotide Polymorphism Database*

*GOI, gene of interest*

*EGL, Emory Genetics Laboratory*

*MAF, minor allele frequency*

*CADD, Combined Annotation Dependent Depletion*

*SIFT, Sorting Intolerant from Tolerant*

## **Financial Support:**

This work was supported by U01 grants from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), (DK103149 [Texas Children's Hospital], DK103140 [University of Utah], DK103135 [The Hospital for Sick Children], DK084575 [Seattle Children's Hospital], DK084538 [Children's Hospital of Los Angeles], DK084536 [Indiana University/Riley Hospital for Children], DK062503 [Johns Hopkins Children's Center], DK062500 [UCSF Children's Hospital], DK062497 [Cincinnati Children's Hospital Medical Center], DK062481 [The Children's Hospital of Philadelphia], DK062470 [Children's Healthcare of Atlanta], DK062466 [Children's Hospital of Pittsburgh of UPMC], DK062456 [The University of Michigan], DK062453 [Children's Hospital Colorado and University of Colorado], DK062452 [Washington University School of Medicine], DK062445 [Icahn School of Medicine at Mount Sinai], and DK062436 [Ann & Robert H. Lurie Children's Hospital of Chicago]). In addition, the project described was supported by UL1 grants from the National Institutes of Health Clinical and Translational Sciences Award (CTSA) program through the National Center for Advancing Translational Sciences (NCATS), (TR001872 [UCSF Benioff Children's Hospital], TR001857 [Children's Hospital of Pittsburgh of UPMC], TR001108 [Riley Hospital for Children at Indiana University], TR002535 [University of Colorado], TR000454 [Emory University School of Medicine],

TR000423 and RR025014 [Seattle Children's Hospital], and TR000077 [Cincinnati Children's Hospital Medical Center], TR000003 [Children's Hospital of Philadelphia]). Additional support to AS, DM and RG was provided by U54 HG003273, and to AIM by the American Gastroenterological Association Research Foundation, Student Research Fellowship Award and TL1TR000456. Support to SJK was provided by *The Cade R. Alpard Foundation for Pediatric Liver Disease, The Spain Foundation and the Meredith Brown Foundation*.

### **Acknowledgments:**

We would like to thank Pamela Parson (Texas Children's Hospital) and Daniel Martinez (Children's Hospital of Philadelphia) for histologic technical expertise, and Dr. Subra Kugathasan (Emory University School of Medicine) for advice and troubleshooting. In particular, we would like to thank all ChiLDReN clinical research coordinators at each of the participating centers for their tremendous time and dedication to this project as well as the study participants and their families without whom this work would not have been possible.

### **Note:**

A portion of this work was presented as an Oral Abstract at the 68<sup>th</sup> Annual Meeting of the American Association for the Study of Liver Diseases: The Liver Meeting in Washington, D.C. on October 23<sup>rd</sup>, 2017. (Hepatology 2017; 66: 1258A-1259A).

### **Abstract**

Biliary atresia (BA) is the most common cause of end-stage liver disease in children and the primary indication for pediatric liver transplantation, yet underlying etiologies remain unknown. Approximately 10% of infants affected by BA exhibit various laterality defects (heterotaxy) including splenic abnormalities and complex cardiac malformations — a distinctive subgroup commonly referred to as the biliary atresia splenic malformation (BASM) syndrome. We hypothesized that genetic factors linking laterality features with the etiopathogenesis of BA in BASM patients could be identified through whole exome sequencing (WES) of an affected cohort. DNA specimens from 67 BASM subjects, including 58 patient-parent trios, from the NIDDK-supported Childhood Liver Disease Research

Network (ChiLDReN) underwent WES. Candidate gene variants derived from a pre-specified set of 2,016 genes associated with ciliary dysgenesis and/or dysfunction or cholestasis were prioritized according to pathogenicity, population frequency, and mode of inheritance. Five BASM subjects harbored rare and potentially deleterious bi-allelic variants in polycystin 1-like 1, *PKD1L1*, a gene associated with ciliary calcium signaling and embryonic laterality determination in fish, mice and humans. Heterozygous *PKD1L1* variants were found in 3 additional subjects. Immunohistochemical analysis of liver from the one BASM subject available revealed decreased PKD1L1 expression in bile duct epithelium when compared to normal livers and livers affected by other non-cholestatic diseases. **Conclusion:** WES identified bi-allelic and heterozygous *PKD1L1* variants of interest in 8 BASM subjects from the ChiLDReN dataset. The dual roles for PKD1L1 in laterality determination and ciliary function suggest that *PKD1L1* is a new, biologically plausible, cholangiocyte-expressed candidate gene for the BASM syndrome.

## Introduction

Biliary atresia (BA) is a severe neonatal cholangiopathy characterized by progressive fibroinflammatory obliteration of both extra- and intra-hepatic bile ducts, generally leading to cholestasis, portal fibrosis, and, ultimately, biliary cirrhosis. Among children, BA is the most common cause of end-stage liver disease worldwide and the primary indication for liver transplantation, yet its etiology (or etiologies) remains unknown (1-4). Hypotheses regarding the pathogenesis of BA include perinatal viral infections or toxins targeting cholangiocytes, chronic inflammatory or autoimmune-mediated bile duct injury, and mutations in specific genes that regulate hepatobiliary development (1). In the biliary atresia splenic malformation (BASM) syndrome, which accounts for ~10% of infants affected with BA, the coexistence of one or more major congenital malformations within a wide spectrum of laterality defects (heterotaxy) along with abnormal biliary tract development suggests a role for genetic contributions to the etiology of the BASM syndrome (5-7).

A developmental cause of BA was first proposed in an early case collection (8) while support for a potential genetic contribution comes not only from pre- and perinatal

evidence of biliary tract dysgenesis in BA (9), but also from several familial case reports of BA (10). However, given the general lack of heritability of BA within families and reports of twin discordance (11-13), recent studies have explored non-genetic etiologies underlying BA, including viral, toxin or immune-mediated mechanisms (2, 14). Nevertheless, the genetic determinants of heterotaxy syndromes often involve mutations in genes essential for ciliary structure or function (15, 16), providing support to re-focus efforts exploring potential roles for variants in key ciliary genes in the pathogenesis of the BASM syndrome (6, 7).

A growing list of genes involved in bile duct development and function have been implicated in the pathogenesis of biliary tract diseases including BA, several of which cause structural and functional alterations in primary cilia (17, 18). Non-motile primary cilia are present in many cell types including the apical surface of biliary epithelium (19, 20). Acting as cellular antennae that sense a wide variety of molecules in the extracellular environment, primary cilia play an integral role in the regulation and transmission of downstream intracellular signaling pathways (15). Moreover, the establishment of normal L-R patterning in vertebrate embryogenesis is dependent on both sensory and motile ciliary functions (15, 16). Support for the potential contribution of ciliary dysfunction in BA comes from several sources: (1) cholangiocyte cilia in BA livers are significantly shorter, abnormal in their orientation, and less abundant compared with other cholestatic disorders and normal livers; (2) mutations in a small number of ciliopathy and laterality genes, including *CFC1*, *NODAL*, and *ZIC3*, have been suggested as a possible etiology for BA in some patients; and (3) several well-characterized mutations in ciliopathy genes lead to developmental biliary tract diseases known as cholangiociliopathies — Caroli syndrome, ductal plate malformations, congenital hepatic fibrosis and polycystic liver diseases (21-25).

Given the central role of cilia in critical developmental pathways of hepatobiliary biogenesis as well as L-R determination during embryogenesis leading to heterotaxy, we hypothesized that ciliary dysgenesis and/or dysfunction may be involved in the pathogenesis of the BASM syndrome. To explore this hypothesis, we performed whole



exome sequencing (WES) in carefully phenotyped BASM subjects enrolled in a large multicenter longitudinal North American study of pediatric cholestatic liver diseases, focusing our analysis on a subset of 2,016 genes related primarily to ciliary structure and function.

## **Materials and Methods**

### ***Subject Selection.***

Participants enrolled between May 2004 – August 2016 in either of two prospective observational cohort studies (A Prospective Database of Infants with Cholestasis [PROBE; NCT00061828] or Biliary Atresia Study in Infants and Children [BASIC; NCT00345553]) within the NIDDK-supported Childhood Liver Disease Research Network (ChiLDReN, <http://childrennetwork.org>) were eligible for inclusion. At the time of selection, 2,001 BA participants, of which 1,488 had DNA available for genetic analyses, including 618 complete child-parent trios, were enrolled in ChiLDReN consortia across 18 clinical sites in the United States and Canada. The diagnosis of BA in each participant was confirmed by review of pertinent diagnostic liver biopsy, radiologic and surgical reports. BA participants for whom DNA was available in the ChiLDReN biorepository, and with at least one reported laterality defect (i.e., splenic abnormalities, intestinal malrotation, abdominal heterotaxy, vascular abnormalities, or congenital heart disease), as defined by the National Birth Defects Prevention Study, or cystic renal dysplasia, were included (26, 27). Sixty-seven BASM participants (58 child-parent trios and 9 duos [child and one parent]) underwent WES. Of note, the clinical phenotype of participants' parents and siblings are not collected in PROBE or BASIC, thus obviating knowledge of familial cardiac, hepatic or laterality abnormalities.

### ***WES and Data Analyses.***

DNA from each trio was sequenced using standard Illumina protocols and NimbleGen SeqCap technology at two sites, The Human Genome Sequencing Center at the Baylor College of Medicine (Houston, TX) and the Northwest Genomics Center (Seattle, WA), with a mean coverage depth of 50-60X in the targeted region. The raw sequence reads were

assembled relative to the Genome Reference Consortium Human GRCh38 (hg38) using the PEMapper software tool and PEPcaller was used to identify single nucleotide variants (SNVs), insertions and deletions (INDELS) (28). These unique variants were functionally annotated using Bystro (<https://bystro.io/>) and ANNOVAR which report the variant's type, functional classification (nonsense, replacement, silent, 5' or 3' UTR, splice junction or other intronic, intergenic), presence in the Single Nucleotide Polymorphism Database (dbSNP), and measures of evolutionary conservation (29, 30).

To explore the potential contribution of ciliary dysgenesis and/or dysfunction underlying the BASM phenotype, we used a pre-specified list of 2,016 Genes of Interest (GOI; Table S1) derived principally from 2 large comprehensive data sets dedicated to ciliomic studies as well as the Emory Genetics Laboratory (EGL) Ciliopathies Sequencing Panel, the EGL Neonatal and Adult Cholestasis Sequencing Panel, and a collated list of putative BA candidate susceptibility genes reported in the literature (18, 23, 27, 31) (Figure 1).

Variants in genes included among the 2,016 GOI were then subjected to further duo and trio analyses using a custom gene prioritization algorithm (Figure S1). In addition to standard quality controls, our candidate gene prioritization method included analysis of variants in coding regions of a gene (i.e. exonic and splicing donor/acceptor sites) weighted towards identification of protein-truncating variants (stop gain/loss, start loss, or frameshift), missense variants, canonical splice-site variants, and in-frame INDELS. Furthermore, we prioritized variants in individuals by preferentially selecting rare variants with a minor allele frequency (MAF) <1% and those with a Combined Annotation Dependent Depletion (CADD) score >10, indicating that the variant is amongst the top 10% of deleterious variants in the human genome, or predicted to negatively impact protein function using the *in silico* Sorting Intolerant From Tolerant (SIFT) algorithm (32, 33). Bi-allelic assignment included variants with CADD scores <10, when an allele with a CADD score >10 was initially identified by application of the screening algorithm. Finally, participants' and their parents' variants were systematically categorized based on four different modes of inheritance: (1) germ-line *de novo* mutations; (2) recessive homozygous genotypes, which were heterozygous in both parents; (3) compound heterozygous; and (4)

hemizygous X chromosome variants.

### ***Liver Histology and Immunohistochemistry.***

Standard hematoxylin and eosin staining was employed in formalin-fixed paraffin-embedded liver tissue. Immunohistochemical (IHC) staining was performed on a Bond Rx automated staining system (Leica Biosystems) utilizing the Bond Refine polymer staining kit (Leica Biosystems). Primary anti-human PKD1L1 antibody incubation (Atlas HPA022424, 1:200 dilution) or anti-human K7 (Dako M7018, 1:100 dilution) proceeded for 1 hour at room temperature, and antigen retrieval was performed with E1 (PKD1L1) and E2 (CK7) (Leica Biosystems) retrieval solution for 20min. Slides were rinsed and dehydrated through a series of ascending concentrations of ethanol and xylene, prior to the placement of coverslips.

## **Results**

### ***Participant Demographics and Clinical Information.***

Sixty-seven BASM ChiLDReN participants were included in the current study. There was a slight female predominance with racially and ethnically diverse backgrounds generally reflective of the overall demographics of the ChiLDReN cohort of BA participants (Table 1). The Kasai hepatoportoenterostomy was performed in 61 participants at an average age of 59 days (range: 21 – 122 days) and 61% of participants had undergone liver transplantation at the time of selection (See Table 1 and Figure S2 for detailed outcomes). At the time of selection, 27% of participants were alive with native liver, similar to previously published series (4,5). Review of PROBE and BASIC case report forms indicated that the majority of BASM participants in this study exhibited at least two laterality features (Figure 2A). The most common abnormalities of L-R patterning were splenic abnormalities (primarily polysplenia and less frequently asplenia) and intestinal malrotation, each present in more than half of the participants. Other common anomalies were congenital heart disease, abdominal heterotaxy, various vascular malformations, and renal anomalies (Figure 2B). The range and distribution of anomalies in this cohort were comparable to previously published collections of BASM patients (6, 7, 26).

### ***WES and Systematic Analyses of Ciliopathy Genes.***

Applying quality control filters to the collated list of 2016 ciliopathy and cholestatic GOIs (Table S1), led to the identification of 226,058 variants in 67 BASM probands. After utilizing our candidate gene prioritization method, 12,190 variants remained and served as the basis for detailed stratifications and inter-subject analyses (Figure S1). No pathogenic variants were identified in genes underlying the cholangiociliopathies (e.g., *PKHD1*, *PKD2*), neonatal sclerosing cholangitis (*DCDC2*), or neonatal cholestasis (e.g., *ABCB11*, *ABCB4*, *CFTR*, *JAG1*), suggesting exclusion of non-BA cholestatic diagnoses and adequacy of clinical phenotyping. Furthermore, no significant pathogenic variants in previously proposed BA candidate genes *CFC1*, *FOXA2*, *INVS*, *NODAL*, and *ZIC3* were identified (see Discussion). However, prioritizing bi-allelic variants led to the identification of several new and promising candidate genes, not previously linked to BA or the BASM subgroup. In particular, *PKD1L1*, which encodes the polycystin 1-like 1 protein (a member of the polycystin protein family), had potentially pathogenic bi-allelic and heterozygous variants in multiple BASM participants. Given the role of *PKD1L1* in ciliary calcium signaling and laterality determination, its interactions with *PKD2*, and as a cause of complex congenital heart disease in children, further investigations of *PKD1L1* variants in this BASM cohort were pursued (34-37).

### ***PKD1L1 Gene Variants and Clinical Features of Heterotaxy.***

A total of 910 *PKD1L1* variants were found in the 67 BASM participants (Table S2). Employing our candidate gene prioritization algorithm led to the identification of bi-allelic *PKD1L1* variants in 5 participants (Subjects 1, 2, 3, 4, and 5), and heterozygous variants of interest in 3 additional participants (Subjects 6, 7, and 8; Tables 2 and S2). Figure 3 schematically depicts the exonic location of the 9 *PKD1L1* variants in this report. MAFs for these 9 variants ranged from 0.0004% - 0.6072%. Of the 9 *PKD1L1* variants (6 missense, 2 splice-site, and 1 chain-terminating), 8 have yet to be associated with a disease phenotype. The splice site mutation found in Subject 3 (c.6473+2\_6473+3delTG) was recently reported as a homozygous variant in 2 siblings with heterotaxy and severe congenital heart disease (35). The variants c.T2399C (p.I800T) and c.C6949T (p.R2317W) were both present in

homozygous form in Subject 1, and present as heterozygous alleles in Subjects 2, 6, and 7 (Tables 2 and S2). Subject 2 was compound heterozygous for p.I800T and c.T7121C (p.I2374T). Subjects 3 and 4 had an allelic variant occurring near a splice junction on one allele, and a missense variant on the other allele. Subject 5 was compound heterozygous for a protein-truncating variant c.C7937G (p.S2646X) and a missense variant c.G8266A (p.D2756N). The variant p.R2317W was found in heterozygous form in 2 additional participants (Subjects 6 and 7); a second potentially pathogenic allelic variant was not readily identifiable in either individual (Table S2). Taken together, 5 participants in this study possessed rare and potentially pathogenic bi-allelic variants in a new candidate gene for BASM — *PKD1L1*.

The protein domains of PKD1L1 have not been mapped in fine detail, but exhibit similarities to portions of existing members of the greater PKD gene family (34, 36-38). The PKD1L1 protein is predicted to be 2,849 amino acids in length, with a large external N-terminal domain comprising approximately half the length of the protein (with PKD and REJ regions), followed by 11 transmembrane domains and ending in a short coiled-coil region at the C-terminus. Four of the 5 participants (Subjects 1, 2, 4, and 5) had at least 1 PKD1L1 coding variant in, or near, the proposed C-terminal coiled-coil region, necessary for interactions with PKD2 (36, 37). Three participants (Subjects 1, 2, and 3) had PKD1L1 variants in the large external N-terminal REJ region. Thus, in addition to the 2 splice site variants, there were 6 potentially deleterious PKD1L1 missense variants and 1 chain-terminating variant in 5 BASM subjects.

Polysplenia, intestinal malrotation, abdominal heterotaxy, and vascular anomalies were each present in 4 of the 5 individuals with bi-allelic *PKD1L1* variants (Table 2).

Polysplenia, abdominal heterotaxy, and vascular anomalies were jointly present in 3 individuals (Subjects 1, 2, and 5) while the concurrence of polysplenia, intestinal malrotation, and vascular anomalies was also reported in 3 individuals (Subjects 2, 3, and 5). Congenital heart disease/heterotaxy was reported in 2 subjects (Subject 1 – dextrocardia; Subject 5 – atrioventricular discordance, left atrial isomerism, left ventricular outflow obstruction, peripheral pulmonary and mitral valve stenosis). Although the clinical

manifestations of heterotaxy in these 5 BASM participants overlapped, there were no clear genotype-phenotype-outcome correlations between individual laterality or hepatic features and specific *PKD1L1* variants. Three of the 5 individuals (60%) with potentially pathogenic bi-allelic *PKD1L1* variants underwent liver transplantation – Subjects 2, 3, and 5 at ages 10, 0.5 and 5 years, respectively. Subjects 1 and 4 are surviving with their native livers at ages 7 and 13 years, respectively. Comparatively, 65% of individuals without notable *PKD1L1* variants from this cohort underwent liver transplantation at an average age of 26 months (range: 3 – 136 months). The limited sample size of this study precludes exploration of connections between *PKD1L1* variants and timing of liver transplantation.

#### ***PKD1L1 Expression in Human Bile Duct Epithelium.***

Protein function and gene expression data for human PKD1L1 are limited. In liver tissue obtained from a normal 3-day-old infant and 2 patients affected by other non-cholestatic liver diseases (carbonyl phosphate synthetase deficiency and hepatoblastoma), PKD1L1 is strongly expressed in cholangiocytes (Figure 4 B-C, G), while expression is absent in liver tissue from a patient with Alagille syndrome with paucity of interlobular bile ducts (Figure 4 D). Liver tissue from one BASM subject with bi-allelic *PKD1L1* variants was available from the ChiLDReN biorepository for immunohistochemical analysis – Subject 1. Notably, the biliary ductal plate profiles in this subject at the time of Kasai portoenterostomy revealed weak or absent expression of PKD1L1 (Figure 4A). Taken together, this is the first demonstration of PKD1L1 in human liver tissue with expression limited exclusively to bile duct epithelium.

## **Discussion**

To date, a firmly established etiology of BA remains elusive. Rather, multiple factors have been implicated in the perinatal biliary injury characteristic of BA that results in progressive intra- and extra-hepatic cholangiopathy evident soon after birth (1, 39). Until this study, a large cohort of BASM patients has not undergone extensive investigation utilizing next-generation sequencing, a technology that has identified disease-causing variants across a diverse spectrum of pediatric and adult diseases in various fields (40).

The application of WES to this cohort of 67 BASM participants, with a focused exploration of variants within a pre-specified list of 2,016 genes associated with ciliopathies, hepatobiliary development, and cholestasis, led to the identification of *PKD1L1* as a new candidate gene for BASM (Figure 3, Tables 2 and S2). Pathogenic variants in several previously-proposed BASM candidate genes, (i.e., *CFC1*, *FOXA2*, *INVS*, *NODAL*, and *ZIC3*) were not observed (Table S3) (41). In primary cilia, PKD1L1 heterodimerizes with PKD2L1 to form a transmembrane ciliary calcium channel that ultimately influences downstream Hedgehog signaling (36, 37, 42). Moreover, PKD1L1-PKD2 heterodimers are required during embryonic development to establish normal L-R patterning, and leads to heterotaxy when this pairing is disrupted (36, 37). These molecular interactions have yet to be explored in cholangiocytes which express PKD1L1 (Figure 4), a crucial step which will help establish the normal functioning of PKD1L1 in bile duct epithelium and the consequences of potentially pathogenic variations in the *PKD1L1* gene.

Support for *PKD1L1* as a plausible candidate gene for BASM includes recently discovered roles for *PKD1L1* variants in humans with heterotaxy and mouse models of laterality (35, 36, 43). Vetrini *et al.* reported the first series of patients with mutations in *PKD1L1* who presented with heterotaxy and severe congenital heart disease (35). Notably, the homozygous *PKD1L1* splice-site mutation, c.6473+2\_6473+3delTG, from that report was present in one allele of Subject 3 (Table 2). This BASM participant had polysplenia and intestinal malrotation without reported abdominal heterotaxy or congenital heart disease. It is not known if any of the 3 individuals from 2 families presented by Vetrini *et al.* had splenic or biliary tract abnormalities.

In both mice and Medaka fish, several *Pkd111* point mutants exhibit disruption of L-R patterning during embryologic development (36-38). However, as in the human report, the presence of any biliary tract abnormalities in these animal models is unknown, perhaps due to the low survival rates of progeny with heterotaxy due to dysfunctional *Pkd111* mutations. Intriguingly, only ~35-45% of mice with homozygous *Pkd111* mutations exhibited heterotaxy, attesting to the variable consequences and penetrance of *PKD1L1* variants on subsequent L-R patterning defects, even in well-defined mouse and zebrafish

genetic backgrounds. The incomplete penetrance observed in human and animal models of heterotaxy is likely the result of fundamental aspects of randomization inherent in L-R determination during embryogenesis (44). Extending this observation to the BASM syndrome, it is possible that the lack of apparent Mendelian inheritance of BASM within families could be due to reduced penetrance and/or variable expressivity of each specific gene variant during embryologic development. In addition, there may be undiagnosed or clinically-insignificant features of heterotaxy in family members, knowledge of which is currently unavailable within this dataset.

In the BASM subgroup, which accounts for ~10% of infants affected with BA, the coexistence of major congenital anomalies and a wide array of laterality defects along with biliary tract dysmorphogenesis suggests underlying genetic abnormalities linking the altered embryologic development of these structures (45). First classified as BASM by Davenport *et al.* in 1993 (5), various aspects of heterotaxy have been associated with individual cases of BA as early as 1892 (8) and 1941 (46) as well as in more recent case series (6, 7, 26). The diverse assortment of thoracic and abdominal anomalies of L-R asymmetry observed in patients with the BASM syndrome overlaps phenotypically with an emerging class of genetic disorders broadly categorized as ciliopathies; however, support for inclusion of BASM as a ciliopathy has, to date, been limited (16, 25). With functional validation and identification of additional ciliopathy genes like *PKD1L1*, BA, and BASM specifically, may ultimately be classified as a cholangiociliopathy (25).

In addition to *PKD1L1* gene variants as a potential contributor to the BASM syndrome, it is possible that reduced or temporally-modified *PKD1L1* RNA expression could play a role. Tsai *et al.* reported a heterozygous deletion of *FOXA2* in a child with BA, intestinal malrotation, and an interrupted inferior vena cava whose father had situs inversus and polysplenia, but not BA (41). The potential link between *FOXA2* and *PKD1L1* gene expression stems from activation of *Pkd1l1* transcription by *Foxa2* in mice (47). We did not identify candidate deleterious *FOXA2* gene variants in our cohort of 67 BASM participants (Table S3).



Whether *PKD1L1* variants reported in this study are relevant to the BASM syndrome awaits detailed functional and developmental analyses. Molecular functional validations of *PKD1L1* mutations as a cause of cholangiopathy are needed and will likely require multifaceted molecular and cellular approaches to investigate and assign normal, and variant, gene and domain functions. We have shown that PKD1L1 is expressed in normal human bile duct epithelium with reduced expression in Subject 1 (Figure 4). From a histological perspective, it is unknown to what degree PKD1L1 is expressed in patients with isolated BA, BASM patients with and without *PKD1L1* gene variants, and other human cholangiopathies, especially since ciliary structures are often blunted in BA bile duct epithelium(22). Moreover, studies of PKD1L1's function will require consideration and incorporation of known heterodimer partners PKD2L1 and PKD2, underscoring the complexities likely to follow detailed explorations of its role in primary ciliary function in cholangiocytes.

In conclusion, a WES-based exploration of variants in a robust ciliopathy gene collection led to the identification of rare and potentially deleterious bi-allelic variants in the *PKD1L1* gene in 5 of 67 BASM participants from the ChiLDReN database. We believe that *PKD1L1*, whose gene product is functionally relevant in primary ciliary calcium signaling, and whose loss-of-function results in heterotaxy in humans and various animal models, emerges as an etiologic candidate gene for BASM, and possibly select cases of BA without splenic malformation. The concept that disordered cholangiocyte ciliary structure and function produces the BASM syndrome seems biologically plausible, particularly given the established role of cilia in sensing and modulating biliary flow and composition, transmission of intracellular signals, and contribution to cholangiopathies (19, 20, 48). Future studies of *PKD1L1* in heterotaxy and BA patients and their families, as well as laboratory-based validations in specific cell and animal-based models, will be needed to determine specific mechanistic roles for *PKD1L1* in biliary tract development, cholangiocyte structure and ciliary signaling.

## Figure Legends

**Figure 1. Construction of the set of 2,016 genes of interest (GOI).** To explore the potential contribution of ciliary and ciliopathy gene variants underlying the BASM phenotype, a collated set of ciliopathy and biliary GOI was derived from 4 large comprehensive data sets: (1) Cildb – a knowledgebase for centrosomes and cilia (27, 49); (2) Simon Fraser University (SFU) Ciliome Database – a summary of ciliomic studies (31); (3) MCIL1 – Emory Genetics Laboratory Ciliopathies Sequencing Panel; and (4) MM340 – Emory Genetics Laboratory Neonatal and Adult Cholestasis Sequencing Panel.

**Figure 2. A.) The number of reported laterality features per participant.** Representation of the number of laterality features (1-6) per participant expressed as a percentage of the total cohort (*n* in parentheses). Note that the majority of participants (48) had two or more laterality defects. Four of the 67 participants had isolated renal anomalies. **B.) The 6 categorical types of reported laterality features.** The most common reported abnormalities of left-right patterning in the 67 BASM participants expressed as a percentage of the total cohort (*n* in parentheses). The majority had splenic abnormalities or intestinal malrotation, with substantial overlap in the types of anomalies between participants. Abbreviation: IVC, inferior vena cava.

**Figure 3. Schematic depiction of the *PKD1L1* variants in this report.** Exonic regions affected by each variant are noted among the 58 *PKD1L1* exons. Adapted from (50).

**Figure 4. *PKD1L1* expression in bile duct epithelium.** Immunohistochemical detection of *PKD1L1* in human liver tissue from: **A)** Subject 1; and representative regions from patients with **B)** carbamoyl phosphate synthetase deficiency; **C)** hepatoblastoma; and **D)** Alagille syndrome (with absence of portal tract bile ducts). *E-G* are from serial sections of liver tissue from a normal 3-day-old infant: **E)** hematoxylin and eosin staining; immunostaining with **F)** keratin 7 and **G)** *PKD1L1*. Bile duct profiles are highlighted and enlarged in *E-G*. Note robust *PKD1L1* expression in cholangiocytes from two livers affected by hepatocellular disease and normal pediatric liver tissue (*B, C, G*), absence in Alagille syndrome (*D*), and weak/absent expression in Subject 1 (*A*).

## References

1. Asai A, Miethke A, Bezerra JA. Pathogenesis of biliary atresia: defining biology to understand clinical phenotypes. *Nat Rev Gastroenterol Hepatol* 2015;12:342-352.
2. Verkade HJ, Bezerra JA, Davenport M, Schreiber RA, Mieli-Vergani G, Hulscher JB, Sokol RJ, et al. Biliary atresia and other cholestatic childhood diseases: Advances and future challenges. *J Hepatol* 2016;65:631-642.
3. Sokol RJ, Shepherd RW, Superina R, Bezerra JA, Robuck P, Hoofnagle JH. Screening and outcomes in biliary atresia: summary of a National Institutes of Health workshop. *Hepatology* 2007;46:566-581.
4. Bezerra JA, Wells RG, Mack CL, Karpen SJ, Hoofnagle JH, Doo E, Sokol RJ. BILIARY ATRESIA: Clinical and Research Challenges for the 21(st) Century. *Hepatology* 2018.
5. Davenport M, Savage M, Mowat AP, Howard ER. Biliary atresia splenic malformation syndrome: an etiologic and prognostic subgroup. *Surgery* 1993;113:662-668.
6. Davenport M, Tizzard SA, Underhill J, Mieli-Vergani G, Portmann B, Hadzic N. The biliary atresia splenic malformation syndrome: a 28-year single-center retrospective study. *J Pediatr* 2006;149:393-400.
7. Guttman OR, Roberts EA, Schreiber RA, Barker CC, Ng VL, Canadian Pediatric Hepatology Research G. Biliary atresia with associated structural malformations in Canadian infants. *Liver Int* 2011;31:1485-1493.
8. Thomson J. On Congenital Obliteration of the Bileducts, 1892: 52.
9. Shen O, Sela HY, Nagar H, Rabinowitz R, Jacobovich E, Chen D, Granot E. Prenatal diagnosis of biliary atresia: A case series. *Early Hum Dev* 2017;111:16-19.
10. Smith BM, Laberge JM, Schreiber R, Weber AM, Blanchard H. Familial biliary atresia in three siblings including twins. *J Pediatr Surg* 1991;26:1331-1333.

11. Werlin SL. Extrahepatic biliary atresia in one of twins. *Acta Paediatr Scand* 1981;70:943-944.
12. Hyams JS, Glaser JH, Leichtner AM, Morecki R. Discordance for biliary atresia in two sets of monozygotic twins. *J Pediatr* 1985;107:420-422.
13. Fallon SC, Chang S, Finegold MJ, Karpen SJ, Brandt ML. Discordant presentation of biliary atresia in premature monozygotic twins. *J Pediatr Gastroenterol Nutr* 2013;57:e22-23.
14. Kilgore A, Mack CL. Update on investigations pertaining to the pathogenesis of biliary atresia. *Pediatr Surg Int* 2017;33:1233-1241.
15. Braun DA, Hildebrandt F. Ciliopathies. *Cold Spring Harb Perspect Biol* 2017;9:a028191.
16. Deng H, Xia H, Deng S. Genetic basis of human left-right asymmetry disorders. *Expert Rev Mol Med* 2015;16:e19.
17. Davit-Spraul A, Baussan C, Hermeziu B, Bernard O, Jacquemin E. CFC1 gene involvement in biliary atresia with polysplenia syndrome. *J Pediatr Gastroenterol Nutr* 2008;46:111-112.
18. Cui S, Leyva-Vega M, Tsai EA, EauClaire SF, Glessner JT, Hakonarson H, Devoto M, et al. Evidence from human and zebrafish that GPC1 is a biliary atresia susceptibility gene. *Gastroenterology* 2013;144:1107-1115 e1103.
19. Larusso NF, Masyuk TV. The role of cilia in the regulation of bile flow. *Dig Dis* 2011;29:6-12.
20. Masyuk AI, Masyuk TV, LaRusso NF. Cholangiocyte primary cilia in liver health and disease. *Dev Dyn* 2008;237:2007-2012.
21. Chu AS, Russo PA, Wells RG. Cholangiocyte cilia are abnormal in syndromic and non-syndromic biliary atresia. *Mod Pathol* 2012;25:751-757.
22. Karjoo S, Hand NJ, Loarca L, Russo PA, Friedman JR, Wells RG. Extrahepatic cholangiocyte cilia are abnormal in biliary atresia. *J Pediatr Gastroenterol Nutr* 2013;57:96-101.
23. Mezina A, Karpen SJ. Genetic contributors and modifiers of biliary atresia. *Dig Dis* 2015;33:408-414.

24. Hartley JL, O'Callaghan C, Rossetti S, Consugar M, Ward CJ, Kelly DA, Harris PC. Investigation of primary cilia in the pathogenesis of biliary atresia. *J Pediatr Gastroenterol Nutr* 2011;52:485-488.
25. Rock N, McLin V. Liver involvement in children with ciliopathies. *Clin Res Hepatol Gastroenterol* 2014;38:407-414.
26. Schwarz KB, Haber BH, Rosenthal P, Mack CL, Moore J, Bove K, Bezerra JA, et al. Extrahepatic anomalies in infants with biliary atresia: results of a large prospective North American multicenter study. *Hepatology* 2013;58:1724-1731.
27. Arnaiz O, Cohen J, Tassin AM, Koll F. Remodeling Cildb, a popular database for cilia and links for ciliopathies. *Cilia* 2014;3:9.
28. Johnston HR, Chopra P, Wingo TS, Patel V, International Consortium on B, Behavior in 22q11.2 Deletion S, Epstein MP, et al. PEMapper and PECaller provide a simplified approach to whole-genome sequencing. *Proc Natl Acad Sci U S A* 2017;114:E1923-E1932.
29. Yang Y, Muzny DM, Xia F, Niu Z, Person R, Ding Y, Ward P, et al. Molecular findings among patients referred for clinical whole-exome sequencing. *JAMA* 2014;312:1870-1879.
30. Kotlar AV, Trevino CE, Zwick ME, Cutler DJ, Wingo TS. Bystro: rapid online variant annotation and natural-language filtering at whole-genome scale. *Genome Biol* 2018;19:14.
31. Inglis PN, Boroevich KA, Leroux MR. Piecing together a ciliome. *Trends Genet* 2006;22:491-500.
32. Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet* 2014;46:310-315.
33. Rentzsch P, Witten D, Cooper GM, Shendure J, Kircher M. CADD: predicting the deleteriousness of variants throughout the human genome. *Nucleic Acids Res* 2018.
34. Yuasa T, Venugopal B, Weremowicz S, Morton CC, Guo L, Zhou J. The sequence, expression, and chromosomal localization of a novel polycystic kidney disease 1-like gene, PKD1L1, in human. *Genomics* 2002;79:376-386.
35. Vetrini F, D'Alessandro LC, Akdemir ZC, Braxton A, Azamian MS, Eldomery MK, Miller K, et al. Bi-allelic Mutations in PKD1L1 Are Associated with Laterality Defects in Humans. *Am J Hum Genet* 2016;99:886-893.

36. Field S, Riley KL, Grimes DT, Hilton H, Simon M, Powles-Glover N, Siggers P, et al. Pkd111 establishes left-right asymmetry and physically interacts with Pkd2. *Development* 2011;138:1131-1142.
37. Kamura K, Kobayashi D, Uehara Y, Koshida S, Iijima N, Kudo A, Yokoyama T, et al. Pkd111 complexes with Pkd2 on motile cilia and functions to establish the left-right axis. *Development* 2011;138:1121-1129.
38. Grimes DT, Keynton JL, Buenavista MT, Jin X, Patel SH, Kyosuke S, Vibert J, et al. Genetic Analysis Reveals a Hierarchy of Interactions between Polycystin-Encoding Genes and Genes Controlling Cilia Function during Left-Right Determination. *PLoS Genet* 2016;12:e1006070.
39. Harpavat S, Finegold MJ, Karpen SJ. Patients with biliary atresia have elevated direct/conjugated bilirubin levels shortly after birth. *Pediatrics* 2011;128:e1428-1433.
40. Virani A, Austin J. Diagnostic clinical genome and exome sequencing. *N Engl J Med* 2014;371:1169-1170.
41. Tsai EA, Grochowski CM, Falsey AM, Rajagopalan R, Wendel D, Devoto M, Krantz ID, et al. Heterozygous deletion of FOXA2 segregates with disease in a family with heterotaxy, panhypopituitarism, and biliary atresia. *Hum Mutat* 2015;36:631-637.
42. Delling M, DeCaen PG, Doerner JF, Febvay S, Clapham DE. Primary cilia are specialized calcium signalling organelles. *Nature* 2013;504:311-314.
43. Vogel P, Read R, Hansen GM, Freay LC, Zambrowicz BP, Sands AT. Situs inversus in *Dpcd/Poll*<sup>-/-</sup>, *Nme7*<sup>-/-</sup>, and *Pkd111*<sup>-/-</sup> mice. *Vet Pathol* 2010;47:120-131.
44. Zaidi S, Brueckner M. Genetics and Genomics of Congenital Heart Disease. *Circ Res* 2017;120:923-940.
45. Yang MC, Chang MH, Chiu SN, Peng SF, Wu JF, Ni YH, Chen HL. Implication of early-onset biliary atresia and extrahepatic congenital anomalies. *Pediatr Int* 2010;52:569-572.
46. Smyth MJ. Congenital obliteration of bile ducts with total transposition of viscera. *British Medical Journal* 1941;1941:84-85.
47. Leordean D, Grimes D, Keynton J, Maier J, Harfe B, Benson M, Gray A, et al. FOXA2 controls Pkd111 expression in the mouse node during left-right determination. *Cilia* 2015;4:P37.

48. Mansini AP, Peixoto E, Thelen KM, Gaspari C, Jin S, Gradilone SA. The cholangiocyte primary cilium in health and disease. *Biochim Biophys Acta* 2017.
49. Arnaiz O, Malinowska A, Klotz C, Sperling L, Dadlez M, Koll F, Cohen J. Cildb: a knowledgebase for centrosomes and cilia. *Database (Oxford)* 2009;2009:bap022.
50. Aken BL, Achuthan P, Akanni W, Amode MR, Bernsdorff F, Bhai J, Billis K, et al. Ensembl 2017. *Nucleic Acids Res* 2017;45:D635-D642.

Author Manuscript

## Conflict of Interest

Dr. Kamath consults for Retrophin, Shire and Mirum.

Dr. Karpen consults for Intercept, Albireo, Retrophin, Mirum and Gemphire.

Dr. Molleston consults for Lilly and received grants from AbbVie, Gilead and Shire.

Dr. Murray received grants and owns stock in Merck. She received grants from Gilead and Shire.

Dr. Rosenthal consults and is on the speakers' bureau for Retrophin. He consults and received grants from Gilead and AbbVie. He consults for Intercept, Alexion, Albireo and Audentes. He received grants from Bristol-Myers Squibb and Roche.

Dr. Thompson consults, advises and owns stock in Generation Bio and Qing Bile. He consults for Albireo, Shire, Mirum and Arcturus.

Author Manuscript



**Table 1. Socio-demographic and clinical characteristics of the 67 BASM participants.**

| <b>Category</b>        | <b>n (%)</b> |
|------------------------|--------------|
| <b>ChiLDReN Study</b>  |              |
| BASIC                  | 43 (64)      |
| PROBE                  | 24 (36)      |
| <b>Sex</b>             |              |
| Female                 | 37 (55)      |
| Male                   | 30 (45)      |
| <b>Race/Ethnicity</b>  |              |
| White                  | 43 (64)      |
| Hispanic               | 10 (15)      |
| Asian                  | 6 (9)        |
| Black                  | 4 (6)        |
| Multiracial            | 4 (6)        |
| <b>Kasai HPE</b>       |              |
| Yes                    | 61 (91)      |
| Mean: 59 days          |              |
| Range: 21-122 days     |              |
| No                     | 6 (9)        |
| <b>Status</b>          |              |
| Liver Transplantation* | 41 (61)      |
| SNL                    | 18 (27)      |
| Unknown                | 6 (9)        |

|       |       |
|-------|-------|
| Died* | 3 (4) |
|-------|-------|

\*Note: One subject died after transplant.

Abbreviations: BASIC, Biliary Atresia Study in Infants and Children; BASM, biliary atresia splenic malformation; ChiLDRen, Childhood Liver Disease Research Network; HPE, hepatoportoenterostomy; PROBE, A Prospective Database of Infants with Cholestasis; SNL, survival with native liver. See Supplemental Figure S2 for a more detailed delineation of status at enrollment.

**Table 2. Clinical and sequence features of the five BASM participants with bi-allelic *PKD1L1* gene variants.**

| Subject | Polysplenia | Intestinal Malrotation | Abdominal Heterotaxy | Congenital Heart Disease | Vascular Anomaly | <i>PKD1L1</i> Variant | Variant Type | HGVS Nomenclature | RefSNP      | CADD | MAF     | Notes                  |
|---------|-------------|------------------------|----------------------|--------------------------|------------------|-----------------------|--------------|-------------------|-------------|------|---------|------------------------|
| 1       | X           | -                      | X                    | X                        | X                | Arg2317Trp            | Hom          | c.6949C>T         | rs139293796 | 31   | 0.1856% | Probable consanguinity |
|         |             |                        |                      |                          |                  | Ile800Thr             | Hom          | c.2399T>C         | rs148011149 | 23   | 0.0211% |                        |
| 2       | X           | X                      | X                    | -                        | X                | Ile800Thr             | Het (P)      | c.2399T>C         | rs148011149 | 23   | 0.0211% | -                      |

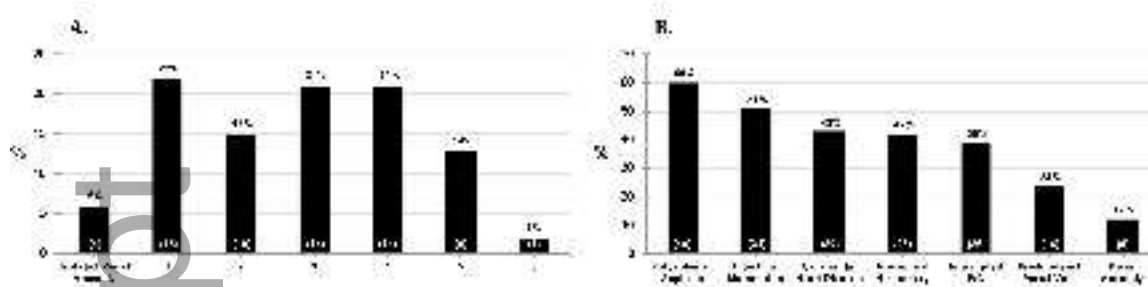
|   |   |   |   |   |   |              |         |                      |             |    |         |  |
|---|---|---|---|---|---|--------------|---------|----------------------|-------------|----|---------|--|
|   |   |   |   |   |   | Ile2374Thr   | Het (M) | c.7121T>C            | rs776420484 | 8  | 0.0012% |  |
| 3 | X | X | - | - | X | Arg874Trp    | Het (P) | c.2620C>T            | rs139858574 | 33 | 0.0154% | * Previously reported pathogenic mutation        |
|   |   |   |   |   |   | Splice Site* | Het (M) | c.6473+2_6473+3delTG | rs528302390 | 23 | 0.0365% |  |
| 4 | - | X | X | - | - | Splice Site  | Het (P) | c.2675+4C>T          | rs143005953 | 4  | 0.6072% | -  |
|   |   |   |   |   |   | Ser2473Phe   | Het (M) | c.7418C>T            | rs140456142 | 23 | 0.1027% |  |
| 5 | X | X | X | X | X | Asp2756Asn   | Het (#) | c.8266G>A            | rs770832954 | 24 | 0.0004% | # Presumed paternal inheritance (duo) or de novo |
|   |   |   |   |   |   | Ser2646Ter   | Het (M) | c.7937C>G            | rs752673990 | 36 | 0.0004% |  |

Abbreviations: BASM, biliary atresia splenic malformation; CADD, Combined Annotation Dependent Depletion; Het, heterozygous; HGVS, Human Genome Variation Society; Hom, homozygous; MAF, minor allele frequency (from gnomAD); M, maternal; P, paternal.



Figure 1. Contribution of the set of 2,016 genes of Interest (GOI). This equation is possible because the studies with a highly gene density under the ERS4 phenotype, a high density of highly variable GOI were derived from large comparisons with data collected by Cildb-939, a well-regarded performance and cell cycle (2013) by Florida University (SFU) and a Database of human gene expression studies (2014) (HNC1 - Enrich, Database Labs, Inc.) (Cildb-939), Supporting Research (1) (MM1 - Enrich, Research, Supporting Research and Data Collection Supporting First

hep\_30515\_f1.tiff



**Figure 2. A.** The number of reported laterality features per participant. Representation of the number of lateralized features per participant expressed as a percentage of the total cohort ( $n = 67$  in parentheses). Note that the majority of participants ( $n = 49$ ) had two or more lateralized features. **B.** Of the 67 participants had lateralized features. **B.1** The 8 categorical types of reported laterality features. The numbers on the top of each bar indicate the number of participants who reported that type of laterality feature as a percentage of the total cohort ( $n = 67$  in parentheses). The majority had explicit, nonnormal lateralized motorization, either bilateral or contralateral to the side of the hand as shown participants. Abbreviations: VC, inferior vena cava.

hep\_30515\_f2.tiff

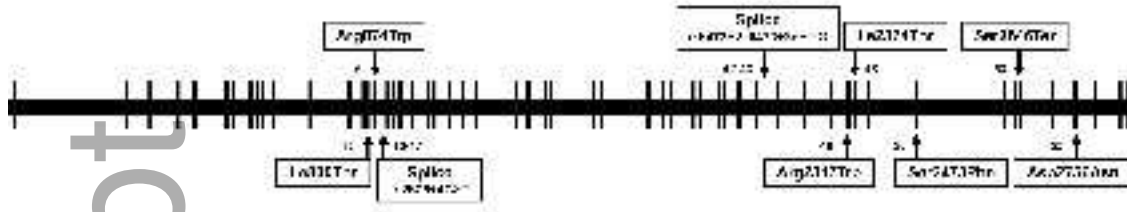


Figure 3. Schematic depiction of the PRQ2LT variants in the repeat. Exact regions of affected sequence are noted among the 28 PRQ2LT variants. (Abbreviations as in Table 1.)

hep\_30515\_f3.tiff

Author Manuscript

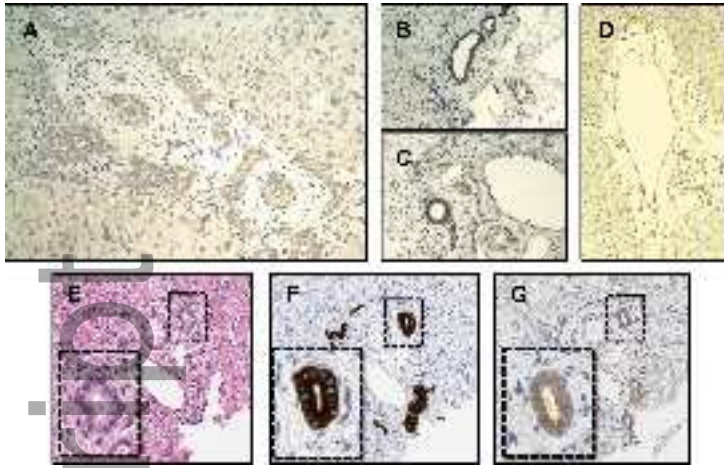


Figure 4. PKD1L1 expression in bile duct epithelium. Immunohistochemical detection of PKD1L1 in human liver tissue from (A) Subject 1 (non-metastatic liver) and from patients with (B) colorectal metastases (C) hepatic metastases and (D) esophageal metastases. Both panels (C) and (D) panel from bile ducts; (E) PKD1L1 from bile ducts of liver tissue from a patient with colorectal liver metastases in and out of bile duct (immunohistochemical staining: hematoxylin (H), Biotinylated DAP (B), DAPI (D)). (F) and (G) immunohistochemical staining and enhanced (H) of Non-metastatic PKD1L1 expression in bile ducts of liver tissue. (H) was obtained by image analysis of the enhanced panel (F) (see Table 1). (I) PKD1L1 immunohistochemical staining in esophageal metastases (E, G, C); also immunohistochemical staining in colorectal (F) and hepatic metastases (B) (immunohistochemical staining in subject 1).

hep\_30515\_f4.tiff

Author Manuscript