Neonatal cholestasis: emerging molecular diagnostics and potential novel therapeutics

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Abstract | Neonatal cholestasis is a group of rare disorders of impaired bile flow characterized by conjugated hyperbilirubinaemia in the newborn and young infant. Neonatal cholestasis is never physiological but rather is a sign of hepatobiliary and/or metabolic disorders, some of which might be fatal if not identified and treated rapidly. A step-wise timely evaluation is essential to quickly identify those causes amenable to treatment and to offer accurate prognosis. The aetiology of neonatal cholestasis now includes an expanding group of molecularly defined entities with overlapping clinical presentations. In the past two decades, our understanding of the molecular basis of many of these cholestatic diseases has improved markedly. Simultaneous next-generation sequencing for multiple genes and whole-exome or whole-genome sequencing now enable rapid and affordable molecular diagnosis for many of these disorders that cannot be directly diagnosed from standard blood tests or liver biopsy. Unfortunately, despite these advances, the aetiology and optimal therapeutic approach of the most common of these disorders, biliary atresia, remain unclear. The goals of this Review are to discuss the aetiologies, algorithms for evaluation and current and emerging therapeutic options for neonatal cholestasis.

Gilbert syndrome
A benign condition caused by a decrease in the activity of UGT1A1 that leads to intermittent elevations in serum unconjugated or indirect bilirubin levels.

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https://doi.org/10.1038/s41575-019-0132-z

Jaundice, a yellow discoloration of the skin, sclera, mucous membranes and bodily fluids, is a common clinical finding in the first 2 weeks of neonatal life, occurring in up to 15% of breastfed infants[1]. In the majority of cases, jaundice is caused by indirect or unconjugated hyperbilirubinemia resulting from physiological jaundice, breastfeeding and breast milk-associated jaundice, red blood cell haemolysis, Gilbert syndrome or rarely Crigler–Najjar syndrome[2]. Unconjugated hyperbilirubinemia often resolves either without intervention or with phototherapy. However, in a minority of infants with jaundice, elevated circulating levels of direct or conjugated bilirubin indicate the presence of cholestasis (impaired bile formation or flow) and importantly might be the presenting sign of serious hepatobiliary or metabolic dysfunction[3] (BOX 1). Jaundice in a formula-fed infant at 2 weeks of age or persistent jaundice beyond 2 weeks of age in a breastfed infant should always alert the clinician to the possibility of cholestasis and should prompt immediate fractionation of the serum bilirubin into a direct or conjugated and indirect or unconjugated portion. Cholestasis is generally defined as a conjugated or direct serum bilirubin level >17 μmol/l (1 mg/dl) when the total bilirubin is <85.5 μmol/l (5 mg/dl) or >20% of the total bilirubin if the total bilirubin is >85.5 μmol/l (REF.[4]). Some studies suggest that during the first 5 days of life, the thresholds to raise the suspicion for cholestasis for direct or conjugated bilirubin could possibly be as low as 5 μmol/l (0.3–0.4 mg/dl) and 10% of the total bilirubin[5–7]. Conjugated hyperbilirubinemia is never physiological or normal and should always prompt further evaluation for an underlying hepatobiliary problem, the urgency and pace of which depend on the age and condition of the infant, physical findings, laboratory values and family history.

Early recognition and expedited evaluation and treatment of an infant with cholestasis are of utmost importance. In certain cholestatic conditions, such as galactosaemia, sepsis or presence of a choledochal cyst, immediate medical or surgical treatment is required. For other aetiologies, early intervention results in improved prognosis. For example, in infants with biliary atresia, the success rate of the hepatoportoenterostomy (HPE) or Kasai procedure in restoring bile flow is highest when surgery is performed in infants before 30–45 days of age[8–10]. All children with cholestasis will benefit from both early nutritional intervention with supplemented breast milk or medium-chain triglyceride (MCT)-containing formula and fat-soluble vitamin supplementation to enhance growth and prevent

Key points

- Early recognition and expedited evaluation of an infant with cholestasis are of utmost importance, as neonatal cholestasis is never physiological and often requires immediate treatment or intervention.
- Cost-effective methods to reliably screen for biliary atresia in the first month of life are needed to improve age at diagnosis and Kasai hepatoportoenterostomy for infants with biliary atresia.
- New genetic causes of neonatal cholestasis are being discovered at a rapid rate owing to the advent of next-generation gene-sequencing technologies and sophisticated bioinformatics.
- Use of genetic testing might enable us to rapidly identify genetic causes of cholestasis without the need for invasive procedures and might lead to new precision treatments.
- Multiple sites exist within the hepatobiliary tree where bile formation or flow can be impaired, resulting in neonatal cholestasis; these sites are potential targets for new pharmacological therapies.

fat-soluble vitamin deficiencies, as well as from medical management to prevent or treat complications of cholestasis such as cholangitis, portal hypertension and/or pruritus. Unfortunately, delayed recognition of neonatal cholestasis and misdiagnosis as physiological jaundice or breastfeeding-related jaundice still remain a problem[11]. Reasons cited for late referral for evaluation of cholestasis include early hospital discharge of newborn babies, inadequate follow-up of persistent jaundice, improved jaundice as physiological indirect hyperbilirubinaemia resolves despite the persistence of cholestasis, a lack of a standard well-child visit at 1 month of life, false reassurance by the appearance of pigmented stool, fluctuating serum bilirubin levels and misdiagnosis of breast milk-associated jaundice[11–14]. Unfortunately, although the average age of diagnosis of biliary atresia and HPE in some countries (for example, Taiwan) has decreased with improved screening techniques[15], the median age at time of HPE in the USA is 63 days and has shown little change over the past 15 years despite many efforts to educate caregivers about the importance of early diagnosis[16]. Moreover, there are few therapeutic approaches that delay or abrogate progression to portal hypertension and liver failure in many of the underlying conditions. However, we are optimistic that this situation will change in the near future for a number of reasons. First, emerging reports suggest that screening all infants for a direct or conjugated bilirubin level in the first week of life might enable early identification of those who have cholestasis and those at risk of biliary atresia[5–7]. Second, there is growing clinical availability of targeted gene panels (TGPs) utilizing next-generation sequencing (NGS) technology or whole-exome sequencing (WES) and whole-genome sequencing (WGS) that can simultaneously and rapidly test for dozens of genetic causes of cholestasis at a relatively low cost[17–21], which might also have potential to discover new aetiologies. Finally, although little attention has been paid over the past 40 years to developing improved therapies for chronic cholestatic conditions, a new pipeline of potential pharmacological agents is now on the horizon. In this Review, we describe the current state of the art for evaluation and management of neonatal cholestasis, emphasizing new concepts and emerging technologies that will advance our evaluation and treatment paradigms for this disease.

Crigler–Najjar syndrome
An autosomal recessive disorder caused by mutations in *UGT1A1* that lead to markedly elevated serum unconjugated or indirect bilirubin levels.

Galactosaemia
An autosomal recessive disorder caused by mutations in *GALT* that result in an inability to metabolize galactose normally, affecting the liver, brain and lens of the eye.

Choledochal cyst
Congenital dilatation of the biliary system.

Biliary atresia
A progressive sclerosing inflammatory process of the extrahepatic and intrahepatic bile ducts in infants under 3 months of age that leads to fibrosis and obliteration of the biliary tree.

Hepatoportoenterostomy (HPE) or Kasai procedure
A surgical procedure for treatment of biliary atresia in which a Roux-en-Y loop of jejunum is connected to the porta hepatitis, enabling bile to flow from the liver to the intestines.

Alagille syndrome
An autosomal dominant disorder in which a mutation in *JAG1* or *NOTCH2* results in phenotypic abnormalities including interlobular bile duct paucity.

Aetiology of neonatal cholestasis

Cholestatic jaundice affects ~1 in every 2,500 infants worldwide[3,14], resulting from a wide variety of disorders including infections, anatomic obstruction of the biliary system, endocrinopathies, genetic and metabolic disorders, toxin and drug exposures, cardiovascular abnormalities, transient neonatal cholestasis (TNC; formerly called idiopathic neonatal hepatitis) and other miscellaneous causes[22] (BOX 1). Of the >100 conditions that result in neonatal cholestasis, the most commonly identifiable aetiologies are biliary atresia (25–55%), a variety of genetic and metabolic diseases including α1-antitrypsin (A1AT) deficiency (10–20%), Alagille syndrome (syndromic paucity of interlobular bile ducts caused by autosomal dominant single mutations in either *JAG1* or *NOTCH2*; 2–14%), hypopituitarism, biliary sludge, preterm birth and TNC[23]. In some racial and ethnic groups, citrin deficiency (East Asian populations) or Niemann–Pick disease type C1 (Hispanic populations) is a more common cause of neonatal cholestasis than A1AT deficiency[24,25]. Infectious causes of neonatal cholestasis outside of urinary tract, herpes simplex virus (HSV) and cytomegalovirus (CMV) infections are uncommon. In preterm infants, cholestasis is more common, occurring at an incidence of 10–20%, and is often multifactorial, resulting from infections, absence of feedings and stimulation of gallbladder emptying, hypotension with ischaemic injury to the liver, intestinal injury or inflammation, medications and the use of parenteral nutrition formulations containing soy lipid emulsions[26]. Overall, 18–67% of infants on >14 days of parenteral nutrition develop liver injury and cholestasis, with the incidence increasing with lower birthweight, longer duration of parenteral nutrition therapy, use of soy-based lipid emulsions, sepsis, intestinal dilatation and bacterial overgrowth and intestinal failure[27–29].

Over the past several decades, major advances in the ability to discover new genetic causes of cholestasis have resulted in a substantial decline in the number of infants who were previously classified as having TNC, which was once the second most common aetiology of neonatal cholestasis[23,30]. The transient nature of the cholestasis in infants with TNC has been linked to heterozygosity of mutations in genes involved in bile transport (*ATP8B1*, *ABCB11* and *ABCB4*)[31–33]. An expanding list of newly discovered genetic causes for cholestasis (BOX 1), including six genes that cause the progressive familial intrahepatic cholestasis (PFIC) phenotype, has shed new light on the physiology and pathophysiology of bile formation. Moreover, these gene discoveries have spawned new diagnostic testing schema and disease classifications, and novel potential therapeutic targets for drug development. With next-generation DNA sequencing now available as a clinical tool, the number of infants with identifiable causes of cholestasis will continue to rise, as will the number of known mutations and genes resulting in cholestasis. This impending revolution in the evaluation and management of infants with cholestasis will almost assuredly lead to the application of precision cholestasis medicine.

Tyrosinaemia type 1
An autosomal recessive disorder caused by a defect in the enzyme fumarylacetoacetate hydrolase resulting in accumulation of toxic intermediates including succinylacetone in tissues and organs, leading to hepatocellular damage, hepatocellular carcinoma and neurotoxicity.

Pathophysiology of cholestasis

There are multiple sites within the hepatobiliary tree, as well as in bile acid synthesis and metabolism pathways, where bile formation or bile flow can be impaired, resulting in neonatal cholestasis (FIG. 1). These sites include perturbations in hepatocyte function and bioenergetics, defects in hepatocellular and biliary secretory pathways and tight junction formation, abnormal bile duct development or injury and mechanical obstruction to bile flow[34]. Reduced generation of bile might result from inherited pathological variants of genes regulating bile acid synthesis (*AKR1D1, AMACR, CYP7B1, HSD3B7, CYP7A1* and *CYP27A1*), bile acid conjugation (*BAAT* and *SLC27A5*) and transport (*ABCB11, ABCB4, FXR* (also known as *NR1H4*), *ATP8B1* (also known as *FIC1*), *ABCC2* and *SLC10A1*), tight junction structure (*CLDN1, TJP2* and *MYO5B*) or cholangiocyte secretion (*CFTR*) or from suppression of these pathways by drugs, toxins (including endotoxins, such as lipopolysaccharides, and the plant sterols present in intravenous soy lipid emulsions) or inflammatory mediators[35]. As the major driving force for bile flow is the ATP-dependent secretion of bile acids through the canalicular bile salt export pump (BSEP), neonatal cholestasis might also be the consequence of mutations in the gene encoding BSEP (*ABCB11*), of mitochondrial dysfunction caused by mutations in nuclear genes that regulate mitochondrial DNA or respiratory chain complexes (for example, *POLG, DGUOK* or *MPV17*) or of ischaemia–reperfusion and birth asphyxia[34,36–38]. Other autosomal recessive genetic disorders might lead to hepatocellular metabolic dysfunction manifested by hepatic steatosis and decreased bile secretion (for example, tyrosinaemia type 1, hereditary fructose intolerance or galactosaemia) or to endoplasmic reticulum stress (A1AT deficiency)[39]. Defective embryogenesis and maintenance of bile duct morphology can result from both genetic variants (for example, mutations in *JAGGED1*, *NOTCH2, DCDC2, ABCB4* or *PKHD1*) and inflammatory or immune-mediated diseases (for example, CMV infection, *ABCB4* mutations or biliary atresia) as well as toxins such as biliatresone[40,41] and plant sterols in parenteral nutrition-associated cholestasis[42]. The new discovery of mutations in a ciliopathy-associated gene, *PKD1L1*, as a cause for biliary atresia in a subset of patients with biliary atresia splenic malformation syndrome opens up the possibility that other ciliopathy genes might be involved in biliary atresia pathogenesis[43].

Obstruction to bile flow might be caused by genetically determined defective cholangiocyte secretion (for example, mutations in *CFTR*)[44] or conditions resulting in anatomic obstruction (for example, biliary atresia, choledochal cyst, neonatal sclerosing cholangitis, spontaneous perforation of the bile duct, tumours and cholelithiasis)[4].

Box 1 | **Causes of neonatal cholestasis**

Infectious
Viruses, bacteria, spirochaetes and parasites

Toxins
Drugs, endotoxins, total parenteral nutrition-associated cholestasis and herbal products

Endocrine
Hypothyroidism and panhypopituitarism

Immune
Gestational alloimmune liver disease

Anatomic obstruction
Biliary atresia, choledochal cyst, cholelithiasis, biliary sludge, inspissated bile, spontaneous perforation of common bile duct and tumour

Other
Idiopathic neonatal hepatitis (transient neonatal cholestasis), cardiovascular and circulatory disorders, haemophagocytic lymphohistiocytosis, malignancy and congenital lupus

Genetic and metabolic[a]

- α1-Antitrypsin deficiency (*SERPINA1*)
- Alagille syndrome (*JAG1* and *NOTCH2*)
- Arthrogryposis–renal dysfunction–cholestasis syndrome (*VPS33B* and *VIPAR*)
- Caroli disease and congenital hepatic fibrosis (*PKHD1*)
- Chromosomal (trisomy 21; Turner syndrome)
- Citrin deficiency (*SLC25A13*)
- Cystic fibrosis (*CFTR*)
- Disorders of bile acid synthesis (*AKR1D1*, *AMACR*, *CYP7B1*, *HSD3B7*, *CYP7A1* and *CYP27A1*)
- Disorders of bile acid conjugation (*BAAT* and *SLC27A5*)
- Fatty acid oxidation defects (*SCAD* and *LCAD*)
- Galactosaemia (*GALT*)
- Glycogen storage disease type IV (*GBE1*)
- Hereditary fructose intolerance (*ALDOB*)
- Mitochondrial respiratory chain disorders (*DGUOK*, *MPV17* and *POLG*)
- Neonatal ichthyosis–sclerosing cholangitis syndrome (*CLDN1*)
- Neonatal sclerosing cholangitis (*DCDC2*)
- Niemann–Pick disease type C (*NPC1* and *NPC2*)
- Peroxisomal disorders (*PEX1*, *PEX6*, *PEX10*, *PEX11B*, *PEX12*, *PEX13*, *PEX14*, *PEX16*, *PEX19*, *PEX2*, *PEX26*, *PEX3*, *PEX5* and *PEX7*)
- Progressive familial intrahepatic cholestasis (*ATP8B1*, *ABCB11*, *ABCB4*, *TJP2*, *NR1H4*, *MYO5B* and *UNC45*)
- Lipid storage diseases (*SCP2*)
- Tyrosinaemia (*FAH*)
- Urea cycle defects

[a]For genetic and metabolic causes, the affected gene or genes are listed in parenthesis when known.

Evaluation of neonatal cholestasis

Initial evaluation of neonatal jaundice. The first decision point in evaluating an infant with jaundice is when to fractionate the serum bilirubin to determine whether the major component is direct or conjugated bilirubin (indicative of a hepatobiliary problem) versus indirect or unconjugated bilirubin (more likely to be benign in origin). As breast milk-associated jaundice (likely caused by an undefined substance in breast milk that inhibits hepatic bilirubin uridine diphosphate glucuronosyl transferase) might be present in up to one in seven 2-week old breast milk-fed infants[1], a typical age for a well-child visit to the primary care provider, there will be ~350 such infants for every infant with cholestasis. Thus, many providers do not evaluate breastfed infants who appear jaundiced at 2 weeks unless they also have acholic stools, hepatomegaly or poor weight gain. The

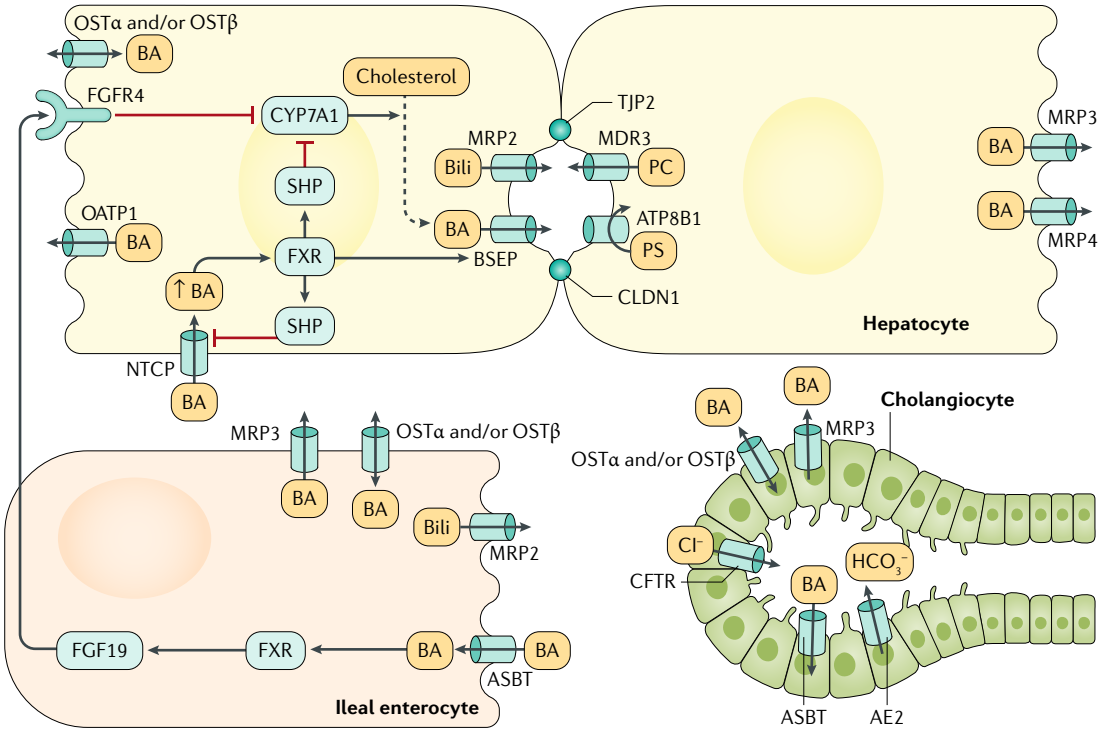

Fig. 1 | **Transport proteins involved in enterohepatic circulation of bile acids.** Multiple sites exist within hepatocytes, ileal enterocytes and cholangiocytes, as well as in bile acid (BA) synthesis and metabolism pathways, in which alterations in transporter regulation and enzyme expression can result in neonatal cholestasis. In hepatocytes, BAs are taken up by sodium taurocholate co-transporting polypeptide (NTCP), organic solute transporter subunit-α (OSTα), OSTβ or organic anion-transporting polypeptide 1 (OATP1) on the basolateral membrane, or they are synthesized from cholesterol by cholesterol 7α-hydroxylase (CYP7A1). Bile salt export pump (BSEP) on the canalicular membrane transports BAs in an ATP-dependent fashion into bile. BAs also bind to farnesoid X receptor (FXR), a nuclear hormone receptor that upregulates mRNA expression of BSEP and downregulates NTCP and CYP7A1 (through small heterodimer partner (SHP)). Other canalicular proteins that participate in bile formation are the conjugated bilirubin (Bili) transporter multidrug resistance-associated protein 2 (MRP2), the phosphatidylcholine (PC) transporter multidrug resistance protein 3 (MDR3) and phospholipid-transporting ATPase IC (ATP8B1, also known as FIC1; a phosphatidylserine (PS) flippase). MRP3 and MRP4 can also transport BAs from the hepatocyte to the sinusoidal space. Tight junction proteins (tight junction protein 2 (TJP2) and claudin 1 (CLDN1)) maintain the canalicular structure and integrity and protect against toxic properties of secreted BAs. In ileal enterocytes, luminal BAs are taken up by apical sodium–bile acid transporter (ASBT) on the enterocyte brush border and can be transported by OSTα, OSTβ or MRP3 from enterocytes into portal blood and back to the liver. Following its conjugation, bilirubin exits the hepatocyte into bile via canalicular MRP2. Absorbed BAs can also activate enterocyte FXR, which leads to secretion of fibroblast growth factor 19 (FGF19) into portal circulation, which then binds to fibroblast growth factor receptor 4 (FGFR4) on the hepatocyte with subsequent suppression of CYP7A1. In cholangiocytes, BAs secreted by hepatocytes can be taken up by ASBT in apical membranes and transported into the portal circulation by OSTα, OSTβ or MRP3 in the basolateral membrane. Chloride (Cl^-) is actively secreted by cystic fibrosis transmembrane conductance regulator (CFTR), and bicarbonate is secreted by the chloride–bicarbonate anion exchanger 2 (AE2) on the apical membrane into bile. Thus, defects in bile formation can result from genetic abnormalities in transporter or tight junction genes or obstructive, inflammatory or drug-induced adaptive changes in transporter expression and function.

Biliary atresia splenic malformation syndrome
A phenotype of biliary atresia in which patients have a combination of laterality (left–right differentiation) defects, including asplenia or polysplenia, midline liver, preduodenal portal vein, interruption of the inferior vena cava, intestinal malrotation, situs inversus or cardiac malformations.

consequence is an unfortunate missed opportunity to diagnose biliary atresia at a young age, as outcomes of HPE surgery in patients with biliary atresia (resolution of jaundice and survival without liver transplantation) are superior if performed before 30–45 days of life compared with after 60–90 days of life[8–10]. In the USA, as opposed to other countries, there is no routine well-child visit at 1 month of age; therefore, fractionation of bilirubin in jaundiced infants is commonly delayed until the 2-month well-child visit, which is already beyond the optimal age for surgical treatment of biliary atresia. For this reason, it is imperative that jaundiced breastfed infants undergo fractionation of their bilirubin at 2–3 weeks of age, and if they are found to meet the conjugated or direct hyperbilirubinaemia criteria described above, they should be immediately referred for expedited evaluation by a paediatric gastroenterologist or hepatologist.

General approach. The general approach to evaluation of an infant with cholestasis (FIG. 2) is to always exclude biliary atresia and other treatable conditions (TABLE 1), to ensure that there are no red flag findings from physical exam, family history or imaging (TABLE 2) to suggest a specific diagnosis and then to evaluate for common and finally rarer conditions. The initial evaluation should

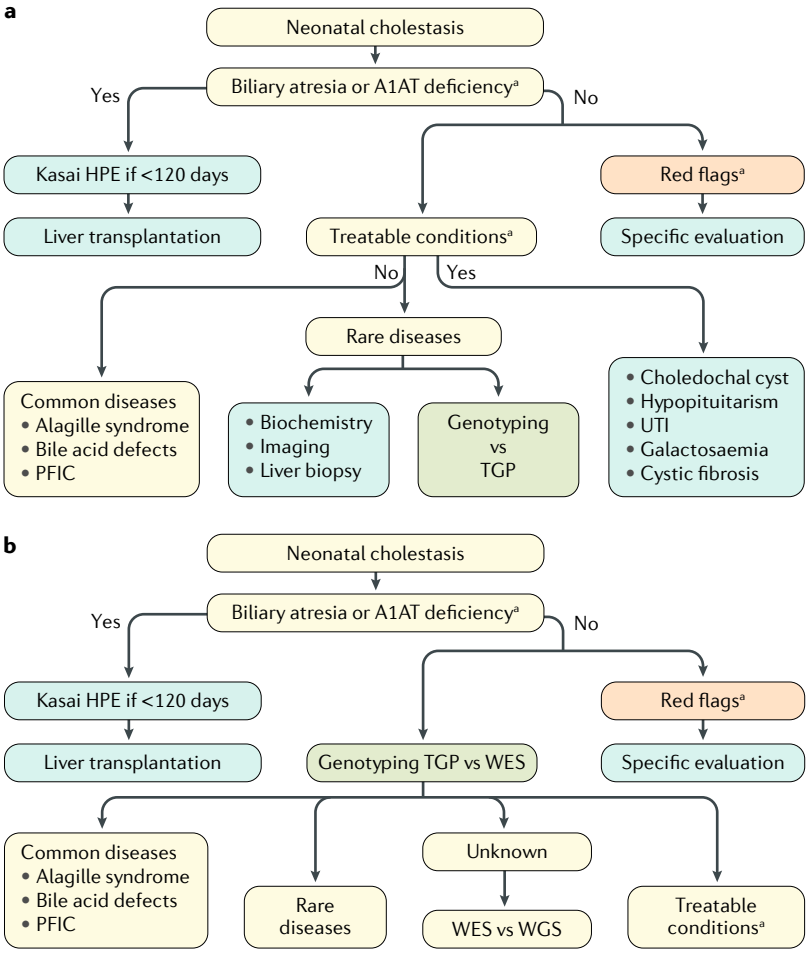

Fig. 2 | **Current and proposed emerging algorithms for evaluation of neonatal cholestasis. a** | Currently, biliary atresia, α1-antitrypsin (A1AT) deficiency and treatable causes of cholestasis (such as choledochal cyst or urinary tract infection (UTI)) are ruled out, which might require a liver biopsy and intraoperative cholangiogram to determine whether biliary atresia is present. If an infant does not have biliary atresia, multiple blood and urine screening tests are performed to evaluate for genetic and metabolic causes of neonatal cholestasis. If a red flag pointing towards an alternative diagnosis (TABLE 2) is present, evaluation for that disease should proceed promptly. If no diagnosis is confirmed, targeted gene panels (TGPs) or specific gene testing is performed. **b** | In the emerging algorithm, once biliary atresia and A1AT deficiency are ruled out, genotyping via whole-exome sequencing (WES) or whole-genome sequencing (WGS) could be utilized to efficiently and effectively evaluate for common and rare genetic causes of neonatal cholestasis. If a red flag pointing towards an alternative diagnosis is present, evaluation for that disease should proceed promptly. HPE, hepatoportoenterostomy; PFIC, progressive familial intrahepatic cholestasis. [a]Can be conducted simultaneously.

include serum levels of liver enzymes (including alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase), which are often elevated in infants with cholestasis but do not discriminate well between the varying aetiologies[3]. A normal or low serum γ-glutamyl transpeptidase (GGT) level (<125 U/l) is, however, not common in infants with cholestasis and should direct the clinician to consider diseases such as PFIC types 1, 2 or 4–6, disorders of bile acid synthesis or metabolism, panhypopituitarism or parenteral nutrition-associated cholestasis. A markedly elevated GGT level (>150–200 U/l) suggests biliary atresia, mechanical bile duct obstruction, paucity of interlobular bile ducts, A1AT deficiency, cystic fibrosis, neonatal sclerosing cholangitis or PFIC type 3. Similarly, an elevated prothrombin time or international normalized ratio (two assays that evaluate the intrinsic pathway of coagulation) that is unresponsive to parenteral vitamin K should alert the clinician to synthetic liver failure, metabolic disease or sepsis. Low serum albumin levels might result from either hepatic synthetic dysfunction or malnutrition consequent to steatorrhea and nutrient malabsorption or poor oral intake. Serum α-fetoprotein levels might be elevated in hereditary tyrosinaemia but also in other conditions[45,46].

Specific aetiologies. Testing for specific aetiologies of cholestasis should include a standard battery of initial tests, although the tests might be guided by clinical history and physical exam. All infants with cholestasis should be evaluated for A1AT deficiency by checking for low serum A1AT levels or the presence of protease inhibitor (PI) ZZ or SZ genotypes on electrophoresis or by rapid genotyping the *SERPINA1* gene before surgical exploration for biliary atresia. The newborn baby metabolic screen should be carefully reviewed (or repeated) to ensure that the infant does not have hypothyroidism, tyrosinaemia or galactosaemia (three treatable causes of cholestasis). If infection is suspected, blood and urine cultures should be obtained in addition to appropriate viral cultures and serologies (keeping in mind that immunoglobulin G-based serologies are suggestive of maternal exposure and not necessarily neonatal infection). Total serum bile acids should be measured, which are typically elevated in cholestasis but are low or normal in the majority of cases of bile acid synthesis disorders[47]. Here, the infant must not be receiving oral ursodeoxycholic acid (UDCA) for 4–5 days prior to this testing, as it can elevate serum bile acid levels. Other serum and urine tests might include urinary-reducing substances or red blood cell galactose-1-phosphate uridyl transferase levels drawn before administration of any blood products (for galactosaemia); urine succinylacetone (for tyrosinaemia if not covered on the newborn baby screen); thyroid function tests; serum amino acids, urine organic acids and acylcarnitine (for metabolic diseases including citrin deficiency and fatty acid oxidation defects); very-long-chain fatty acid levels (for peroxisomal disorders); and a sweat test, serum immunoreactive trypsinogen or *CFTR* genotyping (for cystic fibrosis). If clinical and family histories or biochemical red flags (TABLE 2) are present that immediately point to a specific diagnosis (FIG. 2a), evaluation for that disease should proceed early in the evaluation process. For example, if butterfly vertebrae, posterior embryotoxon, heart murmur or triangular facies are present, Alagille syndrome should be evaluated, or if there is a family history of tyrosinemia or A1AT deficiency, appropriate testing should be expedited. For cholestatic preterm infants receiving parenteral nutrition who are believed to probably have parenteral nutrition-associated cholestasis, practitioners often struggle with how much testing should be performed given the limited blood volume of the infant. We recommend obtaining liver blood tests, GGT levels, blood and urine cultures, A1AT level testing, thyroid

Posterior embryotoxon
A prominent Schwalbe's line, in which the corneal endothelium and the uveal trabecular meshwork join, that is seen commonly in patients with Alagille syndrome.

Table 1 | **Treatable causes of cholestasis**

Cause of cholestasis	Intervention
Infection (viral or bacterial)	Antibiotic or antiviral agents
Galactosaemia	Galactose-free diet
Tyrosinaemia type 1	Low tyrosine or phenylalanine diet and NTBC
Hereditary fructose intolerance	Fructose-free or sucrose-free diet
Hypothyroidism	Thyroid hormone replacement
Cystic fibrosis	Pancreatic enzymes
Hypopituitarism	Thyroid, growth hormone and cortisol replacement
Bile acid synthesis defects	Cholic acid or ursodeoxycholic acid supplementation
Biliary atresia	Hepatoportoenterostomy (Kasai procedure)
Choledochal cyst	Choledochoenterostomy
Spontaneous perforation of the common bile duct	Surgical drainage
Inspissated bile or stone in the common bile duct	Biliary tract irrigation
Parenteral nutrition-associated cholestasis (intestinal failure-associated cholestasis)	Intravenous lipid emulsion modification, advance enteral feedings

NTBC, 2-(2-nitro-4-trifluoromethylbenzol)-1,3-cyclohexanedione.

tests, urine succinylacetone levels and abdominal ultrasonography as a minimum. If the infant has pale stools, if the GGT is markedly elevated, or if hepatosplenomegaly is evident, then further testing with hepatobiliary scintigraphy and/or a liver biopsy might become necessary to establish the diagnosis of biliary atresia or a metabolic disease.

Imaging evaluation. All infants with cholestasis should also undergo abdominal ultrasonography to assess liver size, position and composition, number and size of spleens (as they can be abnormal in biliary atresia splenic malformation syndrome) (FIG. 3a,b) and presence of ascites and, most importantly, to identify causes of extrahepatic obstruction (including a choledochal cyst, mass, gallstone or obstructing sludge). Several findings from ultrasonography (absent or abnormal gallbladder, triangular cord sign, hepatic artery diameter-to-portal vein diameter ratio, hepatic subcapsular flow on colour Doppler ultrasonography or findings of heterotaxia and laterality defects) (FIG. 3a) might suggest biliary atresia; however, none of these features are diagnostic, and if absent, they do not exclude biliary atresia as the aetiology of cholestasis[3,48]. Hepatobiliary scintigraphy with a technetium-labelled iminodiacetic acid analogue can assist in confirming biliary tract patency and thus exclude biliary atresia[3,49] (FIG. 3c). However, although the sensitivity of hepatobiliary scintigraphy for biliary atresia is high (83–100%), the specificity is low (33–80%), as a non-excreting hepatobiliary scintigraphy result is seen not only in infants with biliary atresia but also in those with marked intrahepatic cholestasis, such as in bile duct paucity, idiopathic neonatal hepatitis and those on parenteral nutrition[49,50]. Hepatobiliary scintigraphy can be useful in excluding biliary atresia in a premature infant with cholestasis for whom there is low suspicion of biliary atresia (for example, in an infant with cholestasis with pigmented stool who is receiving parenteral nutrition) and for whom liver biopsy might be of substantial risk[49]. In some centres with specialized expertise, endoscopic retrograde cholangiopancreatography, magnetic resonance cholangiopancreatography or percutaneous transhepatic cholecystocholangiography has been used to define bile duct patency; however, these techniques require general anaesthesia in infants[51–54] and have not replaced intraoperative cholangiography as the definitive diagnostic test for biliary atresia in the large majority of centres.

Hepatobiliary scintigraphy
A diagnostic imaging technique that evaluates hepatocellular function and patency of the biliary system by following a radiolabeled tracer into the liver and out through the biliary system into the small intestine.

Histopathological evaluation. Percutaneous liver biopsy remains an essential diagnostic tool in evaluating neonatal cholestasis and can generally be performed safely in experienced hands, even in the smallest infants. In several single-centre studies as well as a multicentre study, a diagnosis of biliary atresia was correctly suggested by liver biopsy sample histological findings in 85–95% of cases[55,56]. Histological features that predicted cases of biliary atresia from non-biliary atresia in multivariate analysis included bile plugs in portal bile ducts or ductules (OR 13.65; 95% CI 5.90–31.60, $P<0.001$), portal stromal oedema (OR 10.20; 95% CI 1.92–53.96, $P=0.063$), no bile duct paucity (OR 4.26; 95% CI 1.64–1.09, $P=0.0029$), absent to rare giant cell transformation (OR 2.20; 95% CI 0.98–4.63, $P=0.$ 0.054) and absent to rare extramedullary haematopoiesis (OR 3.49; 95% CI 1.41–8.62). These five features when observed together in a biopsy sample resulted in a concordance index of 0.89 (95% CI 0.84–0.93) for the diagnosis of biliary atresia compared with non-biliary atresia[57] (FIG. 3d). However, it should be emphasized that other causes of cholestasis can mimic the histological appearance of biliary atresia; for example, A1AT deficiency, parenteral nutrition-associated cholestasis, multidrug resistance protein 3 (MDR3; encoded by *ABCB4*) deficiency and cystic fibrosis. Liver biopsy can also be helpful in identifying other causes of cholestasis including A1AT deficiency (periodic acid Schiff-positive, diastase-resistant hepatocyte globules), Alagille syndrome (bile duct paucity), neonatal sclerosing cholangitis (necroinflammatory duct lesions), metabolic liver disease (steatosis and pseudoacinar formation of hepatocytes), PFIC and storage diseases (electron microscopy findings), TNC (multinucleated giant cells, extramedullary haematopoiesis and hepatocellular cholestasis without portal tract involvement) and viral infection (HSV and CMV inclusions on immunohistochemistry). It is important to remember that several disease processes resulting in neonatal cholestasis are dynamic in nature and that characteristic liver biopsy sample findings might evolve over time and might not all be present at initial biopsy in infants under 4–6 weeks of age (including in biliary atresia).

Noninvasive liver stiffness evaluation. Given that hepatic fibrosis is a universal feature of biliary atresia and that rapid progression to cirrhosis early in life is somewhat unique to this disease[58], it has been proposed that noninvasive methods to evaluate liver stiffness as

Table 2 | **Red flag findings that suggest a specific cause of cholestasis**

Red flag	Disease
Prenatal history	
Ultrasonography abnormality	Choledochal cyst, cystic biliary atresia or gallstone
Cholestasis of pregnancy	PFIC or mitochondrial disease
Acute fatty liver of pregnancy	LCHAD
Maternal infection during pregnancy	Congenital infection
Physical finding	
Alcoholic stool	Biliary atresia, choledochal cyst, gallstone or biliary sludge
Palpable mass in the right upper quadrant	Choledochal cyst
Heart murmur	Alagille syndrome or biliary atresia
Butterfly vertebrae	Alagille syndrome
Ascites	Spontaneous perforation of the bile duct
Dysmorphic facies	Alagille syndrome, Zellweger syndrome or chromosomal abnormality
Microcephaly	Congenital infection
Posterior embryotoxon	Alagille syndrome
Chorioretinitis	Congenital infection
Cataracts	Congenital infection or galactosaemia
Vision or eye movement abnormalities	Septo-optic dysplasia with panhypopituitarism
Splenomegaly	Niemann–Pick disease type C
Lax skin	Arthrogryposis
Limb or long bone abnormalities	Arthrogryposis or congenital syphilis
Purpura	Congenital infection
Hypotonia	Mitochondrial disorder or peroxisomal disorder
Neurological abnormalities (irritability, lethargy, poor feeding, hypotonia or seizures)	Sepsis, intracranial haemorrhage, metabolic and mitochondrial disorders or panhypopituitarism
Petechiae or thrombocytopenia	Congenital infection or congenital Lupus
Family history	
Early emphysema	α1-Antitrypsin deficiency
Liver disease in siblings or stillbirths	Genetic or metabolic liver disease
Consanguinity	Autosomal recessive genetic liver disease

LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency; PFIC, progressive familial intrahepatic cholestasis.

Dubin–Johnson syndrome
A benign autosomal recessive disorder caused by mutations in *ABCC2* that result in isolated elevated serum conjugated or direct bilirubin levels and a dark coloured liver.

Niemann–Pick disease type C
A lysosomal storage disease associated with mutations in *NPC1* and *NPC2*, which results in cholesterol and lipid accumulation in the lysosomes of hepatocytes, causing cholestasis, and affects the spleen and brain.

an estimate of fibrosis (such as acoustic radiation force impulse (ARFI), transient elastography or sheer wave elastography) could be used to hasten the suspicion for biliary atresia, avoid liver biopsy and, in turn, shorten the time to intraoperative cholangiogram and HPE. In a study of 20 children with cholestasis suspected of having biliary atresia, spleen size and ARFI correlated with the degree of liver fibrosis on biopsy ($r > 0.70$, $P < 0.001$)[59]. Likewise, in 11 children <1 year of age with suspected liver disease, there was a significant difference in mean liver shear wave speed between the biliary atresia group and the non-biliary atresia group ($P < 0.0001$)[60]. In another study using transient elastography in 48 infants with cholestasis from Taiwan (15 had biliary atresia), a liver stiffness measurement >7.7 kPa was found to be predictive of biliary atresia (area under the receiver operated characteristic curve of 85.3%) with an odds ratio of 128 ($P < 0.001$)[61]. Larger multicentred studies are needed to determine the diagnostic specificity and usefulness of these modalities for distinguishing biliary atresia from other cholestatic disorders.

Emerging genetic evaluation. Advances are being made in our ability to establish a molecular diagnosis in infants with genetic diseases, which will certainly change the paradigm for evaluation of infants with cholestasis. NGS is performed by several high-throughput platforms using massively parallel processing of spatially separated amplified DNA templates[62]. Although careful analysis of clinical features, biochemical markers such as low serum GGT levels and histopathology can often narrow the diagnostic possibilities of neonatal cholestasis, the traditional evaluation paradigm can be time consuming and expensive[19,23,35,63]. Thus, evaluation of infants with cholestasis who do not have biliary atresia and other relatively common causes remains challenging, as there are multiple genetic syndromes with low individual prevalence, many of which have overlapping clinical phenotypes. TGPs, WES, WGS and bioinformatics pipelines are now clinically available tools in many centres and countries that can identify all known gene variants that have been associated with cholestatic diseases, including *JAG1* and *NOTCH2* (Alagille syndrome), *ATP8B1* (PFIC type 1), *ABCB11* (PFIC type 2), *ABCB4* (PFIC type 3), *SERPINA1* (A1AT deficiency), *ABCC2* (Dubin–Johnson syndrome) and *SLC25A13* (neonatal or infantile intrahepatic cholestasis caused by citrin deficiency), as well as many other rarer conditions[17–21] (BOX 1). In addition, NGS and bioinformatics analyses provide the capacity to discover new genetic causes of neonatal cholestasis.

TGPs, WES and WGS can provide the ability to rapidly and simultaneously screen large panels of genes that cause cholestasis; however, they require a coordinated effort between a sequencing facility and someone with robust bioinformatics expertise for interpretation. Many research and commercial laboratories are working towards being able to provide this analysis in a timely and cost-effective manner so that NGS can be considered earlier in the diagnostic pathway for neonatal cholestasis. NGS can be particularly useful to establish a genetic diagnosis in the absence of other definitive diagnostic testing, confirm the clinical diagnosis (especially in infants with rare diseases or atypical presentations), potentially decrease the need for invasive procedures such as laparotomy or laparoscopy for intraoperative cholangiography, enable early initiation of treatment (for example, in galactosaemia and tyrosinaemia), identify infants that might be eligible for new and emerging therapies early in their course, identify those children for whom liver transplantation might be the only long-term therapeutic option or alternatively identify those whose diseases might be contraindications for liver transplantation (for example, in mitochondrial disease and Niemann–Pick disease type C) and to enable families to receive specific genetic counselling and risk estimation for future offspring[17]. In one study

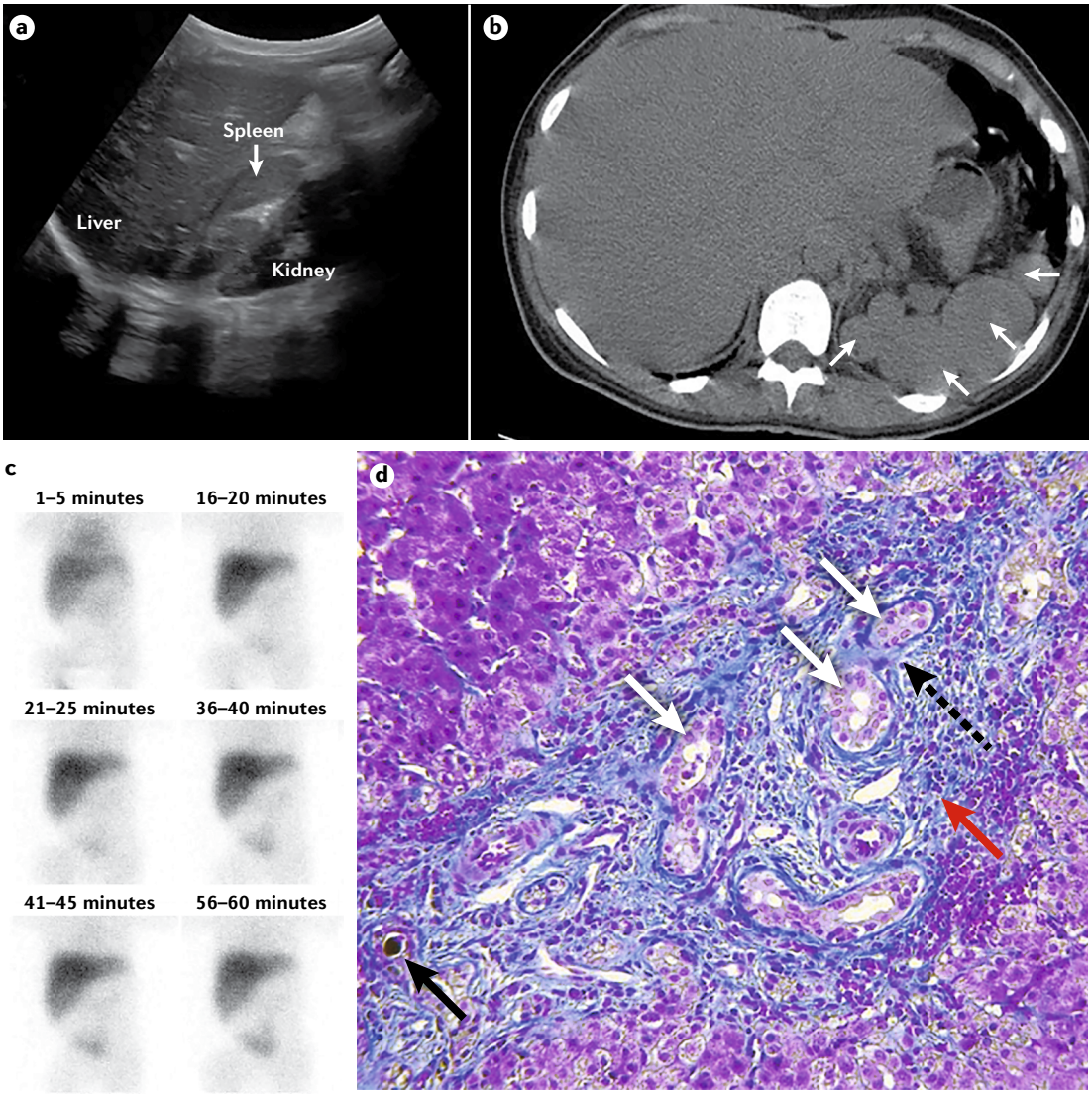

Fig. 3 | **Imaging and histopathological findings of biliary atresia. a** | Ultrasonography image showing situs inversus of the spleen (white arrow), which can be seen in biliary atresia splenic malformation syndrome. **b** | CT scan showing polysplenia (white arrows), which can be associated with biliary atresia. **c** | Hepatobiliary iminodiacetic acid scan with technetium mebrofenin. The radioactive tracer is taken up well by the liver but is not excreted into the intestine over 60 minutes. Similar findings were present 24 hours after radiotracer infusion. A normal, small amount of radiotracer is secreted by the kidneys into the bladder. **d** | Trichrome-stained liver biopsy sample showing features of biliary atresia including a portal tract bile duct plug (solid black arrow), bile duct proliferation (solid white arrows), inflammation (dotted black arrow) and portal fibrosis (red arrow).

utilizing a multi-gene panel that sequenced the 886 coding exons of 61 cholestasis-related genes as well as 25 related known insertion and/or deletion mutations (indels), 22% of 141 patients with cholestasis received a potential genetic diagnosis[18]. In a separate study of 51 infants with cholestasis of undefined aetiology, gene sequence analysis resulted in a molecular diagnosis in 27% of patients[19]. In a Japanese study of 109 patients with neonatal cholestasis, a molecular genetic diagnosis was made for 28 patients (26%) using NGS[20]. In a study of 716 infants and older children from North and South America with cholestasis or hepatitis of unknown aetiology, a 66-gene panel was able to identify 11.7% of patients as positive or likely positive for a genetic disorder and another 12.7% of patients with a pathogenic or likely pathogenic variant[21]. Taken together, these results support the utility of multi-gene testing in diagnosing neonatal cholestasis. However, caution is advised in over-interpreting variants of unknown significance (VOUS) or single allele variants or pathologic variants that have not undergone functional genomics verification. Consultation for interpretation of these types of variants with expertise at the genetics laboratories performing these tests is strongly recommended.

As the cost and turnaround time of TGPs and WES continue to decrease (in the largest study, the median time from receipt of samples in the laboratory to return of reports to clinicians was 18 days)[21], they could replace individual blood and urine screening tests (FIG. 2a) and become an earlier step in evaluation of infants with cholestasis after biliary atresia is excluded and as other treatable disorders are evaluated. FIGURE 2b shows a

proposed emerging algorithm for evaluating neonatal cholestasis that incorporates emerging molecular diagnostics. Prospective multicentre studies that collect and review VOUS identified on multi-gene panel testing might lead to identification of new diseases or elucidate the pathogenesis of certain causes of neonatal cholestasis. This dramatic shift in the cholestasis evaluation paradigm is underway in many centres. Although not yet been reported, earlier genetic diagnosis might be very cost-effective.

Treatment of neonatal cholestasis

The therapeutic approach to neonatal cholestasis should be holistic and consider the needs of the growing child. We have divided the trajectory of cholestasis and recommended interventions into three temporal stages of disease (FIG. 4): the early stage, chronic stage and end stage. Early identification and initiation of therapy for treatable causes of cholestasis are crucial to limit liver damage and fibrosis and to prevent injury to the brain and other organs (TABLE 1). This understanding is best illustrated by the timing of HPE in infants with biliary atresia, in which short-term outcomes are best (up to 80% achieving bile flow, normalization of serum bilirubin and restoration of pigmented stools) if HPE is performed at <30–45 days of life[8,9]. Conversely, <20% achieve bile flow if HPE is performed after 90–120 days of life[8]. Longer-term transplant-free survival mirrors short-term outcomes in biliary atresia to some extent; however, the majority of children (>80%) will eventually develop portal hypertension and require liver transplant before reaching adulthood[64], even those that achieve

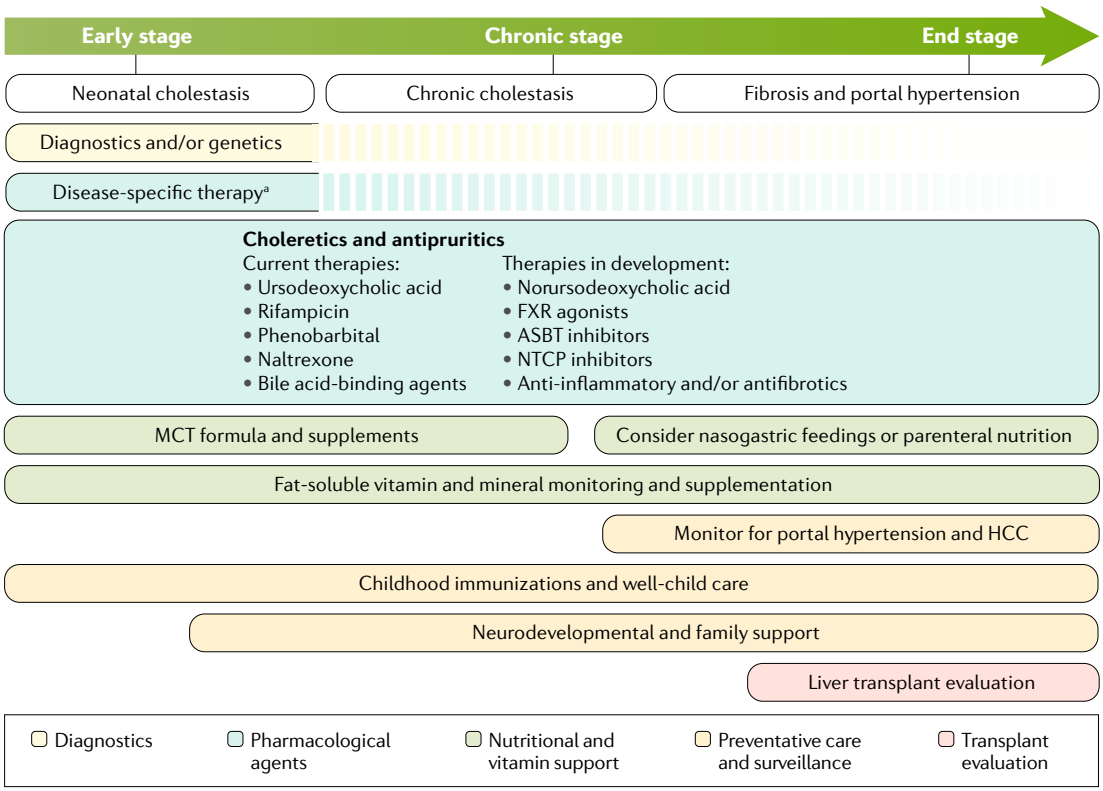

Fig. 4 | **A stage-based approach to the treatment of neonatal cholestasis.** Infants with neonatal cholestasis can progress through different stages of disease. As the cholestasis becomes chronic (>6 months duration), portal fibrosis progresses and synthetic liver failure or complications of portal hypertension lead to end-stage liver disease. Diagnostic testing (if not definitive) should be continued as new aetiologies (especially genetic causes) are being discovered each year. Once the cause of cholestasis is established, disease-specific therapy (if available) should be initiated and general measures instituted. Medical therapies can be utilized to improve or treat pruritus, cholangitis and portal hypertension. If gastrointestinal bleeding occurs, endoscopic variceal surveillance and management might be needed. In the future, new choleretic, antifibrotic, anti-inflammatory and bile acid-modifying agents might be available. Infants with cholestasis are at risk of malnutrition and fat-soluble vitamin deficiency secondary to increased energy requirement and impaired bile flow causing steatorrhea and should receive medium-chain triglyceride (MCT)-containing infant formula and fat-soluble vitamin supplementation as necessary, either via oral or nasogastric tube feedings. Some children might require parenteral nutrition before transplantation to optimize nutritional status. Immunizations should be accelerated when possible (specifically, administration of live vaccines at 6–9 months of age) in anticipation of possible liver transplantation. All children with cirrhosis should have screening for hepatocellular carcinoma (HCC) using α-fetoprotein levels and serial imaging. Assessing neurodevelopment and instituting developmental and family support are essential for long-term optimal functional capacity. ASBT, apical sodium–bile acid transporter; FXR, farnesoid X receptor; NTCP, sodium taurocholate co-transporting polypeptide. [a]For example, hepatoportoenterostomy for biliary atresia, NTBC (2-(2-nitro-4-(trifluoromethyl)benzoyl)cyclohexane-1,3-dione or nitisinone) for tyrosinaemia, cholic acid for bile synthesis defects and diet for galactosaemia and hereditary fructose intolerance.

good short-term bile flow, emphasizing the need for new therapeutic strategies for this disease. In other treatable causes, early initiation of therapy will prevent brain injury (hypopituitarism and hypothyroidism), kidney disease (tyrosinaemia), hypoglycaemia or even death (bacterial infection or sepsis)[65]. Prophylaxis against ascending cholangitis is standard for biliary atresia, and the use of choleretic agents and treatment of pruritus and portal hypertensive complications are necessary in most children with ongoing cholestasis, fibrosis or cirrhosis (FIG. 4). Liver transplantation in biliary atresia and other cholestatic liver diseases has excellent outcomes[66,67], although the requirement for immunosuppression might lead to long-term consequences.

Specific therapies are used in selected cholestatic disorders (TABLE 1). Oral cholic acid, approved by the FDA in the USA in 2015, effectively treats several disorders of bile acid synthesis by replacing the primary bile acid and shutting down synthesis of toxic bile acid intermediates[68]. NTBC (2-(2-nitro-4-(trifluoromethyl)benzoyl) cyclohexane-1,3-dione or nitisinone) blocks 4-hydroxyphenylpyruvate dioxygenase and prevents the accumulation of toxic intermediates such as maleylacetoacetic acid and fumarylacetoacetic acid in hereditary tyrosinaemia type 1 (REF.[69]); therefore, it is initiated as soon as the diagnosis of tyrosinaemia is made. Avoidance of lactose and galactose in children with galactosaemia and avoidance of fructose, sucrose and sorbitol in children with hereditary fructose intolerance also prevent disease progression[70]. Finally, in PFIC type 1, PFIC type 2 and Alagille syndrome, surgical interruption of the enterohepatic circulation by partial external biliary diversion, internal biliary diversion or ileal exclusion leads to relief of pruritus, stabilization or improvement of cholestasis and improved growth in many of those patients without advanced hepatic fibrosis[71].

Regardless of aetiology, nutritional therapy is essential for all cholestatic infants. In the early stage, infants with milder degrees of cholestasis or those that achieve bile flow and clearance of jaundice following HPE might gain weight and grow adequately while being breastfed or receiving standard infant formula[72]. Those not growing well or with more severe cholestasis will require infant formulas containing MCTs or MCT oil supplements, which are absorbed well from the small intestine in the absence of intraluminal bile acid concentrations above the threshold for formation of mixed micelles[73]. Owing to ongoing steatorrhea and increased oxygen consumption[74], infants who have cholestasis often require a minimum target for energy intake of between 125% and 140% of the recommended caloric requirement based on ideal body weight[74], often necessitating nasogastric tube drip feedings to achieve this goal. When nasogastric tube feedings are not successful, home parenteral nutrition has been shown to effectively achieve adequate growth[75], especially in infants in the end stage of cholestasis who are awaiting liver transplantation. Achieving optimal growth is not only important for brain growth and the general health of the infant. Notably, liver transplantation outcomes are directly related to nutritional status at the time of the procedure, with growth failure being an independent risk factor for pre-transplant mortality, post-transplant mortality and graft failure[76,77]. Infants with cholestasis require vitamin supplementation and careful monitoring of fat-soluble vitamin status (vitamins A, D, E and K) to prevent deficiencies that can be life-threatening (for example, intracranial haemorrhage from vitamin K deficiency coagulopathy) or that can lead to major skeletal, muscular, cutaneous and central and peripheral nervous system morbidities[74]. Childhood immunization schedules should be accelerated, and live vaccines should be administered early at 6 months of age if the disease is reaching end stage and liver transplantation is inevitable[78–81]. Care providers must pre-emptively monitor for portal hypertension, ascites, pruritus and hepatocellular carcinoma through the chronic stage and treat accordingly. As developmental delays and impaired quality of life are common in many of the cholestatic diseases and persist after liver transplantation, which have been best studied in biliary atresia[82,83], attention to the neurodevelopment and emotional status of an infant with cholestasis, as well as the well-being of the family, is also essential. Early interventions, therefore, have potential to improve these critical clinical outcomes.

Future therapeutic approaches

Understanding the molecular mechanisms of normal bile formation, secretion and flow has not only advanced our understanding of the pathophysiology of cholestasis but also has identified new potential therapeutic targets for cholestasis (FIG. 5). One of the underlying premises of cholestasis is that the accumulation of bile acids in the cholestatic hepatocyte initiates cell death and inflammatory pathways that promote liver injury and fibrosis[84] and that preventing hepatocyte retention of bile acids would be therapeutically beneficial. Currently, there are no FDA-approved drugs for the treatment of paediatric cholestasis, although UDCA is approved for treatment of adults with the cholestatic disease primary biliary cholangitis (PBC)[85–90]. UDCA is proposed to improve cholestasis by stimulating canalicular efflux pumps (multidrug resistance-associated protein 2 (MRP2; encoded by *ABCC2*) and BSEP) and basolateral export pumps (MRP3 (encoded by *ABCC3*) and MRP4 (encoded by *ABCC4*)) as well as by diluting the pool of toxic bile acids, thereby reducing bile acid toxicity, in addition to potentially having cytoprotective, anti-inflammatory and antifibrotic properties[91–93]. Although commonly used as an off-label choleretic agent to promote bile flow in childhood cholestatic disorders, there is little evidence of the clinical effectiveness of UDCA in these conditions. Importantly, there is evidence that excessively large doses of UDCA (>28 mg/kg/day) in adults with primary sclerosing cholangitis might contribute to more advanced liver disease and its complications[94]. Nor-UDCA, a side chain shortened UDCA derivative that lacks a methylene group and has resistance to amidation, can enhance cholehepatic shunting of secreted bile acids from within the bile duct lumen back to the hepatocyte[95]. This process is attractive, as it induces a bicarbonate-rich hypercholeresis that counteracts intrinsic bile acid toxicity to biliary epithelia, the so-called bicarbonate umbrella[96], and might particularly be of benefit in cystic fibrosis-associated liver

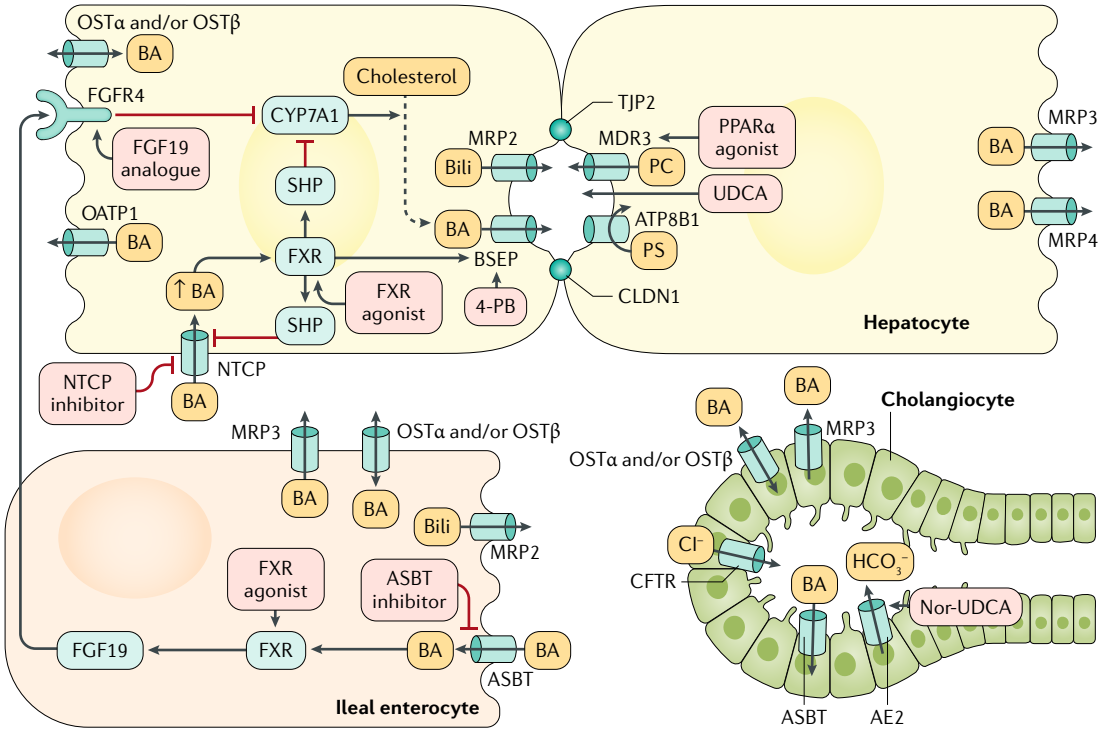

Fig. 5 | **Targets for new therapies in development for the treatment of cholestasis.** Multiple sites exist within hepatocytes, ileal enterocytes and cholangiocytes where pharmacological agents (shown in pink) could potentially improve cholestasis. In hepatocytes, downregulating hepatocyte basolateral membrane transporters (for example, sodium taurocholate co-transporting polypeptide (NTCP)) by specific inhibitors can reduce the bile acid (BA) burden and subsequent toxicity to the hepatocyte. Stimulating canalicular and hepatocyte BA efflux pumps (bile salt export pump (BSEP), multidrug resistance-associated protein 2 (MRP2), MRP3, MRP4, organic solute transporter subunit-α (OSTα) and OSTβ) using farnesoid X receptor (FXR) agonists, ursodeoxycholic acid (UDCA), molecular chaperones (4-phenylbutyrate (4-PB) for BSEP) or other agonists might similarly reduce the BA burden of the hepatocyte and improve bile flow and fat absorption. Stimulating canalicular multidrug resistance protein 3 (MDR3) expression by peroxisome proliferator-activated receptor-α (PPARα) agonists can increase phosphatidylcholine (PC) secretion into the canaliculus and protect against BA toxicity to cholangiocytes. Strategies that would inhibit BA synthesis by suppressing cholesterol 7α-hydroxylase (CYP7A1) (for example, using fibroblast growth factor 19 (FGF19) agonists, FXR agonists or short interfering RNAs) can reduce BA toxicity to the hepatocyte. In the ileal enterocyte, inhibiting ileal BA reuptake with apical sodium–bile acid transporter (ASBT) inhibitors, which will increase faecal excretion of BAs, can lower the BA pool size, change BA composition and alter enterocyte FXR signalling. FXR agonists can also activate ileal enterocyte FXR and increase FGF19 secretion, with subsequent inhibition of CYP7A1 in the hepatocyte. In the cholangiocyte, nor-UDCA acts on the bicarbonate–chloride anion (Cl^-) exchanger 2 (AE2) to increase bile pH and protect the cholangiocyte from BA injury. ATP8B1, phospholipid-transporting ATPase IC (also known as FIC1); Bili, conjugated bilirubin; CFTR, cystic fibrosis transmembrane conductance regulator; CLDN1, claudin 1; FGFR4, fibroblast growth factor receptor 4; OATP1, organic anion-transporting polypeptide 1; PS, phosphatidylserine; SHP, small heterodimer partner; TJP2, tight junction protein 2.

disease. In addition, nor-UDCA has anti-inflammatory, anti-lipotoxic, antiproliferative and antifibrotic qualities[95,97–99] and is currently undergoing trials in adult cholestatic liver diseases.

Another promising class of drugs for the treatment of cholestasis is the farnesoid X receptor (FXR; also known as NR1H4) agonists (for example, 6-ethylchenodeoxcholic acid or obeticholic acid (OCA)). FXR is a nuclear hormone receptor within hepatocytes that, when activated by bile acids (chenodeoxycholic acid and cholic acid), transactivates expression of BSEP and MRP2, indirectly downregulates bile acid synthesis through small heterodimer partner (SHP; also known as NR0B2) inhibition of cholesterol 7α-hydroxylase (CYP7A1) and CYP8B1 expression and downregulates sodium taurocholate co-transporting polypeptide (NTCP; encoded by *SLC10A1*) to prevent hepatocyte uptake of circulating conjugated bile acids[100]. The combined effect of these downstream targets of FXR is to reduce hepatocyte bile acid retention and toxicity. Therapeutic FXR agonists could theoretically overcome reduction in bile flow by activating BSEP (increasing bile acid-dependent bile flow) and MRP2 (increasing bile acid-independent bile flow)[101,102], reducing intrahepatocyte bile acid concentrations, altering bile composition and the intestinal microbiome and modifying the gut–liver axis that is believed to potentiate cholestatic liver injury[103–105]. To our knowledge, FXR agonists have yet to be trialled in neonatal cholestatic conditions, although one (OCA) is FDA-approved as a second line drug for adults with PBC.

Other nuclear receptor (for example, PXR, CAR and peroxisome proliferator-activated receptor-α (PPARα)) agonists and bile acid receptor (for example, TGR5 (also known as GPBAR1)) agonists are also attractive targets for therapy of cholestasis, as these pathways might play a role in controlling bile acid homeostasis and enterohepatic circulation, and probably affect hepatic inflammation and fibrosis[106]. For example, PPARα agonists, which are in clinical trials for PBC, increase MDR3 expression and its subcellular redistribution to the canalicular membrane and, in turn, stimulate biliary phospholipid secretion, reduce bile acid synthesis (via suppression of CYP7A1 and CYP27A1), induce bile acid detoxification (via induction of CYP3A4) and have anti-inflammatory, antifibrotic and antipruritic properties[105].

Another pharmacological target for treatment of cholestasis (based on the effectiveness of surgical biliary diversion in PFIC and Alagille syndrome) is the inhibition of the ileal bile acid transporter by apical sodium–bile acid transporter (ASBT) inhibitors (for example, maralixibat)[71,107]. These drugs bind to ASBT, interrupting the enterohepatic circulation by increasing faecal excretion of bile acids, thereby reducing hepatic bile acid levels that in turn stimulate bile acid synthesis and alter the composition of bile[102,108,109]. Moreover, inhibition of ileal bile acid uptake reduces ileal enterocyte FXR activation, thereby suppressing intestinal fibroblast growth factor 19 (FGF19) synthesis and secretion into the portal circulation[110]. FGF19, through binding to fibroblast growth factor receptor 4 (FGFR4) and/or BKL on the hepatocyte basolateral membrane, normally initiates signalling that also suppresses CYP7A1. Thus, the combined effect of ASBT inhibitors is both a reduction in the bile acid pool and potentially increased bile acid synthesis and secretion by the hepatocyte. In maralixibat trials in Alagille syndrome and PFIC, preliminary reports demonstrate reduction in pruritus, serum bile acid levels or both in a distinct subset of patients[107,111,112]. Similarly, inhibitors of the hepatocyte basolateral bile acid uptake protein NTCP (also known as SLC10A1), such as myrcludex B, could also reduce the bile acid burden in the liver[113]. Bile acid sequestrants that are already in clinical use to prevent hypercholesterolaemia and pruritus (that is, cholestyramine or colesevelam) might also be beneficial in enhancing faecal bile acid excretion, although they have not been shown to alter the course of the liver disease itself and might be impalatable[114].

For the cholestatic disorders caused by genetic variants that impair synthesis or trafficking of canalicular transport proteins, small-molecule chemical chaperones might be one therapeutic option. Chaperones, such as 4-phenyl butyrate, can theoretically bind reversibly to the active site of a missense mutant enzyme, correcting protein misfolding and enhancing delivery of the protein to the correct target[115]. Identification of potential chaperones, and testing of these molecules, is underway for PFIC types 1 and 2 and other diseases that result in cholestasis[116–119]. As our ability to easily and rapidly identify specific genetic mutations continues to improve, we will likely be able to target individualized therapy for each child with these disorders on the basis of their genotype.

Finally, for infants with cholestasis with inflammatory–fibrotic disorders, such as biliary atresia, anti-inflammatory or antifibrotic agents might be beneficial[120]. Unfortunately, the high-dose corticosteroid intervention used in the START trial in biliary atresia did not demonstrate a short-term or long-term benefit after HPE[121], and interrupting innate immunity pathways through intravenous immunoglobulin administration in biliary atresia was also not beneficial[122]. Nevertheless, it is possible that, for a subset of patients with biliary atresia (identified in the future through cellular and molecular immunotyping), anti-inflammatory agents might be of benefit. There are also several anti-inflammatory or antifibrotic agents currently under investigation in adult liver diseases (for example, the CC-chemokine receptor 2 (CCR2) or CCR5 antagonist cenicriviroc)[123]. Therapeutic use of gene-editing and gene transfer strategies is under investigation in a number of animal models of genetic diseases; however, safety and efficacy in children with underlying cholestasis will need to be established if these therapies reach the human clinical trial stage.

Screening and prevention

Despite the clear importance of early detection and diagnosis, the median age at diagnosis of biliary atresia and HPE in the USA, Canada and France remains ≥60 days, by which time the infant has less chance to respond to HPE and might already have developed irreversible cirrhosis[16]. The challenge for caregivers remains to distinguish infants with cholestasis (a rare occurrence in only 0.04% of live births) from infants with physiological and breast-milk-associated indirect hyperbilirubinaemia[3]. Early hospital discharge of newborn babies and lack of standard 1-month well-child visits likely contribute to late referral for evaluation of cholestasis in the United States.

Given that biliary atresia is an important public health problem, that the disease has a clearly defined natural history with a detectable early stage and that early diagnosis and HPE improve outcomes, biliary atresia seems to be an ideal disease for which to implement a screening tool[124]. One strategy that has been utilized in Taiwan, Japan and Switzerland is the use of an infant stool colour card (provided to the mother at hospital discharge after delivery) to identify acholic stools, a clue that an infant has cholestasis and not physiological jaundice. In Taiwan, beginning in 2004, an infant stool colour card was placed into the newborn child's health booklet, and mothers were instructed to notify a provider if their infant's stool colour matched the acholic pictures on the card[15]. In addition, providers reviewed the cards with the parents at the standard 1-month health supervision visit. This programme reduced the average age at diagnosis of biliary atresia from 47 days to 43 days, increased the national rate of the HPE operation performed before 60 days of age from 49% to 66%, increased the 5-year jaundice-free survival rate from 27% to 64% and increased 5-year overall (including liver transplant outcomes) survival (from 56% to 89%)[15,125,126]. To make this programme successful in its current form in the USA,

Box 2 | **Unanswered questions and future directions**

- Are there biomarkers or screening tests that can be utilized in the first month of life to reliably identify infants with biliary atresia?
- Can noninvasive modalities to assess progression of hepatic fibrosis (such as transient elastography) reliably distinguish stages of fibrosis in infants and young children and be used in clinical trials?
- Can whole-exome or whole-genome molecular diagnostic testing be performed and interpreted rapidly enough to become a standard early diagnostic test in the algorithm for evaluating neonatal cholestasis?
- Are there prognostic parameters that can be used to predict long-term success after Kasai hepatoportoenterostomy?
- Will the new pipeline of therapies for cholestasis be effective and well-tolerated in infants and children with neonatal cholestatic disorders?
- Will genetic editing and therapy become feasible and safe modalities to cure genetic causes of cholestasis?

a standard 1-month infant health provider visit seems to be required, which is currently not the standard practice. A cost-effective analysis of implementation of a stool card programme in the USA showed that such a screening programme would result in lower costs and better outcomes (for a stool card, 20-year cost of $133 million, 71 deaths and 147 liver transplants versus no screening with a 20-year cost of $142 million, 74 deaths and 158 liver transplants)[127]. Implementation of a province-wide stool colour card screening programme in British Columbia, Canada, has shown promise, with successful colour card identification of five of six infants with biliary atresia. However, the programme failed to result in early referral and HPE in two of the five infants with biliary atresia[128]. Modifications of the programme to increase early referral are now being evaluated. Alternatively, a mobile health application (such as PoopMD), utilizing a smartphone camera and colour recognition software to identify acholic stools, might offer a method to provide this critical education and screening without necessitating an in-person visit with a physician. In a pilot test of such software, PoopMD was able to accurately differentiate acholic from normal colour stool across users, smartphone type and light source[129].

Ideally, biomarkers should be identified that can quickly and accurately identify biliary atresia at a young age. In one study, serum level of MMP7 was identified as a highly discriminatory diagnostic biomarker for biliary atresia when used in conjunction with GGT levels at the time of evaluation for cholestasis (AUC of 0.98 for MMP7 plus GGT (95% CI 0.94–1.00)[130]. It is unknown whether MMP7 is elevated at a young age in biliary atresia, which would be required for it to be used as a screening test. Data from Harpavat et al.[5–7] suggest that infants with biliary atresia could potentially be identified before discharge home in the newborn period[5–7]. Total and conjugated or direct bilirubin levels were examined from samples obtained during the first 5 days of life in 34 infants later identified as having biliary atresia and compared with those from 300 control infants without cholestasis born in the same hospitals[5]. Remarkably, conjugated or direct bilirubin was above 5 μmol/l (0.3 mg/dl) in all infants with biliary atresia but in none of the controls[5]. At 24–48 hours of life, infants with biliary atresia had mean direct bilirubin levels significantly higher than those of controls (1.40 ± 0.43 mg/dl or 23.90 ± 7.35 μmol/l versus 0.19 ± 0.08 mg/dl or 3.20 ± 1.30 μmol/l; $P < 0.0001$), and there was no overlap between those with biliary atresia and controls[5]. In a follow-up study, an elevated direct or conjugated bilirubin level in the newborn period was shown to have a sensitivity of 100% and specificity of 98.2% for biliary atresia[6,7]. Specificity could be improved further by using 99% reference intervals (direct bilirubin of 0.3 mg/dl or >10% of the total bilirubin level) and/or repeat testing[6,7]. A potential drawback of this approach is the fact that these slight elevations of direct or conjugated bilirubin are close to the variance of the assays, and therefore, each hospital would need to carefully establish normal newborn baby values. However, as the American Academy of Pediatrics currently recommends a total serum bilirubin level be obtained in all infants before discharge from the hospital to monitor for indirect hyperbilirubinaemia, simply adding the reporting of the conjugated or direct bilirubin seems to be feasible without increasing cost. Future studies are needed to determine the feasibility, utility and cost-effectiveness of direct or conjugated bilirubin or of mass spectroscopy testing in the newborn period, mobile health applications and stool colour card programmes alone or in combination. Focus then needs to be on a broad education campaign and implementation of a national screening programme so that the age at diagnosis and HPE outcomes for infants with biliary atresia can be improved.

Conclusions

Over the past two decades, many of the molecular mechanisms and genetic defects causing neonatal cholestasis have been unravelled. New concepts and advanced technologies continue to emerge that will evolve our evaluation and treatment paradigms and will produce needed evidence for unanswered questions (BOX 2). In the near future, NGS (TGPs, WES and WGS) will have an increasingly important role in early detection of disease-causing pathogenic variants and discovery of new genetic aetiologies, decreasing the need for invasive procedures and enabling the initiation of individualized treatments for some infants with cholestasis. Unfortunately, the aetiology and pathogenesis of biliary atresia, the most common cause of cholestasis, remain a challenge, and many infants with biliary atresia continue to have late diagnoses resulting in progressive disease requiring liver transplantation. Even with early HPE and successful bile drainage, most infants will develop progressive portal hypertension and eventually require liver transplantation. The need for a newborn baby screen for cholestasis (and biliary atresia) has never been greater, and several advances in this regard are promising. The explosion in therapeutic development for cholestatic conditions, based on our new understanding of the molecular pathophysiology, also provides hope that new precision treatments and cures for these conditions might be forthcoming.

Published online: 22 March 2019

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Acknowledgements
R.J.S. is supported in part by US National Institutes of Health (NIH) grants U01 DK062453 and UL1 TR002535. A.G.F. is supported by an NIH and National Center for Advancing Translational Sciences Clinical and Translational Science Award (KL2 TR002534) and a Children's Hospital Colorado Research Scholar Award.

Author contributions
Both authors contributed equally to the preparation of this manuscript.

Competing interests
R.J.S. has consulted with Albireo, Alexion, Retrophin and Shire. A.G.F. declares no competing interests.

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