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# Study of Acute Liver Failure in Children Using Next Generation Sequencing Technology

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**Objective** To use next generation sequencing (NGS) technology to identify undiagnosed, monogenic diseases in a cohort of children who suffered from acute liver failure (ALF) without an identifiable etiology.

**Study design** We identified 148 under 10 years of age admitted to King's College Hospital, London, with ALF of indeterminate etiology between 2000 and 2018. A custom NGS panel of 64 candidate genes known to cause ALF and/or metabolic liver disease was constructed. Targeted sequencing was carried out on 41 children in whom DNA samples were available. Trio exome sequencing was performed on 4 children admitted during 2019. A comparison of the clinical characteristics of those identified with biallelic variants against those without biallelic variants was then made.

**Results** Homozygous and compound heterozygous variants were identified in 8 out of 41 children (20%) and 4 out of 4 children (100%) in whom targeted and exome sequencing were carried out, respectively. The genes involved were *NBAS* (3 children); *DLD* (2 children); and *CPT1A, FAH, LARS1, MPV17, NPC1, POLG, SUCLG1,* and *TWINK* (1 each). The 12 children who were identified with biallelic variants were younger at presentation and more likely to die in comparison with those who did not: median age at presentation of 3 months and 30 months and survival rate 75% and 97%, respectively.

**Conclusions** NGS was successful in identifying several specific etiologies of ALF. Variants in *NBAS* and mitochondrial DNA maintenance genes were the most common findings. In the future, a rapid sequencing NGS workflow could help in reaching a timely diagnosis and facilitate clinical decision making in children with ALF. *(J Pediatr 2021;■:1-7).*

Historically, reports from Europe and North America of children with acute liver failure (ALF) identified infectious, metabolic, and drug-related etiologies but the cause remained unexplained in approximately one-half of all cases.[1-3] Despite the progress made over the last 40 years the rate of indeterminate cases remains ~30%.[4] Therefore, challenges remain when evaluating the indeterminate cohort who have a lower chance of spontaneous survival, and higher rates of liver transplantation and death when compared with other diagnostic categories.[4,5]

The rapid increase in the use of next generation sequencing (NGS) since its advent in 2004 has transformed the way children with rare diseases such as ALF are evaluated. This is particularly relevant when considering the more recently identified monogenic disorders such as those caused by variants in *NBAS*,[6] *SCYL1*,[7] and *RINT1*.[8] Therefore, the purpose of this study was to use NGS technology to identify undiagnosed, monogenic diseases in children who received a diagnosis of indeterminate ALF and to gain insight into how this information may assist us in making therapeutic decisions for affected children in the future.

## Methods

This study was carried out at the Pediatric Liver, GI and Nutrition Centre at King's College Hospital, London. The department is an international referral center for children with liver disease. Children under 10 years of age were included in the study as they are considered more likely to suffer from ALF

| | |
|---|---|
| ALF | Acute liver failure |
| CNV | Copy number variant |
| DLD | Dihydrolipoamide dehydrogenase |
| INR | International normalized ratio |
| MDS | mtDNA depletion syndromes |
| mtDNA | Mitochondrial DNA |
| NGS | Next generation sequencing |

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because of a monogenic disease.[9] Targeted sequencing was carried out at King's College Hospital in a historical cohort of children admitted between 2000 and 2018 in whom stored blood was available. Trio exome sequencing was carried out in 3 patients admitted after 2019 at the Exeter Genetics Laboratory at Royal Devon and Exeter Hospital, Exeter, under the United Kingdom Genomics Medicine Rapid Exome Sequencing for acutely unwell children with a likely monogenic disorder service.[10] One patient had exome sequencing performed at the Molecular Genetics Laboratory at Guy's Hospital, London.

The Pediatric Acute Liver Failure study group definition for ALF was used: (1) children with no known evidence of chronic liver disease, (2) biochemical evidence of acute liver injury, and (3) hepatic-based coagulopathy defined as an international normalized ratio (INR) ≥1.5 not corrected by vitamin K in the presence of clinical hepatic encephalopathy or an INR ≥2.0 regardless of the presence or absence of clinical hepatic encephalopathy.[2]

Clinical data was collected retrospectively using the electronic health records on all children who underwent sequencing. Statistical analysis was carried out using GraphPad Prism v 9.1 for Windows (GraphPad Software). Mann-Whitney *U* test was used to compare medians of the clinical characteristics of those who were identified with biallelic variants vs not. The Fisher exact test was used to compare any categorical data.

Children were categorized as ALF of indeterminate etiology when the diagnosis was not established despite thorough, age-appropriate investigations as previously described (**Appendix 1**; available at www.jpeds.com).[2] Blood samples previously collected for research purposes were retrieved after institutional review board/ethics approval and parental consent (18/WA/0009). Genomic DNA was extracted from peripheral leukocytes using standard procedures.

### Targeted NGS: Custom ALF Panel

For the target enrichment sequencing method, a custom ALF panel of 64 genes that are known to cause ALF or a liver based metabolic disorder was constructed (**Appendix 2**; available at www.jpeds.com) using the Agilent eArray web portal (https://earray.chem.agilent.com/earray/). The final design consisted of 14 720 probes covering 276.07 kbp. The enrichment of target regions and library preparation was performed using the SureSelectQXT Target Enrichment Kit (Agilent Technologies). Library quantity and quality were measured using 2200 TapeStation (Agilent Technologies). The pooled libraries were paired-end sequenced (2 × 300 bp) on a flow cell on a MiSeq instrument (Illumina). Demultiplexing and binary base call file conversion to FASTQ was performed by CASAVA (Consensus Assessment of Sequence and Variation). Sequencing quality control and primary analysis were performed using the Illumina Real Time Analysis Software. The FASTQ files were imported and annotated using the CLC Genomics Workbench (Qiagen) where the minimum coverage was set at 30.

### Exome Sequencing

The affected child and unaffected parents were sequenced for the trio exome analysis, which were carried out at the aforementioned laboratories where slightly different methods were employed.[11] Exome library capture was performed using the SureSelectXT Human All Exon Kit V5 (Agilent Technologies). Paired-end sequencing was performed on an Illumina NextSeq 550 or HiSeq 2500. The resulting reads were aligned to the hg19/GRCh37 reference genome with BWA. Variants were called with GATK Unified Genotyper and annotated using ANNOVAR where the minimum coverage was set at 20.

### NGS Data Analysis

Sequence variants were filtered to identify potentially disease-causing mutations using Ingenuity Variant Analysis (Qiagen) or Alamut (Interactive Biosoftware). Variants with a minor allele frequency of >0.01 in the public databases (ExAC, HGMD, dbSNP, 1000 Genomes, Allele Frequency Community, Inova Genomes, EVS and DGV) were excluded. Nonsynonymous (missense, stop gain or loss, frameshift, small insertion and deletion) and intronic variants within 5 bp of exon-intron junctions were included. The filtered variants were investigated for pathogenicity considering the effect on the protein as predicted by Grantham distance, combined annotation dependent depletion score, PolyPhen-2, SIFT, CentoMD, Ingenuity Knowledge Base, VISTA, OMIM, and Clinvar.

## Results

Of 148 children less than 10 years of age admitted with ALF without an identifiable etiology between 2000 and 2018, targeted sequencing was carried out in 41. Exome sequencing was carried out in 4 children admitted in 2019 (**Figure 1**). The 45 children (26 male) from 45 families who were sequenced were primarily of European ancestry: United Kingdom (21 children), Middle East (7), Africa (5), Asian (6), and other (6). Consanguinity was defined by the parents being second cousins or closer and was reported in 9 but none had a family history of ALF in children. The median age at presentation for the full cohort was 23 months (range, 7 days to 9 years and 9 months).

Targeted sequencing in the 41 children achieved a read depth of > × 30 in >99.9% of regions. Variant annotation identified a total of 17 452 unfiltered variants, which was narrowed down to 69 variants in 40 genes in 29 children after the above filtering strategy was applied. Among them, 8 children were found to harbor compound heterozygous or homozygous variants including 1 child with a copy number gain: 3 children in *NBAS*; 1 each in *TWINK, CPT1A, MPV17*, and *DLD*; 1 child harbored compound heterozygous variants in both, *POLG* and *SUCLG1* (**Table I**). Sixty monoallelic

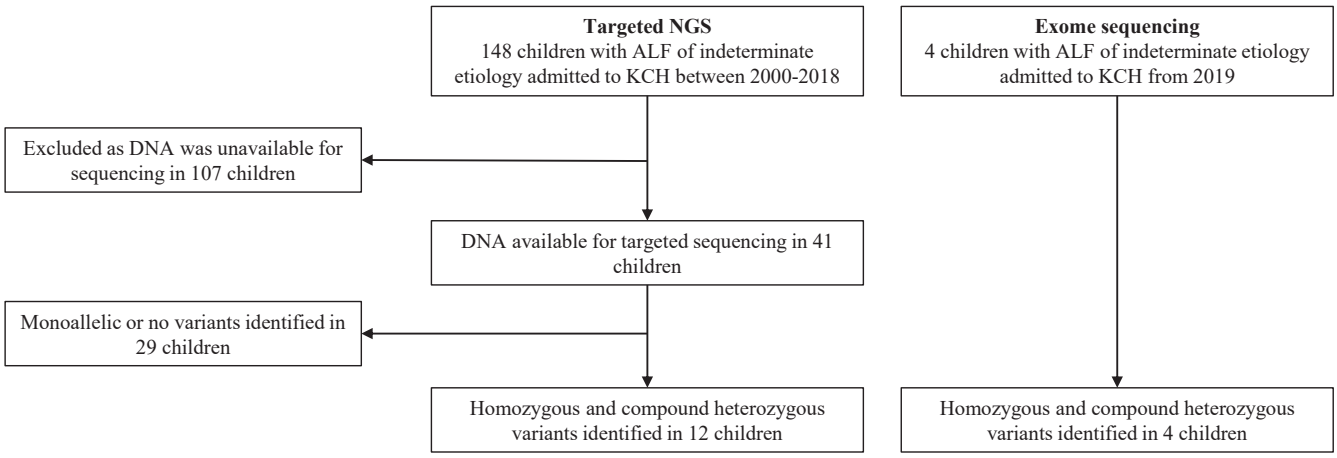


**Figure 1.** NGS in patients with ALF without etiology at King's College Hospital (*KCH*).

variants in 27 children were also identified: 48 missense (24 children), 2 deletion, 1 duplication, 1 nonsense, and 8 splice site (Figure 2). In addition, a monoallelic copy number gain was identified in 2 children. With this information, further interrogation seeking a second pathogenic variant was carried out but was unrevealing.

Exome sequencing achieved a read depth of >×20 in >95% of regions. All 4 children who had exome sequencing were identified with compound heterozygous or homozygous variants: 1 child each in *LARS1*, *FAH1*, *NPC1*, and *DLD* (Table I).

A comparison of the clinical characteristics between those who were identified with biallelic variants vs those who remained indeterminate identified that those with biallelic variants were younger at presentation and more likely to die ($P < .05$; Table II). There was no difference in the other clinical characteristics including biochemistry.

Overall, 3 children were found to harbor compound heterozygous or homozygous variants in *NBAS*. Patient 3 suffered from recurrent ALF associated with intercurrent infections before returning to the Middle East without liver transplantation. He was identified with the homozygous variant, c.4731_4733dup; p.Tyr1578dup. Patient 9 suffered from recurrent ALF and received microbead-encapsulated hepatocytes followed by liver transplantation and remains well on routine follow up. A copy number variant (CNV) ratio of 2 was identified in exon 17-19 in *NBAS*, which was confirmed by quantitative-polymerase chain reaction. Quantitative- polymerase chain reaction spanning the same region on the unaffected parents' DNA revealed a CNV ratio of 1.5 for each parent demonstrating their heterozygous status. A CNV in *NBAS* has not been observed in population databases nor reported previously in affected individuals. Patient 12 suffered from ALF at 3 years of age and recovered without liver transplantation. Twelve years later, he remains well without suffering from further episodes of ALF. He was found to harbor compound heterozygous missense variants in *NBAS*, c.191G>A, p.Arg64His; c.1702G>A, p.Val568Ile.

Four children were found to harbor compound heterozygous or homozygous variants in genes involved in mitochondrial DNA (mtDNA) maintenance: *MPV17, POLG*, *TWINK*, and *SUCLG1*. Patient 8 presented with ALF at 10 weeks of age. A mtDNA depletion syndrome (MDS) was clinically suspected but diagnostic NGS technology was not available at the time and he underwent liver transplantation 6 weeks after presentation. The child is now 7 years of age and attends mainstream school, although requires extra speech and language support. He was found to harbor compound heterozygous, missense variants in *TWINK*: c.1471C>G, p.Ser491Thr; c.1697A>G, p.Lys566Arg. Patient 18 presented at 17 months of age with ALF and underwent auxiliary liver transplantation. The patient is now 17 years old and is under follow-up for mild, cardiac left ventricular hypertrophy. Compound heterozygous missense variants were identified in 2 genes, *SUCLG1* and *POLG*. The variants in *SUCLG1* were c.601A>G, p.Arg201Gly; c.236G>A, p.Gly79Asp. The variants in *POLG* were c.153_158delGCAGCA, p.Gln54_Gln55del; and c.803G>C, p.Gly268Ala. Patient 17 presented with ALF at 18 months of age and received liver transplantation. He is now 19 years old and leads a semi-independent life with mild learning difficulties. Homozygous missense variants were found in *MPV17*: c.3338C>T, p.Pro98Leu.

In patient 43, urinary and plasma succinylacetone were negative at the time of ALF. This was later deemed a false-negative result for tyrosinemia type 1 following detection of succinylacetone by tandem mass spectrometry of the dried blood spot retrieved from day 5 of life.

Two children were identified with biallelic variants in *DLD* including a 1 child with the variants c.826A>T, p.Thr276Ser; and c.911T>C, p.Ile304Thr. She presented at 1 month of age with ALF and received liver transplantation and remains well at 15 years of age. To further interrogate the pathogenicity of this variant, pyruvate dehydrogenase activity was measured using the patient's skin fibroblasts. However, the result of 0.76 nmol/mg protein/minute (normal range 0.60-0.90) went against the possible diagnosis suggested from the genetics.

**Table I.** Clinical and genetic data on patients with homozygous and compound heterozygous variants after filtering

| ID | Sex | Age at presentation | Clinical | Sequencing method | Gene | Genotype | Protein | MAF | CADD/pathogenicity | LT | Outcome | Age at last visit |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pat 1 | F | 1 mo | ×1 ALF | Targeted NGS | *DLD* | c.826A>T | p.Thr276Ser | 5.42E-04 | 25 | Yes | Alive | 15 y |
| | | | | | | c.911T>C | p.Ile304Thr | 1.57E-04 | 26 | | | |
| Pat 3 | M | 6 mo | Recurrent ALF | Targeted NGS | *NBAS* | c.4731_4733dup | p.Tyr1578dup | Not reported | Pathogenicity likely | Yes | Lost to follow up | |
| Pat 8 | M | 2 mo | ×ALF | Targeted NGS | *TWNK* | c.1471C>G | p.Ser491Thr | 3.99E-06 | 24 | Yes | Alive; NDD | 7 y |
| | | | | | | c.1697A>G | p.Lys566Arg | 3.99E-04 | 22 | | | |
| Pat 9 | M | 6 mo | Recurrent ALF | Targeted NGS | *NBAS* | c.exons 17-19 dup | p.? | Not reported | Pathogenicity likely | No | Alive | 6 y |
| Pat 12 | M | 3 y | ×ALF | Targeted NGS | *NBAS* | c.1702G>A | p.Val568Ile | 0.0012 | 15 | No | Alive | 15 y |
| | | | | | | c.191G>A | p.Arg64His | 6.30E-05 | 19 | | | |
| Pat 17 | M | 18 mo | ×ALF | Targeted NGS | *MPV17* | c.338C>T | p.Pro98Leu | 5.31E-05 | 25 | Yes | Alive; NDD | 19 y |
| Pat 18 | M | 17 mo | ×ALF | Targeted NGS | *SUCLG1* | c.601A>G | p.Arg201Gly | 1.99E-05 | 30 | Yes | Alive; cardiomyopathy | 17 y |
| | | | | | | c.236G>A | p.Gly79Asp | 0.0013 | 27 | | | |
| | | | | | *POLG* | c.153_158delGCAGCA | p.Gln54_Gln55del | 0.0036 | * | | | |
| | | | | | | c.803G>C | p.Gly268Ala | 0.0039 | 27 | | | |
| Pat 19 | M | 2 y | ×ALF | Targeted NGS | *CPT1A* | c.2198A>G | p.Asn733Ser | 0.0018 | 16 | No | Alive | 6 y |
| Pat 42 | M | 1 wk | ×ALF | Exome sequencing | *LARS1* | c.1292T>A | p.Val431Asp | 3.47E-04 | 29 | No | Dead | NA |
| | | | | | | c.312G>T | p.Leu104Phe | 4.01E-06 | 26 | | | |
| Pat 43 | F | 1 mo | ×ALF | Exome sequencing | *FAH* | c.974C>T | p.Thr325Met | 4.02E-06 | 26 | No | Dead | NA |
| Pat 44 | M | 2 wk | ×ALF | Exome sequencing | *NPC1* | c.3718G>A | p.Gly1240Arg | 3.98E-06 | 25 | No | Dead | NA |
| Pat 45 | M | 9 y | Recurrent ALF | Exome sequencing | *DLD* | c.685G>T | p.Gly229Cys | 3.10E-04 | 35 | No | Alive | 17 y |

*CADD*, combined annotation dependent depletion; *LT*, liver transplantation; *MAF*, minor allele frequency; *NA*, not applicable; *NDD*, neurodevelopmental delay; *Pat*, patient.
Homozygous and compound heterozygous variants were found in 8 out of 41 children (20%) and 4 out of 4 children (100%) in whom targeted and exome sequencing were carried out, respectively.
*Polyglutamine tract expansion.

## Discussion

Acute liver failure is defined in children as a multisystem disorder in which there is severe impairment of liver function, with or without encephalopathy, with hepatocellular necrosis in a patient with no reported underlying chronic liver disease.[12] This in an inclusive description which inevitably covers numerous, underlying etiologies. Reaching a diagnosis can be difficult[13] and efforts to reduce the rate of indeterminate cases remain a challenge.[4] Monogenic disorders causing metabolic disease are thought to contribute to 10%-28% of these cases[2,14] and are thought to be more likely in younger children or infants.[9] Compound heterozygous or homozygous variants of varying pathogenicity were identified in 8 out of 41 (20%) and 4 out of 4 (100%) children in whom targeted and exome sequencing were carried out, respectively. Among these 12 children with compound heterozygous or homozygous variants, 10 were boys although this apparent discrepancy in sex compared against the group without biallelic variants did not amount to statistical significance (**Table I**). However, children in this group were younger at presentation and more likely to die. Subsequently, the genetic information was then compared against their disease phenotype and thought to provide the diagnosis with confidence in 9 (patients 3, 8, 9, 17, 18, and 42-45). These included disorders where diagnosis is primarily based on genetic sequencing (disease causing mutations in *NBAS* and *LARS1*) or those in which conventional diagnostic methods may take too long for children with ALF (filipin staining or pyruvate dehydrogenase activity measurement in cultured fibroblasts, for Niemann Pick C disease or dihydrolipoamide dehydrogenase [DLD] deficiency, respectively). In 1 infant (patient 43), exome sequencing uncovered a false negative result of a conventional diagnostic test, a rare but recognized case of succinylacetone negative tyrosinemia.[15,16] However, in others the significance of the genetic findings was less clear. In patient 1, DLD enzymology using skin fibroblasts demonstrated normal enzyme activity going against a diagnosis of DLD deficiency. Furthermore, in patient 19, repeat profiles of plasma acylcarnitine suggested that the carnitine transporter was not impaired. A possible interpretation of these results is that susceptibility to ALF was increased genetically, but the variants were not the cause of a monogenic disease.

These findings demonstrated that, although the genetic findings were helpful in reaching a diagnosis in some cases, in others it raised further questions. Challenges will remain in the future with regards to determining the pathogenicity and relevance of the genetic findings with enough confidence to influence therapeutic decision making.

Targeted, as well as exome, sequencing have been successful in providing diagnoses for children with rare diseases[17,18] both of which were employed in this study. However, whole genome sequencing using trio testing when possible is being used with superior diagnostic rates and cost-

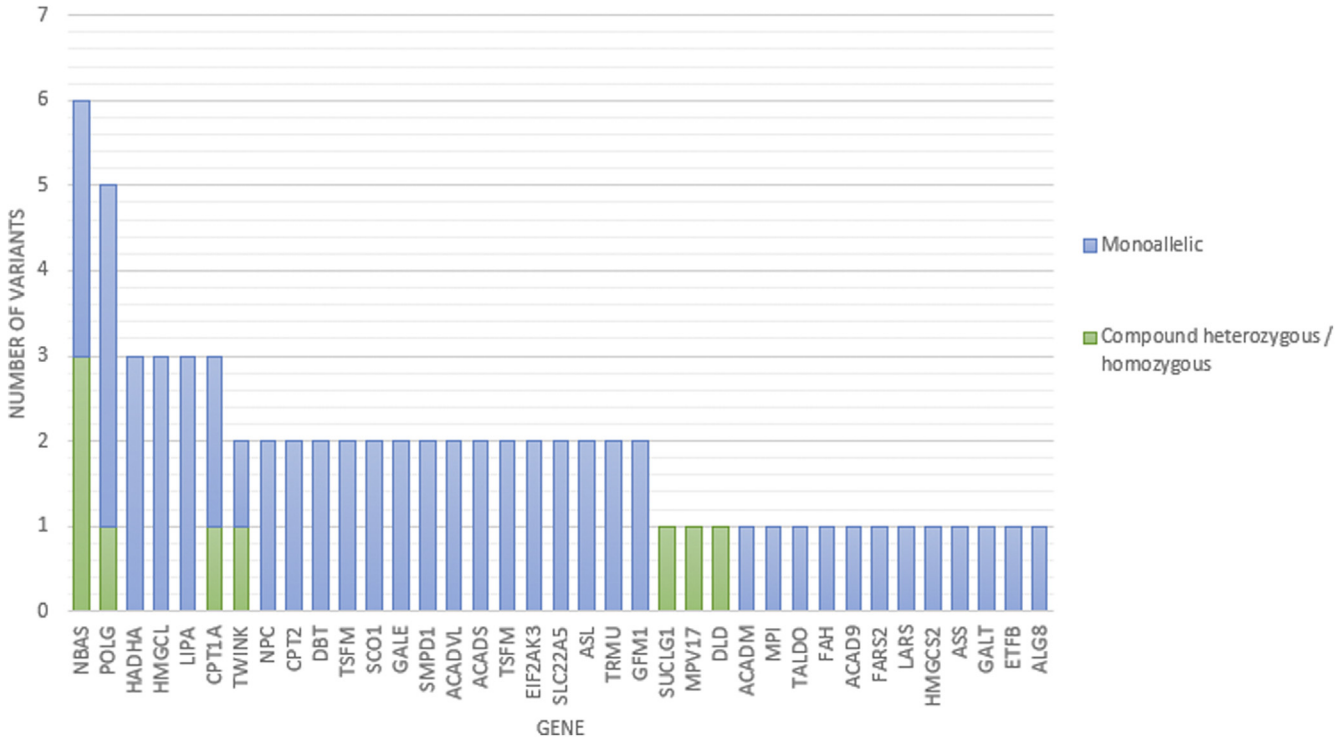


**Figure 2.** Targeted NGS in 41 children: genes in which variants of potential pathogenicity were identified after filtering. Eight children were found to harbor compound heterozygous or homozygous variants. Sixty monoallelic variants in 27 children were also identified. Without specific functional studies it is difficult to comment on the significance of monoallelic variants although it is possible that they contributed to the disease phenotype.

effectiveness.[19,20] Children with ALF pose specific challenges because of the short time frame between presentation to therapeutic decision making as they can lose hepatic function within days.[2,14] In this respect, whole genome sequencing has the added advantage of a shorter library preparation time than is required for targeted or exome sequencing. Efforts to develop rapid sequencing pipelines for critically ill children have demonstrated a turnaround time of 2-3 weeks.[19,20] Future efforts should concentrate around building the appropriate technology, infrastructure, and resources to reduce the turnaround time even further to maximize the clinical utility of NGS in children with ALF.

**Table II. Clinical characteristics of 41 children who underwent targeted NGS**

| Patient characteristics | | Biallelic (n = 12) | Indeterminate (n = 33) | *P* value |
|---|---|---|---|---|
| Demographics | Median age at presentation | 3 mo (min, 7 d to max, 9 y and 9 mo) | 30 m (min, 7 d to max, 8 y and 8 mo) | <.05* |
| | Male sex | 3 (25%) | 17 (41%) | ns† |
| Ethnicity | White | 4 (33%) | 18 (55%) | ns† |
| | Arab | 3 (25%) | 4 (12%) | ns† |
| | Pakistan | 1 (8%) | 3 (9%) | ns† |
| | Other | 4 (33%) | 8 (24%) | ns† |
| Clinical | History of consanguinity | 4 (33%) | 5 (15%) | ns† |
| | History of fever at presentation | 4 (33%) | 10 (24%) | ns† |
| | Max AST (IU/L) | 2125 (range, 22 532) | 2188 (range, 32 040) | ns* |
| | Max total bilirubin (mg/dL) | 15.9 (range, 29.4) | 13.3 (range, 31.2) | ns* |
| | Max INR | 4.6 (range, 15) | 4.8 (range, 11.7) | ns* |
| | Max ammonia (μmol/L) | 98 (range, 201) | 99 (range, 286) | ns* |
| Liver | Recurrent ALF | 3 (25%) | 0 | ns† |
| | Resolution without LT | 4 (33%) | 11 (33%) | ns† |
| | Liver transplantation | 5 (42%) | 20 (60%) | ns† |
| Outcome | Alive | 9 (75%) | 32 (97%) | <.05† |
| | Neurodevelopmental delay | 5 (42%) | 7 (21%) | ns† |

*AST*, aspartate aminotransferase; *ns*, not significant.
Children who were identified with biallelic variants were younger and more likely to die ($P < .05$). There were no other differences in the patient characteristics including biochemistry.
*Mann-Whitney U test.
†Fisher exact test.

### Mitochondrial Hepatopathies and Liver Transplantation

MDS are caused by nuclear gene defects affecting genes responsible for the maintenance and replication of the mtDNA.[21] Liver transplantation for MDS is controversial because of the possibility of offering an organ transplant to a child with a multisystemic, life-limiting disorder. In this study, 3 children were found to have compound heterozygous or homozygous variants in mtDNA maintenance genes all of whom received liver transplantation and are alive at follow-up. In a series of 29 patients with *MPV17*-associated hepatocerebral MDS, liver transplantation was performed in about 40% in whom one-half died afterward.[22] It has been suggested that the degree of mtDNA depletion, in the most commonly identified p.R50Q variant, may give an indication of a milder severity of the disease whilst nonsense, splice site, and frameshift variants as well as deletions lead to death in infancy or early childhood.[22] In *POLG*-associated MDS, progressive neurologic deterioration ensues starting in the teens even if they were to survive with liver transplantation.[23,24] To our knowledge, there are no published reports of liver transplantation in *TWINK* or *SUCLG1* deficiency.

With the increasing use of NGS so too will there be an increase in the number of reported variants of potential pathogenicity. Nevertheless, genotyping is far from providing the phenotypic clarity that is required for therapeutic decision making in MDS; a risk is the possibility of overinterpreting genotypic information. Therefore, conventional biochemical and enzymatic tests should, for the time-being, continue to be carried out in parallel to help complement the genetic findings.

### ALF and NBAS Deficiency

Over 100 cases of ALF and liver dysfunction secondary to NBAS deficiency have been described world-wide in children and adults. Typically, the onset of liver failure is in the first 2 years of life with fever as a triggering factor.[6,25-27] Notably, the biochemical derangement is hyperacute with significant elevations in aspartate aminotransferase and L-alanine aminotransferase.[28] In similar fashion, the INR elevates sharply, typically ranging between 4 and 20.[29] Affected children can suffer multiple episodes of liver failure although, with age, the occurrence of crises seems to diminish.[29] Treatment is dependent on the severity of the ALF: some children recover with supportive therapy whilst others undergo liver transplantation during the acute episode of ALF or as a preventative measure against future episodes.

In our study, 3 patients were found to have biallelic variants in *NBAS*. One patient (patient 9) received intraperitoneal transplantation of alginate-embedded human hepatocytes during an episode of ALF and recovered successfully.[30] The diagnosis was not known at the time and he did undergo liver transplantation 6 months later, after suffering from another episode of ALF. This case highlights the potential for hepatocyte transplantation as rescue therapy for ALF secondary to NBAS deficiency, thereby, gaining time until adulthood when affected children may grow out of these episodes; it also avoids conferring a child to the life-long risks associated with organ transplantation.

This study demonstrated the benefits of using NGS in improving diagnostic rates and how this can help clinicians make therapeutic decisions and support affected families. However, there will be challenges to its practical use given the speed in which ALF in children progresses and the wide range of possible etiologies. Future work should focus on close collaboration with laboratories using conventional, diagnostic methods to help interpret the genetic findings as well as developing rapid, NGS pipelines to meet the timeframe required for therapeutic decision making. ■

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