Diagnostic Yield of an Algorithm for Neonatal and Infantile Cholestasis Integrating Next-Generation Sequencing

Emanuele Nicastro, MD, PhD[1], Angelo Di Giorgio, MD, PhD[1], Daniela Marchetti, PhD[2], Chiara Barboni, MD[1], Anna Cereda, MD[3], Maria Iascone, PhD[2], and Lorenzo D'Antiga, MD, FEBT[1]

Objective To evaluate the performance of a diagnostic protocol for neonatal/infantile cholestasis in which the main clinical patterns steered the early use of different genetic testing strategies.

Study design An observational study was conducted between 2012 and 2017 in a tertiary care setting on a prospective cohort of children with cholestasis occurring at ≤1 year of age and persisting ≥6 weeks, to measure the detection rate of underlying monogenic diseases. After the exclusion of biliary atresia, a clinically driven genetic testing was performed, entailing 3 different approaches with different wideness: confirmatory single-gene testing; focused virtual panels; and wide search through trio whole-exome sequencing.

Results We enrolled 125 children (66 female, median age 2 months); 96 (77%) patients had hypocholic stools and were evaluated rapidly to exclude biliary atresia, which was the final diagnosis in 74 (59%). Overall, 50 patients underwent genetic testing, 6 with single confirmatory gene testing, 38 through panels, and 6 with trio whole-exome sequencing because of complex phenotype. The genetic testing detection rate was 60%: the final diagnosis was Alagille syndrome in 11, progressive familial intrahepatic cholestasis type 2 in 6, alpha-1-antitrypsin deficiency in 3, and progressive familial intrahepatic cholestasis type 3 in 2; a further 7 genetic conditions were identified in 1 child each. Overall, only 18 of 125 (14%) remained with an indeterminate etiology.

Conclusions This protocol combining clinical and genetic assessment proved to be an effective diagnostic tool for neonatal/infantile cholestasis, identifying inherited disorders with a high detection rate. It also could allow a noninvasive diagnosis in children presenting with colored stools. *(J Pediatr 2019;211:54-62)*.

Neonatal/infantile cholestasis, affecting 1 in 2500 children at term, is defined as an impairment in bile formation or flow presenting early in life, resulting in the retention of biliary substances within the liver.[1] It can be caused by biliary or hepatocellular diseases (genetic and metabolic defects disrupting bile composition and transport).[1,2] Prompt identification of the underlying etiology is important to initiate appropriate surgical or medical treatment. The most common cause of neonatal/infantile cholestasis is biliary atresia, in which an early surgical referral is needed to optimize the success rate.[3] In treatable metabolic conditions, a timely diagnosis certainly warrants a prompt specific treatment and a better outcome.

In recent decades, it has been clarified that the vast majority of the previously unexplained cases referred to as "idiopathic neonatal hepatitis" are monogenic liver disorders.[4,5] Nevertheless, the diagnosis of such inherited liver diseases has relied on extensive biochemical and histologic characterization, which is expensive, time-consuming, limited by local availability and mastery, and affected by the stage of the disease.

The advent of the next-generation sequencing (NGS) has allowed us to generate and analyze massive sequence and high-resolution data, in a rapid, cost-effective, and high-throughput fashion, from panels of a few genes to the exome to the whole human genome.[6,7] This offers the opportunity to move genetic testing upwards in the diagnostic algorithm.[8]

Nearly 20% of pediatric liver transplants are performed in children with monogenic, mostly cholestatic, liver disorders, and genetic characterization has important implications for liver transplantation. Therefore, an institutional protocol was designed to implement the use of genetic testing—mainly based on the NGS—in a stepwise process, in which clinical and laboratory data are used to select type and wideness of genetic approach. The aim of this study was to evaluate the overall diagnostic accuracy

A1AT	Alpha-1 antitrypsin
AGS	Alagille syndrome
ARC	Arthrogryposis-renal dysfunction-cholestasis
CP	Cholestasis panel
GGT	Gamma-glutamyl transpeptidase
MLPA	Multiplex ligation-dependent probe amplification
NGS	Next-generation sequencing
PFIC	Progressive familial intrahepatic cholestasis
WES	Whole-exome sequencing

From the [1]Pediatric Hepatology, Gastroenterology and Transplantation, [2]Medical Genetics Laboratory, and [3]Clinical Genetics, Hospital Papa Giovanni XXIII, Bergamo, Italy

The authors declare no conflict of interest.

0022-3476/$ - see front matter.
https://doi.org/10.1016/j.jpeds.2019.04.016

and the genetic detection rate of such protocol in children with neonatal/infantile cholestasis referred to a pediatric hepatology and liver transplantation unit.

Methods

All children with cholestasis persisting for >6 weeks with onset in the first 12 months of age referred to the Pediatric Hepatology Gastroenterology and Transplantation Unit at Hospital Papa Giovanni XXIII, Bergamo, between January 2012 and June 2017 were enrolled and followed for at least 6 months. Cholestasis was defined in presence of serum-conjugated bilirubin level >1 mg/dL when the total bilirubin was <5 mg/dL or a conjugated component >20% of the total when the total bilirubin was >5 mg/dL.[9] Newborns with prematurity, sepsis, parenteral nutrition lasting ≥2 weeks, and endocrine abnormalities were excluded.

The diagnostic protocol for neonatal/infantile cholestasis was discussed, revised, and approved in October 2011 by an institutional board including pediatric hepatologists and geneticists of our center. All demographic, auxologic, biochemical, genetic, and histologic data from all the patients enrolled were recorded anonymously. A written informed consent approved by the ethical committee of our hospital was obtained before genetic testing. The ethical committee of our institution approved the study.

General Protocol and Use of Virtual Gene Panels

The protocol is illustrated in **Figure 1** (available at www.jpeds.com). Children initially received a comprehensive first-tier laboratory characterization and were stratified according to stool appearance and serum gamma-glutamyl transpeptidase (GGT) concentration.

Patients with pale (hypocholic) stools rapidly underwent liver biopsy to rule out biliary atresia after exclusion of alpha-1 antitrypsin (A1AT) deficiency by serum enzymatic activity. In the presence of a consistent histology (ductular proliferation, portal fibrosis, ductular cholestasis with bile plugs), biliary atresia was confirmed by cholangiography.

The stool appearance was evaluated according to the stool color card published by Hsiao et al.[10] The reference ranges for GGT in the first year of life are reported by Cabrera-Abreu and Green.[11] Three different targeted virtual gene panels (cholestasis panel [CP] 1, CP2, and CP3) were designed and selected according to patients' features (**Table I**; available at www.jpeds.com). Patients with hypocholic stools were tested with CP1 when histologic examination showed signs of intrahepatic cholestasis (giant cell transformation, canalicular cholestasis, cholestatic rosettes); when histology was consistent with biliary atresia but intraoperative cholangiography showed a patent biliary tree; and when histology was consistent with biliary atresia but surgery was not indicated due to diagnostic delay. Patients with normocholic stools were further divided according to serum GGT activity: those with elevated GGT were tested with CP1; those with low or normal GGT and elevated serum bile acids were tested with CP2; and those with low or normal GGT and low total serum bile acids were tested with CP3 (targeted to the bile acid synthesis defects) + urine mass spectrometry for bile acid profile. In case CP2 and CP3 did not achieve any molecular diagnosis, the analysis was extended to the remaining genes of CP1.

The choice of virtual (exome sequencing followed by bioinformatically selected panel) rather than physical panels allowed to update the loci as new cholestasis-related genes were identified. At the last revision, the virtual panels include a total of 37 genes associated with 33 cholestatic liver diseases. All the patients were evaluated by a clinical geneticist and a pediatric neurologist before undergoing genetic testing. Neurologic and clinical genetic assessment addressed family history, general appearance including dysmorphic features, developmental milestones, complete neurologic examination including posture, and muscle tone.

Genetic Confirmation of a Strong Clinical Suspicion

Patients in whom the clinical phenotype suggested a specific diagnosis such as Alagille syndrome (AGS) or A1AT deficiency on a low serum concentration were considered for a prioritized evaluation of the specific locus (eg, *JAG1*, *NOTCH2*, *SERPINA1*).

Use of Wide Genetic Analysis as the First Step (Direct trio-Whole-Exome-Sequencing)

A wide molecular approach through a first-tier Whole-exome-sequencing (WES) on the case−parents trio was employed as the first step in patients with complex phenotypes (liver disease associated with major malformations and/or minor anomalies and/or intellectual disabilities and/or other medical problems) but no clear clues to a definite etiology or when a condition was suspected but the clinical presentation was not typical or complete.

Biochemical studies and second-tier trio-WES

Table II displays the baseline laboratory evaluation and the extensive biochemical studies for underlying infectious, endocrine, and metabolic causes that are performed in the patients with neonatal/infantile cholestasis. The first-tier workup is carried out in all patients, whereas the second-tier workup is carried out in patients without a definite diagnosis, following an inconclusive molecular study. In the presence of clear suspicion of a specific etiology, relevant tests were performed promptly, especially if the suspected disease was not previously ruled out by newborn screening or the referring center. In undiagnosed patients, a second-tier trio-WES was not offered routinely but only in selected cases in whom the family history was strongly suggestive of a genetic condition (eg, ≥2 affected siblings and/or consanguinity).

DNA Extraction, Enrichment, Sequencing, and Analysis

After genetic counseling, genomic DNAs of patients and parents (when the suggested test is trio-WES) were extracted from peripheral blood using commercial DNA extraction

Table II. Biochemical investigation protocol in infants with cholestasis

Protocols	Details
First-tier (at enrollment)	
General workup	Serum/blood: transaminases, GGT, total/conjugated bilirubin, ALP, CK, complete blood count, Coombs test, glucose, albumin, electrolytes, ferritin, A1AT AFP, ammonia, gas analysis and acid−base balance, liver Doppler ultrasound.
Second-tier (if negative genetic testing)	
Endocrine workup	Cortisol (ACTH) stimulation test if indicated), TSH, and thyroxine.
Virologic workup	Serology (IgG and IgM): HBV, HCV, (EBV, EBNA IgG, and EBV-VCA IgM), CMV, HSV-1/2, *Toxoplasma gondii*, Rubella. Nucleic acids: EBV-DNA, CMV-DNA, HSV-1/2-DNA
Metabolic workup	Plasma and urine amino acids, urine organic acids, serum total bile acids + mass spectrometry urine analysis, transferrin isoelectric focusing, acylcarnitine profile, lactic acid, red blood cell galactose-1-phosphate uridyl transferase activity, urine galactose, urine fructose, lysosomal acid lipase, and acid sphingomyelinase on dried blood spot mass spectrometry.
Other investigations	Echocardiography, spine radiograph, eye examination, bone marrow aspiration (if lysosomal storage disease is suspected).

ALP, alkaline phosphatase; *ACTH*, adrenocorticotropic hormone; *AFP*, alpha-fetoprotein; *CK*, creatine kinase; *CMV*, Cytomegalovirus; *EBV*, Epstein–Barr virus; *HBV*, hepatitis B; *HCV*, hepatitis C; *HSV-1/2*, herpes simplex virus-1 and-2; *TSH*, thyroid-stimulating hormone.

kits (QIAGEN, Valencia, California). Exonic regions and the splice sites flanking regions of all human exons were enriched using an Agilent SureSelect Clinical Research Exome enrichment kit (Agilent, Santa Clara, California) followed by sequencing with 150-bp paired-end reads on the NextSeq500 platform (Illumina, San Diego, California). Reads were mapped to the human genome and analyzed by BWA Enrichment tool (Illumina) and a second independent homemade pipeline.[12]

The variant call file including single-nucleotide substitution and indels was annotated using available databases. including the Genome Aggregation Database (http://gnomad.broadinstitute.org/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), and Human Gene Mutation Database Professional. The variant call file, when indicated, was filtered according to the suggested gene panels (CP1, CP2, CP3). The remaining variants were prioritized using a sequential strategy, based on the potential effect on protein and transcript (splicing, missense, nonsense, and frameshift), the minor allele frequency compatible with the rarity of the disease, and all available information.

When trio-WES was suggested, in case of a negative family history, the filtering of variants was based on an autosomal-recessive, de novo filtering strategy or X-linked recessive when the proband was a male subject, and their classification and the potential pathogenic effect is focused on the patient's phenotype, according to the Human Phenotype Ontology classification (www.human-phenotype-ontology.org/).

Variants were classified on the basis of American College of Medical Genetics and Genomics guidelines.[13,14] The potential pathogenic variants were evaluated and visual inspected with Alamut Visual Software (http://www.interactive-biosoftware.com/alamut-visual/) and subsequently confirmed by Sanger sequencing using a second independent DNA sample including both parents.

In patients with a strong suspicion of AGS but no pathogenic variants in *JAG1* and *NOTCH2* genes, multiplex ligation-dependent probe amplification (MLPA) analysis of *JAG1* gene was performed (SALSA MLPA probemix P184-C1/C2/C3 JAG1; MRC-Holland, Amsterdam, The Netherlands) to identify large deletions of this gene. In case of suspicion of A1AT deficiency, a focused sequencing analysis on exons 5 and 7 of the *SERPINA1* gene was performed for the 2 most common alpha 1-antitrypsin alleles (PiZ and PiS).

Statistical Analyses

The Student *t* test, ANOVA, the χ^2 method, or the Fisher exact test were performed when appropriate for statistical analysis, to compare continuous and categorical variables. Univariate logistic regression was performed to analyze factors predicting the achievement of a genetic diagnosis; statistically significant variables were then included in a multivariate model adjusted for covariates to identify independent risk factors. A *P* value lower than .05 was chosen as a cutoff for significance. Data were analyzed with SPSS (IBM Corp Released 2011, IBM SPSS Statistics for Mac, Version 20.0; IBM Corp, Armonk, New York).

Results

Of 170 children referred to our center for neonatal/infantile cholestasis in the study period, 45 in whom cholestasis was considered related to prematurity or judged to be multifactorial or fading by 6 weeks from onset, all with colored stools, were excluded. The remaining 125 children (66 female, 109 white), with a median age of 2 (1-11) months at first visit, were enrolled in the study and followed for a median time of 15 (6-98) months. Twenty-five children were referred for liver transplantation from foreign hospitals, where initial management was performed in close collaboration with our unit. Their clinical and laboratory features are shown in Table III. Consanguinity was present in 5 (4%), and in 4 (3%) a history of liver disease in the first-degree relatives was documented. Coagulopathy (international normalized ratio >1.2) was present in 32% of the children.

Median total and conjugated bilirubin were 10.6 mg/dL (range 1.9-54.4) and 8 mg/dL (1-50), and median GGT and alanine aminotransferase were 306 IU/L (14-2200) and 135

Table III. Demographic, clinical, and laboratory features of 125 children with neonatal-infantile cholestasis

Demographic and clinical characteristics	n/proportion of subjects
Sex, female/male, n	66/59
Gestational age, wk, median (range)	40 (29-41)
Age at visit 1, mo, median (range)	2 (1-11)
Birth weight, g, mean ± SD	3135 ± 547
Ethnicity, n (%)	
White	109 (87%)
Black	12 (10%)
Asian	4 (3%)
Consanguinity, n (%)	5 (4%)
Liver disease in first-degree relatives, n (%)	4 (3%)
Dysmorphisms/malformations, n (%)	27 (22%)
Hypocholic stools*, n (%)	96 (77%)
Coagulopathy†, n (%)	35 (32%)‡

Biochemistry	mean ± SD	Ref. range
Total bilirubin, mg/dL	13.06 ± 10.6	0.2-1.2
Conjugated bilirubin, mg/dL	10.9 ± 9.6	
AST, IU/L	292 ± 226	3-46
ALT, IU/L	179 ± 146	2-50
GGT, IU/L	531 ± 780	§
Serum bile acids, mmol/L	179 ± 133	0-6
Total cholesterol, mg/dL	238 ± 241	20-199
Triglycerides, mg/dL	199 ± 188	40-170
Vitamin A, μg/dL	41 ± 116	20-80
Vitamin 25(OH)D, ng/mL	18 ± 18	>30
Vitamin E, μg/dL	468 ± 507	300-1200
PT INR	1.32 ± 0.46	0.8-1.25

ALT, alanine aminotransferase; *AST*, aspartate aminotransferase; *PT INR*, prothrombin time international normalized ratio.
*Clay-colored, pale yellowish, and light yellowish stools according to published color card (Hsiao et al[10]).
†Patients with PT INR >1.2.
‡9 missing values.
§According to reference levels for age (Cabrera-Abreu and Green[11]).

IU/L (36-925), respectively. Dysmorphisms or major malformations were present in 27 (22%). Facial dysmorphisms and cardiac abnormalities were present in 12 patients each, embryotoxon was observed in 6, and abnormalities of the digestive tract and situs inversus were present in 3 and 2 patients, respectively. Microcephaly, cutaneous, and limb defects were present in 1 patient each. The results of the diagnostic protocol are shown in **Figure 2**.

Patients with Hypocholic Stools

Ninety-six (77%) patients had hypocholic stools. These children were rapidly evaluated to rule out biliary atresia: liver biopsy was performed in all but 4 patients, 2 with low serum A1AT and 2 older than 3 months bearing features highly suggestive of AGS, and the diagnosis was rapidly confirmed by *SERPINA1* prioritized testing and *JAG1* mutation found at CP1, respectively. Of 92 patients undergoing liver biopsy, 84 had predominant portal fibrosis and ductular proliferation. A diagnosis of biliary atresia was made in 72 of them by intraoperative cholangiography at time of Kasai portoenterostomy; in another 2 of them biliary atresia was presumed, laparotomy was omitted due to old age at referral, and the diagnosis eventually was confirmed at liver transplantation. Of the remaining 10 patients with biliary atresia–like histology, 3 children had extrahepatic biliary malformation, autoimmune sclerosing cholangitis, and neonatal sclerosing cholangitis, 1 each, and 7 children were tested through CP1.

In 8 patients, liver biopsy samples suggested intrahepatic cholestasis with predominant giant cell transformation; therefore, they were tested through CP1. Of note, 1 patient with giant cell hepatitis ultimately was determined to be affected by septo-optic dysplasia on clinical and laboratory ground.

As for the child with the unusually early presentation of autoimmune sclerosing cholangitis (onset was at 10 months of age), the diagnosis was based on the presence of severe portal and periportal lymphohistiocytic infiltrate and portal fibrosis on histology and multifocal biliary strictures on magnetic resonance cholangiopancreatography. The child dramatically responded to steroid therapy; he is currently 5 years old and had a recent relapse after long remission while on minimal steroid treatment. A screening for primary immunodeficiency and Langerhans cell histiocytosis yielded no result. Overall, biliary atresia was the underlying disease in almost 60% of the enrolled children.

Patients with Normal Stool Color

Twenty-nine children had normally colored stools. Of them, 17 had an elevated GGT, and 12 had low or normal GGT, all with elevated serum bile acids. In all of them, clinical evaluation guided the choice of genetic testing. Four children with high-GGT cholestasis and features suggesting AGS underwent confirmatory focused analysis of *JAG1* and *NOTCH2*; of the remaining 13 children with elevated GGT, 11 underwent targeted sequencing through CP1; finally, 8 of 12 children with low GGT received first-step sequencing through CP2.

In the remaining 6 patients, neurologic and medical genetic assessment defined a complex phenotype, leading us to choose trio-WES as the first-tier genetic testing (patients 25-29 in **Table IV**; available at www.jpeds.com).

In a 3-month-old boy of North African origin presenting with end-stage liver disease, severe hepatosplenomegaly, and hypotonia, WES did not identify any variant explaining the phenotype. Patient 25 was a 2-month-old white girl with hepatosplenomegaly, failure to thrive, apneas, neurodevelopmental delay, and opisthotonos. Homozygous mutations in the *GBA* gene allowed a diagnosis of acute neuronopathic Gaucher disease. In patient 26, a 4-month-old white infant born abroad, and therefore without newborn screening, admitted in severe clinical conditions with severe cholestasis and liver failure, failure to thrive, hepatosplenomegaly, severe neurodevelopmental delay, and seizures, homozygous mutations in *FAH* (tyrosinemia type 1) were found.

In patient 27, a 2-month-old boy with hepatomegaly, lactic acidosis, hypoglycemia, and hypotonia, WES identified biallelic mutations in the *TRMU* gene responsible for a rare mitochondrial infantile liver disease. In patient 28, a newborn girl with low birth weight, limb anomalies, failure to thrive, hypotonia, renal tubular acidosis, and low-GGT cholestasis,

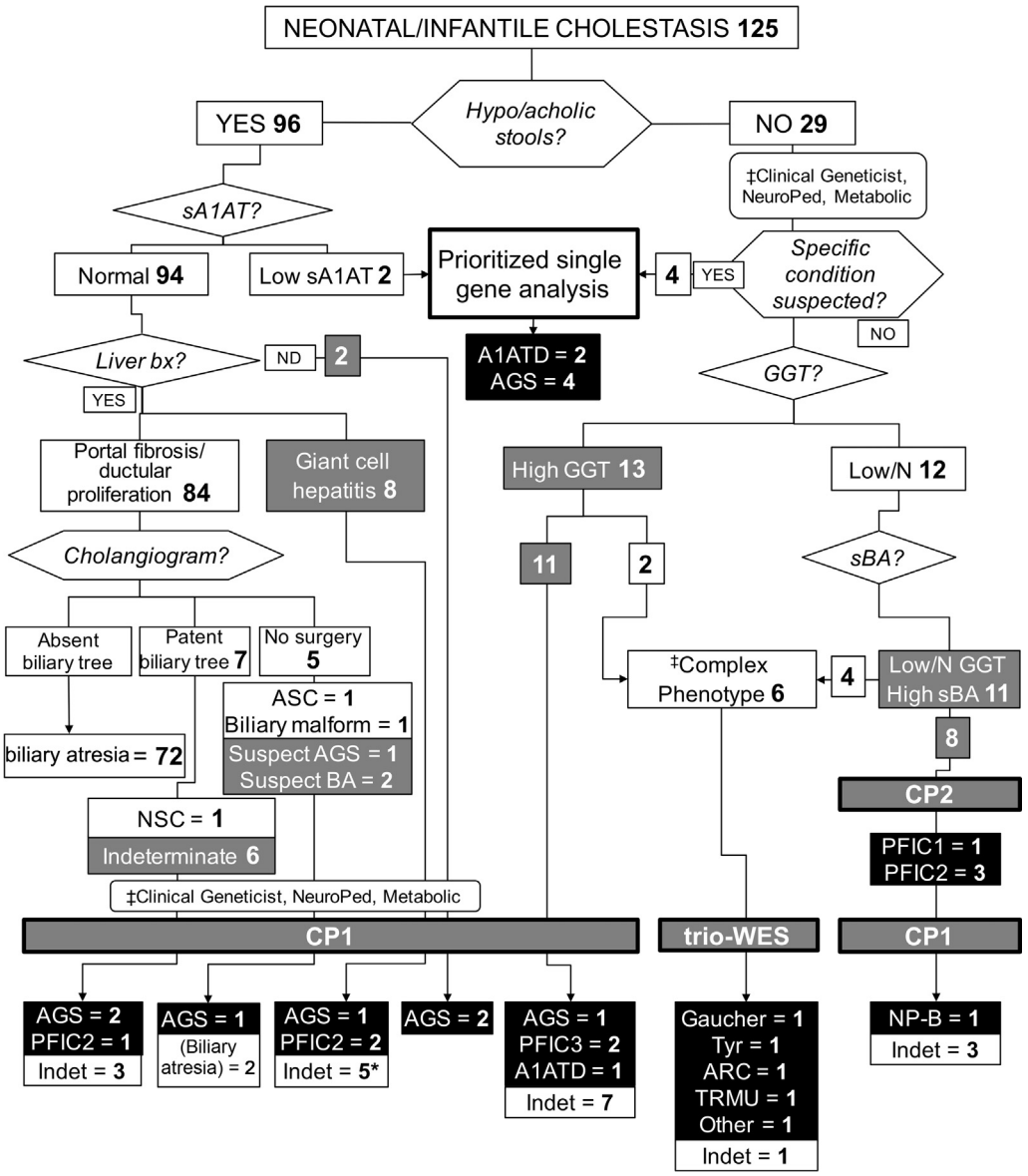

Figure 2. Results of the adopted diagnostic protocol for neonatal/infantile cholestasis. *ASC*, autoimmune sclerosing cholangitis; *NP-B*, Niemann–Pick disease type B; *NSC*, neonatal sclerosing cholangitis; *TRMU*, TRMU gene-related transient liver failure; *Tyr*, tyrosinemia type 1. ‡Clinical genetic consultation (+ advice by pediatric neurologist and/or inherited metabolic disorders, if needed) was obtained for phenotype characterization and to choose the appropriate genetic testing; *one child with negative genetic result was diagnosed as affected by sept-optic dysplasia on clinical and instrumental ground.

WES analysis led to the diagnosis of arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome.

Patient 29 was a 6-month-old girl referred because of severe cholestasis, hypotonia and cortical atrophy, central hypothyroidism, laryngotracheomalacia, and Coombs-negative hemolytic anemia. WES identified a compound heterozygosity for disease-causing mutation in *ATP7B* (p.His1069Gln + p.Gln7fs). Her mother was found homozygous for p.His1069Gln in *ATP7B* and was cirrhotic, and the father was heterozygous for the frameshift mutation (p.Gln7fs). These results triggered further studies that demonstrated biochemical and histologic evidence of copper overload that might have occurred prenatally. Mother and child fulfilled the Leipzig criteria for the diagnosis of Wilson disease.

Genetic Testing and Protocol Diagnostic Performance

Overall, 50 children were investigated with genetic testing, including 2 with biliary atresia who did not undergo portoenterostomy and were confirmed at transplantation. A targeted gene analysis was the test of choice in 38; 30 received CP1 as the first step. Eight patients with low/normal GGT underwent CP2 as the first step, followed by CP1 in 4 of them, of

whom 3 remained without a molecular diagnosis. No patient was studied with CP3.

Six patients underwent genetic testing that successfully confirmed a clinical suspicion of AGS (4 patients) and A1AT deficiency (2 patients). The remaining 6, studied through direct trio-WES, have been described previously in this article.

Overall, genetic testing allowed a final diagnosis in 29 of 48, with a 60% detection rate and a median diagnostic turnaround of 43 (38-55) days. The disease-causing mutations identified are shown in **Table IV**. AGS, progressive familial intrahepatic cholestasis (PFIC) type 2, and A1AT deficiency were the most common genetic causes (30%, 13%, and 6%, respectively); PFIC3 was diagnosed in 2 cases, and PFIC1, Niemann−Pick type B, Gaucher disease, tyrosinemia type 1, ARC syndrome, and *TRMU*-related liver disease were present in 1 child each (**Figure 3**; available at www.jpeds.com). In 1 boy with the histologic picture of giant cell transformation, in whom the CP1 failed to identify gene variants, septo-optic dysplasia was diagnosed after few months on the basis of clinical, endocrinologic, and imaging data.

Overall, only 18 of 125 (14%) patients remained with indeterminate etiology, and the protocol diagnostic yield was as high as 86%. The 18 children with indeterminate etiology received extensive biochemical evaluation, and 6 of them were further studied with a second-tier trio-WES because of consanguinity or familial history of liver disease, but no other pathogenic variants possibly responsible for the clinical picture were identified.

The clinical and laboratory features of the children according to the most frequent diagnoses are shown in **Table V**. The proportion of children small for gestational age was greater in AGS (54%) and low-GGT PFICs (28%, $P < .01$), and dysmorphisms or malformations were invariably present in the patients with AGS in comparison with 0%-20% of the others ($P < .01$). Of 11 cases of AGS, 2 did not fulfill the clinical criteria and were identified by genetic testing. The overall outcomes were not different between the groups.

Overall, 60 children (48%) received liver transplantation after a median time of 13 months (range 4 months to 5 years), 38 (31%) remained with compensated cholestatic disease, 6 developed severe portal hypertension, 7 normalized the indexes of cholestasis, and 4 were lost to follow-up. Ten children died, 7 from complications of liver transplantation, 2 from disease progression in Gaucher disease and ARC syndrome, and 1 from cerebral hemorrhage in AGS. In 1 patient with clinically evident AGS but without pathogenic variants at CP1, MLPA allowed to identify a large deletion of *JAG1*. No demographic, clinical, or anamnestic feature was correlated to genetic etiology, as judged by univariate logistic regression.

Discussion

A few studies have assessed a comprehensive genetic testing approach mainly based on high-throughput sequencing for neonatal/infantile cholestasis. In a previous study, a NGS targeted panel of 93 genes potentially associated with inherited cholestatic diseases was used to define the underlying defect in 6 children with neonatal/infantile cholestasis of indeterminate etiology.[15] In 4, novel variants in *PKHD1*, *ABCB11*, and *NPC1* were identified.

Togawa et al tested an 18-gene NGS custom panel in children with intrahepatic cholestasis, with an overall detection rate of 28%.[16] This low diagnostic yield was probably related to the patient selection, because the authors included children with probable nongenetic causes such as prematurity, infections, and metabolic or hormonal system abnormalities. Another study assessed prospectively a large panel of 61 genes associated with cholestasis and 25 known recurrent large insertions or deletions, allowing a diagnosis of genetic cholestasis in approximately 42% of the tested subjects.[17]

A greater rate of detection was observed in another study from Saudi Arabia, in which, probably due to the high rate of consanguinity, a genetic cause was identified in 61% of patients with advanced cholestatic disease.[18] In the

Table V. Characteristics of the children according to final diagnosis

Characteristics	Biliary atresia	AGS	PFIC1/2	Indeterminate	Other	*P*
Sex, male (%)	31 (42)	7 (64)	3 (43)	10 (56)	8 (53)	.58
Consanguinity, n (%)	2 (3)	0 (0)	1 (14)	2 (11)	0 (0)	.17
Prematurity, n (%)	7 (10)	1 (9)	0 (0)	1 (6)	0 (0)	.88
SGA, n (%)	6 (8)	6 (54.5)	2 (29)	2 (11)	1 (7)	<.01
Dysmorphisms/malformations, n (%)	10 (13.5)	11 (100)	0 (0)	3 (17)	3 (20)	<.01
Hypocholic stools, n (%)	74 (100)	6 (54.5)	3 (43)	8 (44)	5 (33)	<.01*
Birth weight, g	3166 ± 556	2675 ± 288	3222 ± 506	3216 ± 559	3205 ± 553	.06*
Age at visit 1, mo	1.8 ± 0.7	2.7 ± 2.4	4.2 ± 3.0	3.1 ± 2.7	3.8 ± 2.5	<.01*
ALT, IU/L	165 ± 148	198 ± 76	207 ± 96	225 ± 194	170 ± 133	.56*
GGT, IU/L	555 ± 520	1134 ± 2039	24 ± 5	334 ± 356	441 ± 617	.02*
TB, mg/dL	14.1 ± 11.5	14.2 ± 7.1	17.4 ± 16	11.3 ± 8.1	7.0 ± 5.1	.12*
sBA, μmol/L	205 ± 172	134 ± 52	166 ± 72	132 ± 68	180 ± 101	.53*
PT INR	1.3 ± 0.4	1.07 ± 0.1	1.9 ± 0.9	1.3 ± 0.4	1.3 ± 0.3	<.01*
Outcome: death/LT, n (%)	42 (56.8)	7 (63.6)	5 (71.4)	8 (44.4)	8 (53.3)	.77

LT, liver transplantation; *SGA*, small for gestational age; *sBA*, serum bile acid; *TB*, total bilirubin.
*ANOVA test.

aforementioned reports, NGS was used to define a diagnosis in cases already classified as "intrahepatic" cholestasis. In contrast, the present diagnostic protocol addresses all newborns and infants with persistent cholestasis, aiming at achieving a timely identification of the extrahepatic causes and simplifying the characterization of the monogenic diseases underlying intrahepatic cholestasis. To do that, we designed a blended clinical–genetic algorithm, in which the genetic testing approach was flexible and adjusted on the basis of the clinical picture.

We demonstrated that a protocol for neonatal/infantile cholestasis embedding a clinically steered use of genetic testing provided good diagnostic accuracy and proved feasible in a tertiary care setting, with a reasonable turnaround time. In fact, no surgical cause of cholestasis was missed by the initial clinical evaluation, and a molecular genetic diagnosis was made in 58% of the remaining patients.

As expected in a liver transplant center, the study population is mainly represented by children with a greater suspicion of surgical causes and/or showing a rapid deterioration toward liver insufficiency. Namely, biliary atresia in this series accounts for almost 60% of the cases, much greater than the 25%-30% observed in the general population of infants with cholestasis.[4,19,20]

In this setting, the pale stool color has demonstrated to be a valuable clue of surgical cause of cholestasis. In children with abnormal (hypocholic) stools, the presence of ductular reaction and portal fibrosis in liver biopsy samples correctly identified the cases amenable to surgical treatment, and conversely, in only 6 of 84 children the invasive cholangiographic evaluation demonstrated a patent biliary tree, ruling out biliary atresia.

In contrast, in children with pigmented stools, liver biopsy, when performed as an optional evaluation or for staging purposes, did not add substantial diagnostic information.

Among the inherited conditions identified, the most frequent genetic diagnoses were AGS, identified in 11 children, and PFIC1-3 in 9. These diagnoses do have implications for liver transplantation. Although AGS could be easily recognized in presence of typical cardiac, vertebral, and ophthalmic stigmata, in children with isolated hepatic involvement and an incomplete phenotype, the diagnosis could be missed, especially in the very young infants. In these children, it is important to identify and possibly treat coexisting pulmonary artery stenosis or intracranial vascular malformations at risk of potentially devastating complications, especially in those requiring liver transplantation.[21] In this series, 1 girl with AGS died from subarachnoid hemorrhage before undergoing the scheduled pre-listing magnetic resonance imaging of the brain.

In the pediatric liver transplant setting, genetic characterization of the "low-GGT" cholestatic diseases has special importance. In fact, each condition has peculiar possible complications, such as post-transplant severe diarrhea, and progressive graft steatohepatitis in PFIC1,[22,23] and the risk of hepatocellular carcinoma in the native liver and of recurrent immune-mediated graft cholestatic disease in PFIC2.[24,25] Genetic diagnosis has a central role in the management of such patients toward medical or surgical bile acid-lowering treatments or transplantation.

In this series, exome sequencing was followed by virtual panel analysis (CP1 or CP2). This strategy allowed us to update and expand the candidate gene list over time and to reanalyze patients when new cholestasis-related genes were discovered.

In patients without a genetic diagnosis, the analysis was expanded to the exome-wide scale only in case of a high index of suspicion for a genetic cause. However, this second-tier trio-WES did not further improve the detection rate. This is in contrast with what has been reported in different clinical fields. Walsh at al reported that, when applied sequentially, WES increased the detection rate of a virtual panel for hereditary peripheral neuropathies from 22% to 60%.[26] Similarly, Wortmann et al showed that WES allowed a molecular diagnosis outside a virtual targeted panel in about 20% of a cohort of patients with clinical and biochemical suspected mitochondrial disease.[27]

The reasons for such different results are diverse and mainly related to protocol design and patients' selection. In fact, in our protocol, the initial clinical evaluation efficiently addressed the complex cases to a first-tier WES, possibly reducing the second-tier exome analysis detection rate, but also reducing costs and turnaround time.

Furthermore, as a transplant referral center, it is possible that a bias toward isolated, rapidly progressive, and fibrogenic forms of cholestasis exists. In fact, our cohort was essentially composed of children with canalicular bile acid transport defects and biliary developmental disorders, and this also explains the limited number of genetic diagnoses despite the large number of genes listed in the panels. It is very likely that, if applied in an unselected cohort of patients and with a less strict clinical selection, this protocol could define a broader spectrum of diagnoses, maintaining a good overall diagnostic performance.

For similar reasons, this work does not address the issue of diagnostic workup in transient cholestasis of newborn/infants. In this study, we purposely selected patients with cholestasis lasting longer than 6 weeks, therefore excluding those with transient neonatal cholestasis. Transient neonatal cholestasis certainly ought to be studied extensively, especially with wide genetic testing, but within a differently designed research frame. Currently, our approach to newborn/infants with neonatal/infantile cholestasis and colored stools having risk factors for transient/multifactorial cholestasis is to maintain a 6-week observation policy before entering the algorithm presented here. In case of hypocholic stools, the patients are investigated promptly.

We used a wide genetic analysis (first-tier trio-WES) in selected cases with high detection rate and sometimes expanding the phenotypic spectrum of known disease genes. Namely, WES allowed us to identify a boy with mutations in the *TRMU* gene, a thiouridylase important to the maturation of mitochondrial tRNA, that has been associated with rare cases of infantile liver failure and a variable degree of

cholestasis.[28] In a girl with complex phenotype in whom it was used as first choice, WES allowed to identify a compound heterozygous state for pathogenic *ATP7B* mutations, born to a mother who in turn resulted affected by cirrhosis due to Wilson disease, raising the hypothesis—confirmed by biochemical tests—that prenatal copper overload could have occurred. This is under further investigations and could expand the *ATP7B* disease spectrum.

Beside the diagnostic performance, we were able to couple a decisional framework—using clinical and biochemical pointers—to a genetic tool that has evolved over the years. We attained a flexible use of genetic testing and a method allowing us to reanalyze the stored bioinformatic data on the basis of novel disease-associated genes, bearing the possibility of future updates, with substantial cost-effectiveness.

In conclusion, this blended protocol for persistent neonatal/infantile cholestasis, in which a comprehensive genetic approach has a pivotal role, proved to be a feasible and effective option in the differential diagnosis of this condition. This approach allowed timely and accurate genetic characterization and, if further implemented, could reduce the complexity of the biochemical and histologic evaluation currently in use. ■

Submitted for publication Jan 21, 2019; last revision received Mar 15, 2019; accepted Apr 9, 2019.

Reprint requests: Emanuele Nicastro, MD, PhD, Pediatric Hepatology, Gastroenterology and Transplantation Unit, Hospital Papa Giovanni XXIII, Piazza OMS, 1, 24127 Bergamo, Italy. E-mail: enicastro@asst-pg23.it

References

1. Fawaz R, Baumann U, Ekong U, Fischler B, Hadzic N, Mack CL, et al. Guideline for the Evaluation of Cholestatic Jaundice in Infants: joint Recommendations of the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition and the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition. J Pediatr Gastroenterol Nutr 2017;64:154-68.
2. Catzola A, Vajro P. Management options for cholestatic liver disease in children. Expert Rev Gastroenterol Hepatol 2017;11:1019-30.
3. Shinkai M, Ohhama Y, Take H, Kitagawa N, Kudo H, Mochizuki K, et al. Long-term outcome of children with biliary atresia who were not transplanted after the Kasai operation: >20-year experience at a children's hospital. J Pediatr Gastroenterol Nutr 2009;48:443-50.
4. Balistreri WF, Bezerra JA. Whatever happened to "neonatal hepatitis"? Clin Liver Dis 2006;10:27-53-v.
5. Balistreri WF, Bezerra JA, Jansen P, Karpen SJ, Shneider BL, Suchy FJ. Intrahepatic cholestasis: summary of an American Association for the Study of Liver Diseases single-topic conference. Hepatology 2005;42:222-35.
6. Metzker ML. Sequencing technologies—the next generation. Nat Rev Genet 2010;11:31-46.
7. Stranneheim H, Wedell A. Exome and genome sequencing: a revolution for the discovery and diagnosis of monogenic disorders. J Intern Med 2016;279:3-15.
8. Nicastro E, D'Antiga L. Next generation sequencing in pediatric hepatology and liver transplantation. Liver Transpl 2018;24:282-93.
9. Moyer V, Freese DK, Whitington PF, Olson AD, Brewer F, Colletti RB, et al. Guideline for the evaluation of cholestatic jaundice in infants: recommendations of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. J Pediatr Gastroenterol Nutr 2004;39:115-28.
10. Hsiao C-H, Chang M-H, Chen H-L, Lee H-C, Wu T-C, Lin C-C, et al. Universal screening for biliary atresia using an infant stool color card in Taiwan. Hepatology 2008;47:1233-40.
11. Cabrera-Abreu JC, Green A. Gamma-glutamyltransferase: value of its measurement in paediatrics. Ann Clin Biochem 2002;39:22-5.
12. Iascone M, Sana ME, Pezzoli L, Bianchi P, Marchetti D, Fasolini G, et al. Extensive arterial tortuosity and severe aortic dilation in a newborn with an EFEMP2 mutation. Circulation 2012;126:2764-8.
13. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17:405-24.
14. Nykamp K, Anderson M, Powers M, Garcia J, Herrera B, Ho Y-Y, et al. Sherloc: a comprehensive refinement of the ACMG-AMP variant classification criteria. Genet Med 2017;19:1105-17.
15. Herbst SM, Schirmer S, Posovszky C, Jochum F, Rödl T, Schroeder JA, et al. Taking the next step forward—diagnosing inherited infantile cholestatic disorders with next generation sequencing. Mol Cell Probes 2015;29:291-8.
16. Togawa T, Sugiura T, Ito K, Endo T, Aoyama K, Ohashi K, et al. Molecular genetic dissection and neonatal/infantile intrahepatic cholestasis using targeted next-generation sequencing. J Pediatr 2016;171:171-7.e1-4.
17. Wang N-L, Lu Y-L, Zhang P, Zhang M-H, Gong J-Y, Lu Y, et al. A specially designed multi-gene panel facilitates genetic diagnosis in children with intrahepatic cholestasis: simultaneous test of known large insertions/deletions. PLoS One 2016;11:e0164058.
18. Shagrani M, Burkholder J, Broering D, Abouelhoda M, Faquih T, El-Kalioby M, et al. Genetic profiling of children with advanced cholestatic liver disease. Clin Genet 2017;92:52-61.
19. Fischler B, Lamireau T. Cholestasis in the newborn and infant. Clin Res Hepatol Gastroenterol 2014;38:263-7.
20. Feldman AG, Sokol RJ. Neonatal cholestasis. Neoreviews 2013;14:e63-73.
21. Pavanello M, Severino M, D'Antiga L, Castellan L, Calvi A, Colledan M, et al. Pretransplant management of basilar artery aneurysm and moyamoya disease in a child with Alagille syndrome. Liver Transpl 2015;21:1227-30.
22. Nicastro E, Stéphenne X, Smets F, Fusaro F, de Magnée C, Reding R, et al. Recovery of graft steatosis and protein-losing enteropathy after biliary diversion in a PFIC 1 liver transplanted child. Pediatr Transplant 2012;16:E177-82.
23. Miyagawa-Hayashino A, Egawa H, Yorifuji T, Hasegawa M, Haga H, Tsuruyama T, et al. Allograft steatohepatitis in progressive familial intrahepatic cholestasis type 1 after living donor liver transplantation. Liver Transpl 2009;15:610-8.
24. Keitel V, Burdelski M, Vojnisek Z, Schmitt L, Häussinger D, Kubitz R. De novo bile salt transporter antibodies as a possible cause of recurrent graft failure after liver transplantation: a novel mechanism of cholestasis. Hepatology 2009;50:510-7.
25. Knisely AS, Strautnieks SS, Meier Y, Stieger B, Byrne JA, Portmann BC, et al. Hepatocellular carcinoma in ten children under five years of age with bile salt export pump deficiency. Hepatology 2006;44:478-86.
26. Walsh M, Bell KM, Chong B, Creed E, Brett GR, Pope K, et al. Diagnostic and cost utility of whole exome sequencing in peripheral neuropathy. Ann Clin Transl Neurol 2017;4:318-25.
27. Wortmann SB, Koolen DA, Smeitink JA, van den Heuvel L, Rodenburg RJ. Whole exome sequencing of suspected mitochondrial patients in clinical practice. J Inherit Metab Dis 2015;38:437-43.
28. Gaignard P, Gonzales E, Ackermann O, Labrune P, Correia I, Therond P, et al. Mitochondrial infantile liver disease due to TRMU gene mutations: three new cases. JIMD Rep 2013;11:117-23.

29. Crosnier C, Driancourt C, Raynaud N, Dhorne-Pollet S, Pollet N, Bernard O, et al. Mutations in JAGGED1 gene are predominantly sporadic in Alagille syndrome. Gastroenterology 1999;116:1141-8.
30. Heritage ML, MacMillan JC, Anderson GJ. DHPLC mutation analysis of Jagged1 (JAG1) reveals six novel mutations in Australian alagille syndrome patients. Hum Mutat 2002;20:481.
31. Pilia G, Uda M, Macis D, Frau F, Crisponi L, Balli F, et al. Jagged-1 mutation analysis in Italian Alagille syndrome patients. Hum Mutat 1999;14:394-400.
32. Colliton RP, Bason L, Lu FM, Piccoli DA, Krantz ID, Spinner NB. Mutation analysis of Jagged1 (JAG1) in Alagille syndrome patients. Hum Mutat 2001;17:151-2.
33. Kamath BM, Thiel BD, Gai X, Conlin LK, Munoz PS, Glessner J, et al. SNP array mapping of chromosome 20p deletions: genotypes, phenotypes, and copy number variation. Hum Mutat 2009;30:371-8.
34. Krantz ID, Colliton RP, Genin A, Rand EB, Li L, Piccoli DA, Spinner NB. Spectrum and frequency of jagged1 (JAG1) mutations in Alagille syndrome patients and their families. Am J Hum Genet 1998;62:1361-9.
35. Wang NL, Li LT, Wu BB, Gong JY, Abuduxikuer K, Li G, et al. The Features of GGT in Patients with ATP8B1 or ABCB11 Deficiency Improve the Diagnostic Efficiency. PLoS One 2016;11:e0153114.
36. Bull LN, van Eijk MJ, Pawlikowska L, DeYoung JA, Juijn JA, Liao M, et al. A gene encoding a P-type ATPase mutated in two forms of hereditary cholestasis. Nat Genet 1998;18:219-24.
37. Nobili V, Di Giandomenico S, Francalanci P, Callea F, Marcellini M, Santorelli FM. A new ABCB11 mutation in two Italian children with familial intrahepatic cholestasis. J Gastroenterol 2006;41:598-603.
38. Shah I, Chilkar S. Progressive Familial Intrahepatic Cholestasis Type 2 in an Indian Child. J Pediatr Genet 2017;6:126-7.
39. Strautnieks SS, Byrne JA, Pawlikowska L, Cebecauerová D, Rayner A, Dutton L, et al. Severe bile salt export pump deficiency: 82 different ABCB11 mutations in 109 families. Gastroenterology 2008;134:1203-14.
40. van Mil SW, van der Woerd WL, van der Brugge G, Sturm E, Jansen PL, Bull LN, et al. Benign recurrent intrahepatic cholestasis type 2 is caused by mutations in ABCB11. Gastroenterology 2004;127:379-84.
41. Davit-Spraul A, Fabre M, Branchereau S, Baussan C, Gonzales E, Stieger B, et al. ATP8B1 and ABCB11 analysis in 62 children with normal gamma-glutamyl transferase progressive familial intrahepatic cholestasis (PFIC): phenotypic differences between PFIC1 and PFIC2 and natural history. Hepatology 2010;51:1645-55.
42. Schatz SB, Jüngst C, Keitel-Anselmo V, Kubitz R, Becker C, Gerner P, et al. Phenotypic spectrum and diagnostic pitfalls of ABCB4 deficiency depending on age of onset. Hepatol Commun 2018;2:504-14.
43. Kubitz R, Bode J, Erhardt A, Graf D, Kircheis G, Müller-Stöver I, et al. Cholestatic liver diseases from child to adult: the diversity of MDR3 disease. Z Gastroenterol 2011;49:728-36.
44. Kidd VJ, Wallace RB, Itakura K, Woo SL. Alpha 1-antitrypsin deficiency detection by direct analysis of the mutation in the gene. Nature 1983;304:230-4.
45. Gluck I, Zeigler M, Bargal R, Schiff E, Bach G. Niemann Pick Disease type A in Israeli Arabs: 677delT, a common novel single mutation. Mutations in brief no. 161. Online. Hum Mutat 1998;12:136.
46. Ferlinz K, Hurwitz R, Weiler M, Suzuki K, Sandhoff K, Vanier MT. Molecular analysis of the acid sphingomyelinase deficiency in a family with an intermediate form of Niemann-Pick disease. Am J Hum Genet 1995;56:1343-9.
47. Stone DL, Tayebi N, Orvisky E, Stubblefield B, Madike V, Sidransky E. Glucocerebrosidase gene mutations in patients with type 2 Gaucher disease. Hum Mutat 2000;15:181-8.
48. Eyal N, Wilder S, Horowitz M. Prevalent and rare mutations among Gaucher patients. Gene 1990;96:277-83.
49. Rootwelt H, Berger R, Gray G, Kelly DA, Coşkun T, Kvittingen EA. Novel splice, missense, and nonsense mutations in the fumarylacetoacetase gene causing tyrosinemia type 1. Am J Hum Genet 1994;55:653-8.
50. Vrabelova S, Letocha O, Borsky M, Kozak L. Mutation analysis of the ATP7B gene and genotype/phenotype correlation in 227 patients with Wilson disease. Mol Genet Metab 2005;86:277-85.
51. Tanzi RE, Petrukhin K, Chernov I, Pellequer JL, Wasco W, Ross B, et al. The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. Nat Genet 1993;5:344-50.
52. Gissen P, Tee L, Johnson CA, Genin E, Caliebe A, Chitayat D, et al. Clinical and molecular genetic features of ARC syndrome. Hum Genet 2006;120:396-409.
53. Zeharia A, Shaag A, Pappo O, Mager-Heckel AM, Saada A, Beinat M, et al. Acute infantile liver failure due to mutations in the TRMU gene. Am J Hum Genet 2009;85:401-7.

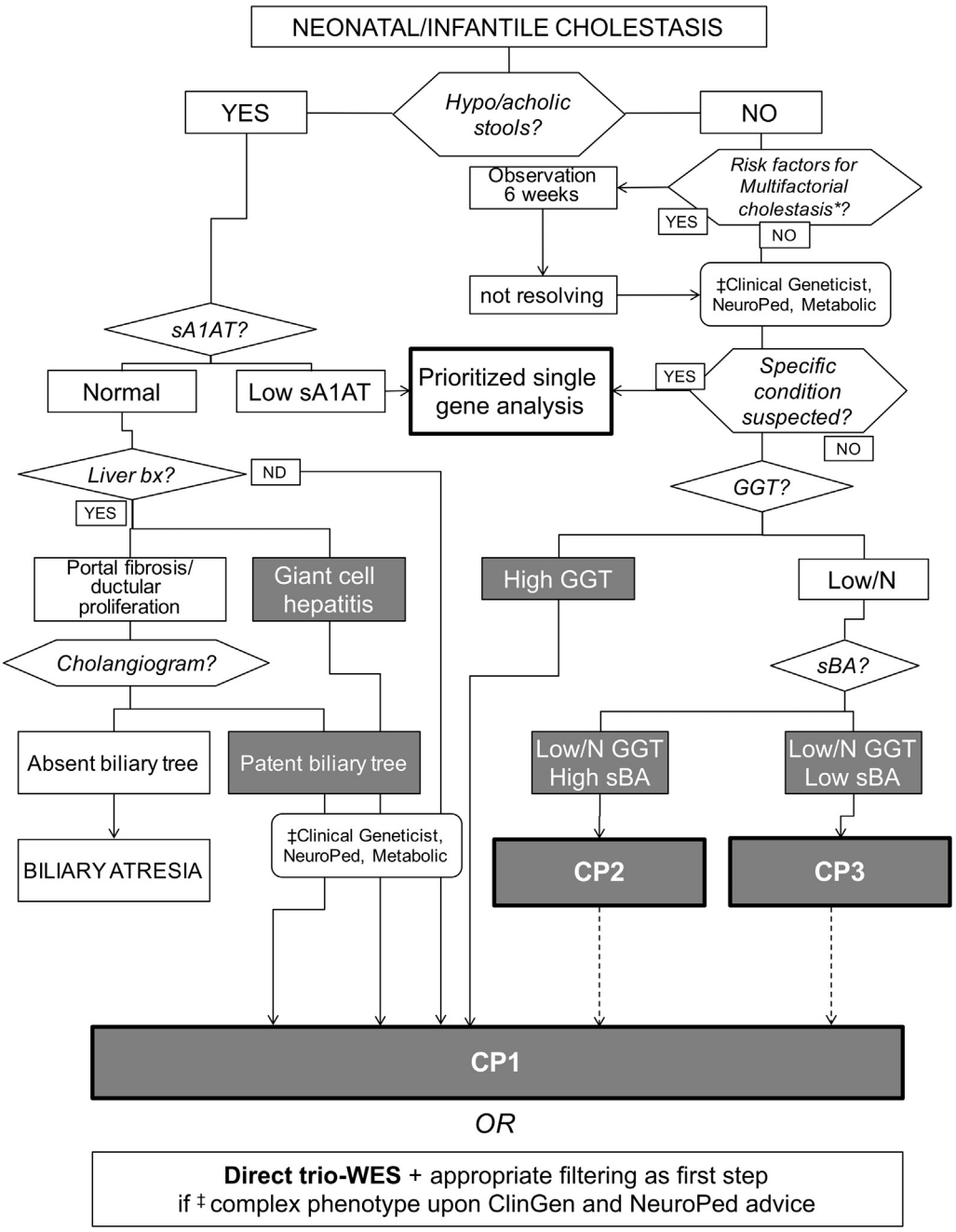

Figure 1. Schematic representation of the adopted diagnostic protocol for neonatal/infantile cholestasis. *Risk factors for multifactorial neonatal cholestasis are at least 1 among prematurity, sepsis, parenteral nutrition lasting ≥2 weeks, and endocrine abnormalities. ‡Clinical genetic consultation (+ advice by pediatric neurologist and/or inherited metabolic disorders, if needed) was obtained for phenotype characterization and to choose the appropriate genetic testing. *Liver bx*, liver biopsy; *ND*, not determined; *sA1AT*, serum alpha-1-antitrypsin; *sBA*, total serum bile acids.

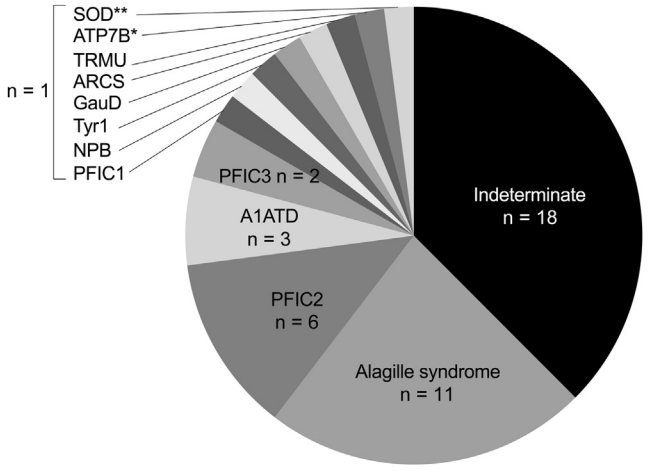

Figure 3. Final etiology of cholestasis in 48 children with neonatal/infantile cholestasis undergoing genetic testing. *ATP7B-related disease in consecutive generations (mother–infant pair both fulfilling the criteria for Wilson disease); **no pathogenic variant identified, diagnosis based on clinical and radiologic findings. *A1ATD*, A1ATD deficiency; *ARCS*, ARC syndrome; *GauD*, Gaucher disease; *SOD*, septo-optic dysplasia; *TyrT1*, tyrosinemia type 1.

Table I. Genes included in the targeted cholestasis panels at our institution

Liver diseases	OMIM	Gene	Locus	Panels
PFIC1	211600	ATP8B1	18q21.31	CP1, CP2
PFIC2	601847	ABCB11	2q31.1	CP1, CP2
PFIC3	602347	ABCB4	7q21.12	CP1
TJP2 deficiency	615878	TJP2	9q21.11	CP1, CP2
AGS	118450	JAG1	20p12.2	CP1
	610205	NOTCH2	1p12	CP1
A1AT deficiency	613490	SERPINA1	14q32.13	CP1
BASD1	607765	HSD3B7	16p11.2	CP3
BASD2	235555	AKR1D1	7q33	CP1, CP3
BASD3	613812	CYP7B1	8q12.3	CP3
BASD4	614307	AMACR	5p13.2	CP3
Cerebrotendinous xanthomatosis	606530	CYP27A1	2q35	CP1, CP2
Familial hypercholanemia	602938	BAAT	9q31.1	CP1, CP2
	607748	EPHX1	1q42.12	CP1
ARC syndrome	208085	VPS33B	15q26.1	CP1, CP2
	613404	VIPAR	14q24.3	CP1, CP2
Transaldolase deficiency	606003	TALDO1	11p15.5	CP1
NICCD	605814	SLC25A13	7q21.3	CP1
Niemann–Pick A, B	257200, 607616	SMPD1	11p15.4	CP1
Niemann–Pick C	257220	NPC1	18q11.2	CP1
	607625	NPC2	14q24.3	CP1
Gaucher disease	230800	GBA	1q22	CP1
Wolman disease, CESD	278000	LIPA	10q23.31	CP1
CDG 1b	602579	MPI	15q24.1	CP1
Galactosemia	230400	GALT	9p13.3	CP1
Hereditary fructose intolerance	229600	ALDOB	9q31.1	CP1
Tyrosinemia	276700	FAH	15q25.1	CP1
MCAD deficiency	201450	ACADM	1p31.1	CP1
MDS (POLG, Alpers type)	203700	POLG	15q26.1	CP1
MDS (DGUOK)	251880	DGUOK	2p13.1	CP1
MDS (MPV17)	256810	MPV17	2p23.3	CP1
OTC deficiency	311250	OTC	Xp11.4	CP1
Neonatal sclerosing cholangitis	617394	DCDC2	6p22.3	CP1
NISCH syndrome	607626	CLDN1	3q28	CP1
BACL deficiency	603314	SLC27A5	19q13.43	CP1, CP2
FXR deficiency	603826	NR1H4	12q23.1	CP1, CP2
MYO5B-related cholestasis	606540	MYO5B	18q21.1	CP1, CP2

BACL, bile acid-CoA ligase; *BASD*, congenital bile acid synthesis defect; *CDG 1b*, congenital defects of glycosylation 1b; *CESD*, cholesteryl-ester storage disease; *FXR*, farnesoid X receptor; *MCAD*, medium-chain acyl-CoA dehydrogenase; *MDS*, mitochondrial depletion syndrome; *NICCD*, neonatal intrahepatic cholestasis caused by citrin deficiency; *NISCH*, neonatal ichthyosis and sclerosing cholangitis; *OMIM*, Online Mendelian Inheritance in Man; *OTC*, ornithine transcarbamylase.

Table IV. Genetic causes of cholestasis identified among the 50 patients who underwent genetic testing.

Cohort	Gene	HGVS nomenclature	References	Diagnosis
Patient 1	*JAG1*	p.[Glu36fs];[Glu36=]	Novel	AGS
Patient 2	*JAG1*	p.[Val1055fs];[Val1055=]	Crosnier 1999[29]	AGS
Patient 3	*JAG1*	c.[886+3A>G];[c.866+3=]	Heritage 2002[30]	AGS
Patient 4	*JAG1*	c.[1395+3A>G];c.[1395+3=]	Pilia 1999[31]	AGS
Patient 5	*JAG1*	p.[Cys714Tyr];[Cys714=]	Colliton 2001[32]	AGS
Patient 6	*JAG1*	entire gene deletion	Kamath 2009[33]	AGS
Patient 7	*NOTCH2*	p.[Lys2009Arg];[Lys2009=]	Novel	AGS
Patient 8	*JAG1*	p.[Cys633Ter];[Cys633=]	Crosnier 1999[29]	AGS
Patient 9	*JAG1*	p.[His209fs];[His209=]	Novel	AGS
Patient 10	*JAG1*	p.[Gln403fs];[Gln403=]	Krantz 1998[34]	AGS
Patient 11	*JAG1*	c.[1395+5A>G];c.[1395+5=]	Novel	AGS
Patient 12	*ATP8B1*	p.[Arg271Ter];c.[2097+2T>C]	Wang 2016[35]; Bull 1998[36]	PFIC1
Patient 13	*ABCB11*	c.[2179_2343del];[2179_2343del] deletion exon 19	Novel	PFIC2
Patient 14	*ABCB11*	p.[Ile541Leu];[Ile541Leu]	Nobili 2006[37]	PFIC2
Patient 15	*ABCB11*	p.[Gly386ter];[Gly386ter]	Shah 2017[38]	PFIC2
Patient 16	*ABCB11*	p.[Arg487Pro];[p.Gly255Glu]	Strautnieks 2008[39]	PFIC2
Patient 17	*ABCB11*	c.[2178+1G>A];p.[Ile112Thr]	van Mil 2004[40]; Davit-Spraul 2010[41]	PFIC2
Patient 18	*ABCB11*	p.[Arg487Pro];[Arg487Pro]	Strautnieks 2008[39]	PFIC2
Patient 19	*ABCB4*	p.[Trp234ter];[Arg582Trp]	Novel; Schatz 2018[42]	PFIC3
Patient 20	*ABCB4*	c.[286+1G>A]; [286+1G>A]	Kubitz 2011[43]	PFIC3
Patient 21	*SERPINA1*	p.[Glu366Lys];[Glu366Lys]	Kidd 1983[44]	A1AT deficiency
Patient 22	*SERPINA1*	p.[Glu366Lys];[Glu366Lys]	Kidd 1983[44]	A1AT deficiency
Patient 23	*SERPINA1*	p.[Glu366Lys];[Glu366Lys]	Kidd 1983[44]	A1AT deficiency
Patient 24	*SMPD1*	p.[Ser192fs];[Trp393Gly]	Gluck 1998[45]; Ferlinz 1995[46]	Niemann Pick B
Patient 25*	*GBA*	p.[His294Gln+Asp448His];[His294Gln+Asp448His]	Stone 2000[47]; Eyal 1990[48]	Gaucher disease
Patient 26*	*FAH*	p.[Gln64His];[Gln64His]	Rootwelt 1994[49]	Tyrosinemia 1
Patient 27*	*ATP7B*	p.[Gln7fs];[His1069Gln]	Vrabelova 2005[50]; Tanzi 1993[51]	ATP7B-related†
Patient 28*	*VPS33B*	p.[Tyr187fs];c.[778+2T>G]	Gissen 2006[52]; novel	ARC
Patient 29*	*TRMU*	p.[Tyr128Cys];[Val279Met]	Novel; Zeharia 2009[53]	Liver failure, transient infantile

A1AT, alpha-1 antitrypsin; *AGS*, Alagille syndrome; *ARC*, artogriposis-cholestasis-renal dysfunction syndrome; *PFIC*, progressive familial intrahepatic cholestasis.
*Identified by direct trio-WES analysis.
†*ATP7B* biallelic disease causing mutations in the patient and her mother in presence of signs of copper overload.