Citation for published version (APA):
Dixon, P. H., Sambrotta, M., Chambers, J., Taylor-Harris, P., Syngelaki, A., Nicolaides, K., Knisely, A. S., Thompson, R. J., & Williamson, C. (2017). An expanded role for heterozygous mutations of ABCB4, ABCB11, ATP8B1, ABCC2 and TJP2 in intrahepatic cholestasis of pregnancy. *Scientific Reports*, 7(1), [11823].
https://doi.org/10.1038/s41598-017-11626-x

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An expanded role for heterozygous mutations of \(\text{ABCB4}, \text{ABCB11}, \text{ATP8B1}, \text{ABCC2}\) and \(\text{TJP2}\) in intrahepatic cholestasis of pregnancy

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Intrahepatic cholestasis of pregnancy (ICP) affects 1/140 UK pregnancies; with pruritus, hepatic impairment and elevated serum bile acids. Severe disease is complicated by spontaneous preterm delivery and stillbirth. Previous studies have reported mutations in hepatocellular transporters (\(\text{ABCB4}, \text{ABCB11}\)). High throughput sequencing in 147 patients was performed in the transporters \(\text{ABCB4}, \text{ABCB11}, \text{ATP8B1}, \text{ABCC2}\) and tight junction protein 2 (\(\text{TJP2}\)). Twenty-six potentially damaging variants were identified with the following predicted protein changes: Twelve \(\text{ABCB4}\) mutations - Arg47Gln, Met113Val, Glu161Gly, Thr175Ala, Glu528Glyfs*6, Arg590Gln, Ala601Ser, Glu884Ter, Gly722Ala, Tyr775Met (x2), Trp854Ter. Four potential \(\text{ABCB11}\) mutations - Glu297Gly (x3) and a donor splice site mutation (intron 19). Five potential \(\text{ATP8B1}\) mutations - Asn45Thr (x3), and two others, Glu114Gln and Lys203Glu. Two \(\text{ABCC2}\) mutations - Glu1352Ala and a duplication (exons 24 and 25). Two potential mutations were identified in \(\text{TJP2}\); Thr62Met (x2) and Thr626Ser. No patient harboured more than one mutation. All were heterozygous. An additional 545 cases were screened for the potential recurrent mutations of \(\text{ATP8B1}\) (Asn45Thr) and \(\text{TJP2}\) (Thr62Met) identifying three further occurrences of Asn45Thr. This study has expanded known mutations in \(\text{ABCB4}\) and \(\text{ABCB11}\) and identified roles in ICP for mutations in \(\text{ATP8B1}\) and \(\text{ABCC2}\). Possible novel mutations in \(\text{TJP2}\) were also discovered.
prevalence of ICP was highest amongst the Native Chilean population. Further evidence of genetic involvement comes from sibling studies that show a 12-fold increase in risk between parous sisters.

Severe childhood liver disease can be caused by homozygous mutations in genes that encode transporters involved in bile formation at the hepatocyte canalicular membrane. These transporters play roles in cholestatic disorders that range in severity from progressive paediatric disease to transient cholestasis associated with drug ingestion or pregnancy (ICP).

Studies of the gene ABCB4, (ATP binding cassette subfamily B member 4) encoding the phosphatidylcholine floppase MDR3 and homozygously mutated in progressive familial intrahepatic cholestasis (PFIC) type 3, identified a homozygous mutation of ABCB4 first in a familial case and then in a sporadic instance of ICP. Subsequent studies have identified a wide range of mutations of ABCB4 in ICP, most commonly missense mutations. Approximately 10% of women with ICP harbour a mutated allele of ABCB4. Mutations of ABCB4 have also been implicated in drug-induced cholestasis and low phospholipid-associated cholelithiasis.

The bile salt export pump (BSEP), encoded by the gene ABCB11, (ATP binding cassette subfamily B member 11) is homozygously mutated in PFIC type 2 and benign recurrent intrahepatic cholestasis (BRIC) type 2. Several studies found ABCB11 mutations in women affected with ICP.

Of note is that in addition to causative mutations of ABCB4 and ABCB11 acting as Mendelian-like alleles, population studies of ICP cohorts have identified common genetic variants at these loci that contribute to disease risk, albeit to a much smaller degree than causative mutations.

The third PFIC (and BRIC) gene, ATP8B1, (ATPase class B8 member 1) encoding a phosphotidylserine flipase (familial intrahepatic cholestasis 1, FIC1), has only been studied to a limited extent (with respect to sequencing) in ICP cohorts, and hence the role of genetic variation at this locus in ICP is not established. A functional interdependence of this flipase with MDR3 in hepatocytes suggests that ATP8B1 remains a viable candidate for involvement in ICP susceptibility, as does the link to ABCB11 function through FXR/PLD2 signalling.

Another canalicular ATP-driven transporter, the multidrug resistance-associated protein 2 (MRP2, encoded by the gene ABCCC2(ATP binding cassette subfamily C member 2) is involved in bile formation. This protein exports bilirubin, some bile acids and many other anion conjugates into bile. Homozygous mutations of ABCCC2 cause the rare liver disorder Dubin-Johnson syndrome, which presents with conjugated hyperbilirubinaemia.

Although common variation and ICP susceptibility have been investigated at this locus in population cohorts, with conflicting results, there are no published analyses of comprehensive sequencing of the coding region of ABCCC2 in an ICP cohort.

Familial forms of severe progressive cholestasis without a confirmed genetic diagnosis also exist. Mutations of another gene, tight junction protein 2 (TJP2) have recently been implicated in some patients with such disease. The encoded protein named zona occludens 2 (ZO-2) is a cytosolic component of a number of types of tight junction. Nonsense and frameshift mutations in TJP2 have previously been reported as pathogenic in cholestatic disease. Three novel mutations are predicted to result in a frameshift leading to a stop codon six missense amino acids thereafter (Glu528Glyfs*6) and two immediate frameshift mutations of TJP2 were identified in these individuals (Table 1). All of the missense variants identified, except where specified, were observed in the heterozygous state and no patient harboured more than one potentially pathogenic variant. The frequency of two potential mutation was identified together with its predicted protein consequence. Clinical findings for each patient in whom a potential mutation was identified are also shown (Table 2). All of the described mutations were observed in the heterozygous state and no patient harboured more than one potentially pathogenic variant. The frequency of two of the previously identified common susceptibility variants for ICP, namely rs2109505 in ABCB4 and rs2287622 in ABCB11, was noted in these individuals (Table 1). All of the missense variants identified, except where specified (Table 1) were predicted to be pathogenic by SIFT and MutationTaster.

ABC4 variants. Consistent with previous findings, the largest number of potential mutations (11 different variants in 12 patients) were identified in the gene encoding the phosphatidyl choline floppase ABCB4 in this patient cohort (Table 1). Two predicted resulting variants, namely Thr175Ala and Arg590Gln, have previously been reported as pathogenic in cholestatic disease. Three novel mutations are predicted to result in a frameshift leading to a stop codon six missense amino acids thereafter (Glu528Glyfs*6) and two immediate frameshift mutations of ABCB4 were identified in these individuals (Table 1). All of the missense variants identified, except where specified (Table 1) were predicted to be pathogenic by SIFT and MutationTaster.
introductions of a premature stop codon (Glu684Ter, Trp854Ter), a type of mutation only very infrequently previously described in ICP15. The remaining 7 mutations are novel missense changes predicted to be pathogenic (Alamut).

**ABCB11 variants.** Fewer potentially pathogenic variants (n = 2, 4 patients) were identified in ABCB11, encoding the bile salt export pump. A recurrent mutation, predicted to yield the substitution Glu297Gly, first described in PFIC2 patients27, and later identified in a study of ICP patients 29, was discovered in 3 separate patients not known to be related. A donor splice site mutation in intron 19 was identified with the predicted consequence of an exon skipping event. A missense variation, resulting in the protein change Asn591Ser (dbSNP: rs11568367), was identified in an additional patient, which has previously been reported in ICP28. However, despite this amino acid being highly conserved, bioinformatic tools predict the variation to be benign; this allele is also relatively common in the South Asian population (MAF 11%). Thus, it seems not likely to be a pathogenic variant.

**ATP8B1 variants.** Several variants of interest were identified in ATP8B1, encoding a phosphatidyl serine flippase. A mutation predicted to be pathogenic by SIFT and Mutation Taster, causing the protein change Asn45Thr, was seen in 3 unrelated individuals. Two further variants predicted to be pathogenic were identified in 2 additional patients, resulting in the protein changes Glu114Gln and Lys203Glu. To clarify the potential pathogenic and recurrent role (in ICP) of the Asn45Thr variant a cohort of 545 cases was screened for this change (see methods above); an additional 3 patients harbouring this variant were identified (Tables 1 and 2).

**ABCC2 variants.** Two significant alterations were identified in ABCC2, encoding MRP2, a transporter of conjugated bilirubin and other organic anions. A single missense substitution, predicted to result in the protein change Glu1352Ala was identified in one patient. Alamut analysis indicated that SIFT and mutationTaster both predict the change to be deleterious. A second identified DNA change, confirmed by RT-PCR dosage methodology, was a genomic duplication of 5299 base pairs, encompassing exons 24 and 25 of the gene and predicted

### Table 1. Potential pathogenic variants identified in this study. Notes: Individuals in italics were identified by second screen of larger cohort. NV - novel variant not currently reported in dbSNP. NT - not typed. 1 Insertion is out of frame. 2 SIFT predicts tolerated, mutation taster predicts disease causing. rs2287622 shows amino acids at this SNP, rs2109505 is nucleotides (SNP is synonymous).
to result in the insertion of a premature stop codon. This copy number variant has been reported once before (database reference esv3423829).

Three patients harboured TJP2 variants of potential interest. Two unrelated individuals had the same DNA mutation causing the protein change Thr62Met. This alteration is predicted to be disease causing by Mutation Taster; SIFT, however, predicts this change to be tolerated. A second missense alteration seen in one patient is predicted to cause the protein alteration Thr626Ser. This change is also assessed as tolerated by SIFT but as disease causing by Mutation Taster. 545 ICP cases (as above) were subsequently screened for the possible recurrent Thr62Met variant, however no further ICP cases carrying this change were identified.

Biochemical and clinical features of ICP cases with variants. Elevations in serum BA and alanine transaminase (ALT) activity values varied among patients and there was no particular trend for greater elevation of specific analytes in women with particular mutations in specific genes. Hyperbilirubinaemia was rare. It did, however, occur in association with ABCB4, ABCB11 or ATP8B1 variants. Six women had elevated bilirubin values (>20µmol/L); all had severe ICP (serum BA >40µmol/L). The two whose bilirubin concentrations were consistent with jaundice (>35µmol/L) harboured ABCB4 mutations. Only two women with mutations (one in ABCB4, one in ATP8B1) were known to have elevated gamma glutamyl transferase (GGT) levels. Of the 26 women with a mutation in a biliary transporter (ABCB4, ABCB11, ATP8B1, ABCC2), 12 (46%) had an established family history of biliary disease (ICP, cirrhosis or gallstones).

### Table 2. Clinical and biochemical findings in the individuals with potential mutations.

| Patient | ALT | ALP | GGT | Bilirubin | Bile Acids | Maternal Biliary Disease | F/H Biliary Disease | Adverse pregnancy outcomes |
|---------|-----|-----|-----|----------|------------|-------------------------|---------------------|---------------------------|
| 1       | 345 | 159 | 20  | 40       | 150        | no                      | no                  | SPL, M                   |
| 2       | 27  | 204 | 13  | 4        | 16         | no                      | no                  | PPH                      |
| 3       | 45  | 280 | 18  | 12       | 39         | no                      | no                  | no                       |
| 4       | 35  | 284 | NP  | 6        | 30         | no                      | no                  | no                       |
| 5       | 172 | 522 | 20  | 13       | 239        | G                       | G, ICP              | no                       |
| 6       | 291 | 244 | 18  | 8        | 39         | G                       | G                   | no                       |
| 7       | 1802| 710 | 10  | 18       | 108        | no                      | ICP*                | M, FHR                   |
| 8       | 58  | 261 | NP  | NP       | 78         | no                      | G                   | no                       |
| 9       | 579 | 270 | NP  | NP       | 112        | G                       | G, M, FHR           | no                       |
| 10      | 115 | 376 | 28  | 7        | 44         | no                      | Cirr                | no                       |
| 11      | 776 | 292 | 57  | 50       | G, C       | G, ICP                 | FHR                 | no                       |
| 12      | 803 | 204 | 66  | NP       | 222        | no                      | G, Cirr             | no                       |
| 13      | 229 | 238 | NP  | 23       | 118        | G, C                   | nk                  | SPL, M, FHR              |
| 14      | 94  | 190 | 12  | 8        | 23         | no                      | no                  | no                       |
| 15      | NP  | 91  | 8   | 4        | 14         | no                      | no                  | no                       |
| 16      | 32  | 302 | 11  | 4        | 44         | G                       | no                  | NNU                      |
| 17      | 410 | 344 | 29  | 16       | 27         | no                      | PPH                 | no                       |
| 18      | 131 | 247 | 38  | 26       | 69         | no                      | no                  | no                       |
| 19      | 707 | 263 | 17  | 20       | 120        | G                       | G                   | FHR                      |
| 20      | 23  | 227 | 36  | 10       | 35         | G                       | G, Cirr             | no                       |
| 21      | 96  | 261 | 33  | 9        | 22         | no                      | no                  | no                       |
| 22      | 506 | 510 | 110 | 16       | 182        | no                      | G                   | SPL                      |
| 23      | 415 | 358 | 32  | 18       | 16         | nk                      | nk                  | no                       |
| 24      | 101 | 323 | 18  | 4        | 17         | no                      | no                  | FHR                      |
| 25      | 14  | 291 | 23  | 11       | 66         | no                      | no                  | no                       |
| 26      | 80  | 257 | NP  | 10       | 73         | no                      | G                   | no                       |
| 27      | 110 | 178 | NP  | 9        | 57         | nk                      | nk                  | no                       |
| 28      | 225 | 435 | 26  | 17       | 42         | no                      | no                  | SB                       |
| 29      | 740 | 527 | NP  | 127      | 127        | no                      | no                  | no                       |

*Based on recollection of maternal aunt.
Discussions
This study is the largest analysis to date of the role of mutations of familial cholestasis genes in ICP susceptibility. Previous studies have identified genetic contributions to the disease primarily focussing on ABCC4. Here, by using a next generation sequencing approach, a panel of genes could be analysed in parallel in a large cohort of patients.

Our analysis identified a number of mutations of ABCC4, encoding the phosphatidyl choline flopase MDR3. ABCC4 has been analysed in a number of ICP cohorts and mutations in this gene represent the largest overall genetic contribution to ICP susceptibility known to date. Several groups have established functional assays for this protein that can incorporate mutant constructs. Such assays help to define the impact of a given variant on protein function; bioinformatics predictors of clinical importance of variants like those used in this study are useful (and improving) but can never be conclusive. Functional assays are being used to classify mutations by mechanism of effect on protein function (e.g., trafficking defect resulting in mis-localisation, loss of flopase activity). This is of value in ABCC4-associated diseases; these data may predict response to therapy intended to rescue flopase function. It is noteworthy that in the ABCC4 mutant carrier group, 8/12 (67%) had a family history of biliary disease (ICP, cholelithiasis or cirrhosis) (Table 2). This is consistent with a study of parous women with LPAC and co-existing ABCC4 mutations, in which approximately 50% developed ICP when pregnant.

A clear role is now established for genetic variation in ABCC11 contributing to the aetiology of ICP, although one seemingly less than that of ABCC4. Our study has confirmed this role; taken together with other data, it suggests that up to 5% of ICP cases harbour a heterozygous mutation of ABCC11. In particular, the two mutant alleles found commonly in the PFIC2/BRIC2 population (Glu297Gly and Asp482Gly) are also present in the ICP population. Functional studies of ABCC11 mutations have been performed in addition to mini-gene construct splicing analysis and immunohistochemical studies to establish genotype/phenotype correlations. Studies of this type are helpful to clarify the clinical relevance of findings of unknown significance. Two of the five women in this group had cholelithiasis (Table 2). Previous studies of ICP patients and ABCC11 mutations have not identified this association.

Analysis of ATP8B1 in ICP has been limited, with the largest study focussing on common SNPs and population risk rather than mutations. In this study we confirmed a small potential role for ATP8B1 variants to contribute to ICP. We examined a larger ICP population for the recurrent variant (Asn45Thr) and identified three further individuals harbouring it. Taken with the other mutations identified in this gene, it is therefore possible that up to 3% of ICP patients may have a heterozygous mutation of ATP8B1. However, the Asn45Thr variant, although predicted to be deleterious, is fairly common in the normal population (global minor allele frequency 0.49%), and has been reported in a study of chronic pancreatitis. Although this does not exclude this variant from having a mildly deleterious effect on protein function, in vitro experimental approaches are warranted to confirm if this variant contributes to disease risk.

That our cohort included two patients with potentially pathogenic variants of ABCC2, a missense mutation predicted to be pathogenic and a large in-frame duplication, is of interest. No published studies report sequencing this gene in ICP; our data suggest a small but potentially overlooked contribution to ICP by heterozygous ABCC2 mutations.

This study is the first to examine TJP2 in an ICP cohort. We have identified some evidence for involvement of this gene, with three occurrences of variants that may predispose to disease. However, as bioinformatics prediction tools did not agree on the clinical relevance of these changes, further analysis is warranted. Development of functional assays for this protein, together with further sequencing of TJP2 in larger patient cohorts, will be key to understanding the role of these variants.

A spectrum of variants have been identified in this and other ICP cohorts. Although some variants will be predicted to have only a mild impact upon disease susceptibility, either from database predictions or using in vitro assays, the cholestatic impact of gestational elevations of reproductive hormones may result in a more severe phenotype in the context of pregnancy.

Heterozygous mutations in the five genes sequenced were identified in nearly 20% of the ICP population being studied. However, the mutant-carrying groups exhibited no clear phenotypic differences (Table 2). Serum BA or ALT values did not vary by gene and raised serum GGT activity was not common in those with a mutation in ABCC4. Biomarkers that indicate which biliary transporter is mutated in a specific ICP patient thus remain to be found. That >50% of the women with identified variants had a family history of biliary disease is noteworthy; this information could be useful in a clinical setting to make decisions about mutation screening. Further studies with deeper phenotyping approaches (especially metabolic profiling/serum lipidomics and reproductive hormone metabolite analysis, based on our knowledge of disease pathophysiology) are warranted to determine if these mutation groups can be distinguished by clinical phenotype. Research into the impact of specific genotypes on drug response, risk of subsequent hepatobiliary disease, resolution of symptoms after delivery, duration of symptoms, and long term maternal and child health will be of particular value.

In this study, we used a candidate sequencing approach to identify mutations with a potentially large impact on protein function. Separate studies have also taken a population genetics approach using candidate single nucleotide polymorphisms and/or tagging SNPs in an attempt to identify common variants that contribute to overall risk. Common variants that make a much smaller contribution to disease risk in ICP populations have been identified at the ABCB4 and ABCC11 loci (in particular rs2109505 (ABCB4) and rs2287622/rs7577650 (ABCB11)). Thus, the genetic architecture of ICP is complex, with rare mutant alleles, often but not exclusively specific to each patient, present in affected women, such as reported in this study, together with common variants at the same loci that represent risk alleles as we have previously described. Although our previous study excluded a large role for common variation of ATP8B1 and ABCC2 in ICP, studies in larger cohorts may identify variants conferring a small degree of risk (or protection), given the potential high-impact mutations of these genes.
genes identified by this study. Common variation around TJP2 causing small alterations in risk (which this study was not powered to detect) may also play a role.

We have previously studied the role of FXR variants in ICP and identified a small number of rare variants with a functional effect\(^6\). When designing this study this gene was not included in the screening panel. However, the recent identification of FXR mutations in progressive familial intrahepatic cholestasis strengthen the case for further FXR analysis in future studies of ICP\(^5\).

The catalogue of ICP-associated mutations that this analysis, and others, have established permits consideration of the possibility of precision genomic medicine. With sequencing costs falling and technology continuing to advance, to contemplate screening all of the candidate genes for ICP is now feasible, enabling genetic diagnosis that in future may allow better targeted treatment and surveillance.

**Methods**

Blood samples were collected in standard EDTA vacutainers and buffy coats were prepared by centrifugation using standard protocols. DNA was extracted from 200 µl of buffy coat using the Qiagen Blood mini kit (Crawley, UK). Extracted DNA was assessed for purity using a ND-1000 Nanodrop spectrophotometer (Thermofisher Scientific, Loughborough, UK) and quantified using a Qubit fluorimeter (Thermofisher).

Selected genes known or hypothesized to be involved in ICP (ABCB4, ABCB11, ATP8B1, ABCC2 and TJP2) underwent targeted next-generation sequencing. Oligonucleotides ("oligos") to screen the entire coding region of each gene were designed using the Illumina web-based sequencing assay design tool Design Studio (http://designstudio.illumina.com/) and used in the Illumina TruSeq custom amplicon v1.5 workflow according to the manufacturer's instructions. This workflow has three main stages: 1) hybridisation of the oligos to 50 ng/µl of genomic DNA; 2) extension and ligation of the oligos; 3) PCR amplification using two unique index primers for sample. Afterwards, sample DNA concentrations were normalised using the Illumina bead-based system and pooled in a final library. The pooled library was then sequenced using a MiSeq Reagent Kit v2, 300-cycle (2 × 150bp), on the MiSeq benchtop sequencing platform (Illumina, Cambridge, UK).

Sequencing data were analysed using a bioinformatics pipeline designed in Biomedical Genomic Workbench 2.5.1 (CLC bio, Aarhus, Denmark). Sequence reads were mapped against the human reference (hg19/GRCh37). Variants were called when they had a minimum coverage of 20 × and a probability of 80% of being different from the reference, in accordance with the Bayesian model incorporated into the software. Putative variants identified were filtered according to the quality and the frequency of occurrence; all changes having an allele read frequency of less than 10% and an average base quality (Phred score) of less than 20 were removed. The variants identified were further annotated using dbSNP v139 and the 1000 Genome Project database. A deeper investigation was also performed using Alamut Visual 2.7.1 (Interactive Biosoftware, Rouen, France), in which each variant was integrated with allele frequency information derived from several single nucleotide polymorphism (SNP) databases, including dbSNP v144, the 1000 Genomes Project, the Exome Aggregation Consortium and the National Heart Lung and Blood Institute Grand Opportunity Exome Sequencing Project and with in silico missense predictions from SIFT (sorting intolerant from tolerant) and MutationTaster. Copy number variants (CNVs) were detected via in-house dosage analysis using the read-counts generated by the NGS analysis. The CNVs were confirmed by RT-PCR using Universal Probe Library (Roche).

Each potential pathogenic variant was confirmed in patient samples using routine Sanger sequencing. Forward and reverse primers were manually designed for the flanking regions and purchased from Integrated DNA Technologies (sequences available on request).

Analysis of potential recurrent mutations of ATP8B1 and TJP2 were performed following primer design ("Primer picker", LGC Genomics, Hoddesdon, UK) using a competitive allele specific PCR SNP system using fluorescence resonance energy transfer (FRET) quencher cassette oligonucleotides (KASP\(^\text{TM}\), LGC Genomics).

**Data Availability.** Any further information on the sequencing data described in this paper is available from the corresponding author on reasonable request.

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Acknowledgements
The authors wish to acknowledge the patients who participated in this study, the doctors and midwives for their help with recruitment and sample collection, and the assistance of the Institute for Liver Studies, the National Institute of Health Research Biomedical Research Centres at Imperial College London and at Guy’s and St Thomas’ NHS Foundation Trust and King’s College London. The views expressed herein are those of the authors and not necessarily those of the NHS, NIHR or the Department of Health. The authors are grateful for helpful discussions with Kenneth Linton and Pierre Foskett. Financial support: The Lauren Page Trust, ICP support, Institute of Liver Studies, Wellcome Trust (P30874), National Institute of Health Research Biomedical Research Centres at the Guy’s and St Thomas’ NHS foundation Trust and the Imperial College Healthcare NHS Trust.

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Additional Information
Competing Interests: The authors declare that they have no competing interests.

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