

Hepatic Parenchymal Injury in Crigler-Najjar Type I

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ABSTRACT

Background: Crigler-Najjar syndrome type I (CNI) arises from biallelic variants of *UGT1A1* that abrogate uridine diphosphate glucuronosyltransferase (UGT1A1) activity resulting in unconjugated hyperbilirubinemia. Historically, liver parenchyma in CNI was considered structurally and histologically normal. Recent review of CNI liver explants revealed fibrosis. Our aim was to investigate the association between hepatic histology and disease phenotype in CNI.

Methods: We extracted data from the medical record at the time of liver transplant from 22 patients with CNI at the Children's Hospital of Pittsburgh, and reviewed explant histology. Continuous data were normally distributed, are presented as mean (± 1 SD), and analyzed using two-tailed Student *t*-test. Categorical data were analyzed using the Chi-square test.

Results: Both alanine transaminase (ALT; mean 87.4 IU/L) and aspartate transaminase (AST; mean 54.6 IU/L) were elevated. Nine (41%) of 22 explants had significant fibrosis. Pericentral ($n=5$), periportal ($n=2$), and mixed ($n=2$) patterns of fibrosis occurred. A significant difference in mean age of subjects with fibrotic versus non-fibrotic livers (16.1 years vs 10.5 years; $P=0.02$) was seen. There were no indices of synthetic liver dysfunction or portal hypertension. Neither a history of gallstone disease nor excess weight appeared to contribute to the development of fibrosis.

Conclusions: For the first time, we report a 41% prevalence of clinically silent, yet histologically significant fibrosis among subjects with Crigler-Najjar type I. Risk for fibrosis appears to accrue with time, indicating that earlier intervention may be prudent whenever considering alternative treatments such as hepatocyte transplant, auxiliary liver transplant, or viral gene therapy.

Key Words: Crigler-Najjar, fibrosis, pediatric, transplant

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What Is Known

- Crigler-Najjar type 1 is a rare genetic disorder due to a deficiency of the bilirubin-uridine diphosphate glucuronosyltransferase enzyme activity resulting in pathological elevation of unconjugated bilirubin which, can have devastating neurological sequelae.
- Orthotopic liver transplantation in Crigler-Najjar type 1 is curative and often pursued once more conservative therapies begin to fail.
- Historically, liver parenchyma in Crigler-Najjar type 1 was considered structurally and histologically normal making Crigler-Najjar type 1 an ideal candidate for orthotopic liver transplantation-avoiding therapies such as hepatocyte transplant, auxiliary liver transplant, or viral gene therapy.

What Is New

- Clinically silent, yet histologically significant fibrosis is prevalent among subjects with uridine diphosphate glucuronosyltransferase deficiency.
- Individuals with fibrosis were notably older than those without fibrosis suggesting that the injury may be incrementally acquired.
- These findings suggest that histological assessment is warranted and earlier intervention may be advantageous whenever looking to optimize the therapeutic benefit of alternative treatment approaches such as hepatocyte transplant, auxiliary liver transplant, or viral gene therapy.

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Crigler-Najjar (CN) is a rare genetic disorder found in less than 1 in 1,000,000 births that results in an unconjugated hyperbilirubinemia (1). It was first reported in 1952 when Crigler and Najjar published a series of 6 hospitalized infants with unconjugated hyperbilirubinemia that developed severe neurological symptoms (2). It is a deficiency of the bilirubin-uridine diphosphate glucuronosyltransferase (UGT1A1) enzyme activity that is inherited in an autosomal recessive manner (3). This defect results in a pathological elevation of unconjugated bilirubin, which can deposit in the basal ganglia of the brain and cause kernicterus, long-term neurological sequela, and even death (4).

There is a spectrum of disease with two described phenotypes, type I (CNI) being the most lethal form of hereditary hyperbilirubinemia with complete absence of enzyme function. CNI often arises from mutations, which result in either premature truncation or deletion of key amino acid sequences on any of the 5 exons of the *UGT1A1* gene; however, pathogenic frameshift and premature stop codons have also been reported (5,6).

The mainstay of treatment for CNI is intensive phototherapy of up to 20 hours per day in infants and 12 hours in older children (7). Compliance is difficult and the efficiency of phototherapy decreases over time because of reduced surface area to volume ratio, skin thickening, and skin lesions (8). Ursodiol therapy is often prescribed and phenobarbital can be effective in patients with less severe enzyme defects. Additional therapeutic avenues include plasmapheresis, albumin infusions, prophylactic cholecystectomy, and the avoidance of medication-induced bilirubin displacement (7,9–11).

Liver transplantation remains the definitive treatment of CNI; however, decisions regarding the optimal timing of transplant are challenging as transplant risks must be weighed against the ability to control jaundice and the subsequent development of neurological sequelae. It is controversial what degree of neurological symptom improvement can be expected after transplantation, although the goal of therapy has been to transplant patients before the development of these symptoms (9).

Other therapies such as hepatocyte transplant and gene replacement are being developed as methods to retain the native liver (12,13). Importantly, in individuals with CNI, these therapies have been pursued under the presumption that whereas the defective *UGT1A1* enzyme is uniquely expressed in the liver, minimal hepatic parenchymal damage is accrued and elevated unconjugated bilirubin levels primarily impact extrahepatic tissues such as the nervous system. To date, the description of abnormal liver histology in patients with Crigler-Najjar is limited to the initial case series. Interestingly, all 6 patients were found to have bile thrombi and half of the patients had slight periportal fibrosis (2). Notably, these biopsies were all obtained within the first year of life and therefore, it is unknown if and how these findings progress over time.

In review of explants from a single center, we have found a significant prevalence of clinically silent fibrosis. Here, we aim to describe the long-term changes on liver histology and investigate associations between histologic findings and disease phenotype in patients who have received liver transplantation for CNI.

METHODS

Subjects

All patients who were transplanted at the Children's Hospital of Pittsburgh of UPMC for the indication of CNI were included in this study. The diagnosis of CNI was either based on genetic mutation or clinical symptoms. Records were analyzed to delineate demographics and pertinent clinical information. Wherever available, genetic testing results were collected. Biochemical data were extracted from the medical record at the time of transplant.

Histology

The histology slides were retrieved and assessment was made of the degree of cholestasis on H&E stain, fibrosis on a Masson Trichrome stain, and wherever available, ductular reaction with a cytokeratin 7 (CK7) immunohistochemical stain (mouse monoclonal, OV-TL12130, DAKO, CA; 1:250). The degree of intrahepatic cholestasis was graded for pericentral and portal areas and a note was made of any hilar bile plugs on explanted liver resections. Presence of isolated pericentral zone 3 cholestasis was graded as 1+, cholestasis of zones 3 and 2 were labelled 2+, and panlobular distribution was labelled as 3+. Degree of portal and hilar bile plugs were also assessed as 0 to 3+ depending on the degree of cholestasis in those areas. The fibrosis was staged for both centrilobular fibrosis and portal fibrosis. Pericentral fibrosis was staged as "0" if there was no evidence of fibrous strands extending from the central vein collagen, "1+" if fine trabeculae of fibrous tissue were seen extending into the sinusoids, "2+" if the fibrosis was more accentuated with extensions into zones 3 and 2, and "3+" if there was evidence of central to central bridging fibrosis. The portal fibrosis was staged 0 to 6 using the Ishak staging system. The CK7 was used to estimate the degree of bile ductular reaction (0–3+: 0, no ductular reaction; 1+, mild; 2+, moderate; and 3+, extensive ductular reaction) as well as to assess the degree of cholestasis with the degree of ectopic hepatocyte staining for CK7 (0–3+ depending on extent of ectopic hepatocyte staining; qualitative). All scoring was done by in a blinded fashion without any knowledge of clinical or laboratory parameters by a single hepatopathologist. Additional pathology findings were documented but not scored.

Fibrosis Group Assignment

In order to explore the associations between data collected from the medical record and the presence of fibrosis, patients were categorized based on the presence or absence of significant histological fibrosis. Fibrosis was determined to be significant if it was appreciated that there was the presence of stage 2 or higher pericentral and/or stage 3 or higher portal/periportal fibrosis.

Statistical Analysis

Continuous data that were normally distributed are presented as the mean plus or minus SD, and were analyzed by the two-tailed Student *t*-test. Chi-square test was used for categorical data. Differences were considered statistically significant if the *P* value was less than 0.05.

All information was gathered on a pre-approved form. Data were de-identified and coded by study number in accordance with the Health Insurance Portability and Accountability Act guidelines. The study was approved by the Institutional Review Board at the University of Pittsburgh.

RESULTS

Patient Characteristics

Twenty-three individuals underwent liver transplantation for the indication of CNI between 1991 and 2016. Patient and graft survival is 96% with mean follow-up of 5.8 years. One patient was excluded from the analysis because of unavailability of explant tissue for histological scoring. Therefore, 22 patients were included in the final analysis (Table 1). Ten of the 22 patients had a genetically confirmed diagnosis (2 of these patients were assigned a genetic diagnosis based on positive genetic testing in a sibling with common parents). The most common genetic mutation

TABLE 1. Patient characteristics of Crigler-Najjar type I transplant recipients (n = 22)

| | |
|------------------------|---|
| Sex: n (%) | Men: 8 (36) Women: 14 (64) |
| Age (years): mean (SD) | 13.4 (5.6) |
| Height (cm): mean (SD) | 145.3 (27.5) |
| Weight (kg): mean (SD) | 28.2 (20.6) |
| Genetic defect | UGT1A1 c.222C>A: n = 7 UGT1A1 c.847C>T heterozygote, 41,429 bp deletion heterozygote: n = 1 Deletion of nucleotide 615 with exon 1 leading to a change of F206S followed by a frameshift and a stop codon 5 amino acids later (F206SfsX5): n = 1 UGT1A1 c.238_239insGTAC mutation in exon 1: n = 1 Unknown: n = 12 |

SD = standard deviation.

identified was the *UGT1A1* c.222C>A Y74TER mutation in exon 1 often found in the Mennonite and Amish populations of Lancaster County, Pennsylvania. Within the analyzed cohort, there were 4 sibling relationships, 3 sibling pairs, and 1 sibling trio.

Histology

Nine of 22 (41%) explant livers of patients with CNJ demonstrated significant fibrosis (stage 2 or higher pericentral and/or stage 3 or higher periportal fibrosis). Both pericentral and portal fibrosis patterns were established with varying phenotypic manifestations (Fig. 1). Five patients demonstrated primarily pericentral fibrosis while two individuals were found to have their fibrosis mostly localized to the portal tracts with maximum Ishak scores of 3 out of 6. The remaining 2 patients demonstrated both pericentral and portal fibrosis. Histology was additionally graded for degree of cholestasis by assessing the pericentral grade as well as documentation of portal and hilar bile plugs (Fig. 2). Canalicular cholestasis to some extent was the usual feature in all patients, that is, scores 1 to 3+, irrespective of degree of fibrosis. Additional findings such as steatosis, hepatitis (all were postsurgical and hence “surgical hepatitis”), ductular reaction, and cytokeratin staining patterns are reported (Table 2).

Disease Phenotype

Aggregate phenotypic characteristics of the CNJ cohort are presented (Table 3). As expected, total bilirubin was elevated in all individuals, which was accompanied by a low direct/conjugated bilirubin consistent with their underlying diagnosis of CNJ. Interestingly, subtle evidence of hepatocellular unrest was present in the cohort with both ALT (mean 87.4 IU/L) and AST (mean 54.6 IU/L) elevations appreciated. There was no biochemical evidence of synthetic liver dysfunction or portal hypertension whenever assessing the cohort as a whole.

To further investigate possible associations between the findings of explant fibrosis and biochemical and/or historical data, additional analyses were performed (Table 3). Remarkably, liver explants with fibrosis tended to come from older individuals (16.1 vs 10.5 years, $P=0.02$) suggesting an accumulative injury. Although alkaline phosphatase differences were found to be significant, higher levels were found in the nonfibrotic, younger group (191.5 vs 122.1, $P=0.02$) suggesting that the elevations were more related to bone growth and development and not hepatobiliary in origin. This is further supported by lack of significance in GGT, which is a marker more often used in children and adolescents to determine biliary injury and cholestasis. No additional markers of hepatobiliary injury or synthetic function could differentiate between the fibrosis and no fibrosis groups. There did not appear to be a confounding effect from excess weight as BMI percentiles were not different between the two groups and average BMIs were within the normal range for both groups (19.2 vs 22.8). A history of gallstone disease or cholecystectomy did not differentiate between those with fibrosis and those without ($P=0.2$, Table 3).

Differences in evidence of portal hypertension, a common complication of hepatic fibrosis, were also absent between the 2 groups. Neither platelet count, white blood cell count, nor the calculated AST-to-platelet ratio index (APRI) score, which has demonstrated efficacy in assessing for hepatic fibrosis in children with liver disease (14,15), were measurably different between the two groups. Splenomegaly, as a clinical indicator of portal hypertension, was not noted on any patient physical exams. Retrospective reviews of abdominal imaging performed within 3 months of transplant reported an enlarged spleen on 3 patients, 2 of whom were noted to have fibrosis on their explant livers. Laboratory and clinical findings were not concerning for portal hypertension in any of these 3 subjects. Finally, the Fibrosis-4 index (FIB-4), a biomarker-based screening tool that has shown efficacy in predicting histology (16,17), did not correlate with the presence of fibrosis seen in the explants (Table 3).

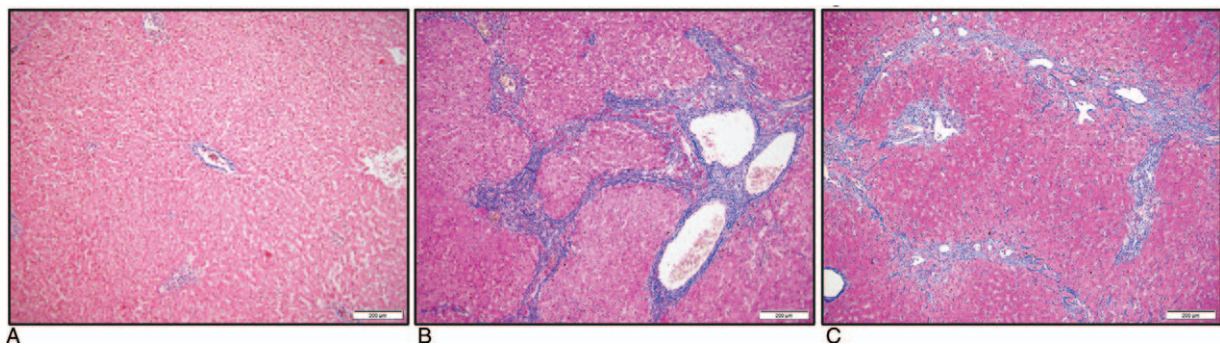


FIGURE 1. Spectrum of fibrosis in Crigler-Najjar Type I. Representative Masson Trichrome stains on liver explants in Crigler-Najjar type I. (A) Subject 12 (Table 2) with normal histology (absent fibrosis with 1+ cholestasis) classically described in patients. (B) Pericentral fibrosis with early central-central bridging from subject 19. (C) Portal fibrosis with portal-portal bridging from subject 14 (Masson Trichrome stain $\times 40$).

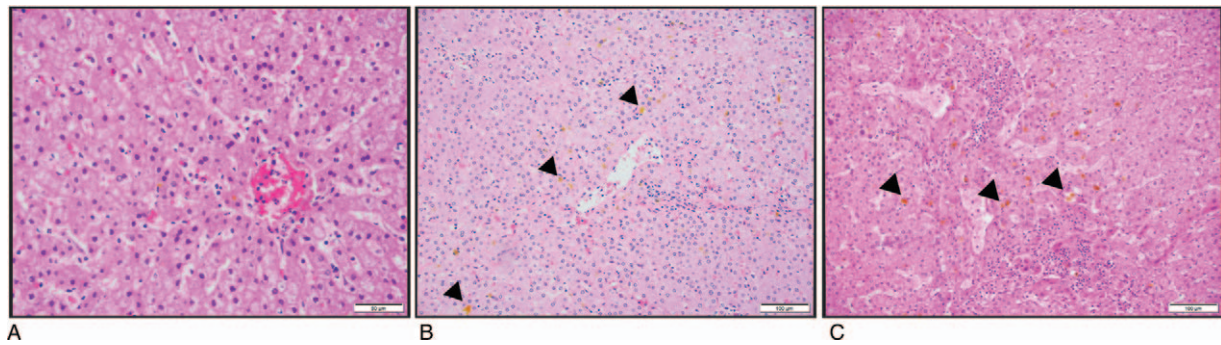


FIGURE 2. Spectrum of cholestasis in Crigler-Najjar Type I. Hematoxylin-eosin stains on liver explants in Crigler-Najjar type I. (A) Mild cholestasis classically described in patients from subject 12. (B and C) Centrilobular cholestasis with canalicular bile plugs (arrow heads) from subjects 14 and 19 (H&E ×100).

TABLE 2. Individual clinical features and histological findings in Crigler-Najjar type I

| Subject ID | Age (years) | Hepatobiliary markers | | | | Histology | | | | | | |
|------------|-------------|-----------------------|-------------------------|------------|-----------------------------|-------------------|--------|-------|----------------|---------|-----------------------|---|
| | | ALT (IU/L) | Total bilirubin (mg/dL) | GGT (IU/L) | Alkaline phosphatase (IU/L) | Cholestasis grade | | | Fibrosis stage | | CK7 ductular reaction | Others |
| | | | | | | Pericentral | Portal | Hilar | Pericentral* | Portal† | | |
| 1 | 19 | 114 | 26 | 61 | 123 | 2+ | 1 | 2+ | 1+ | 2 | 0 | Surgical hepatitis; centrilobular swelling |
| 2 | 11 | 63 | 16.5 | 19 | 249 | 1+ | 0 | 0 | 1+ | 2 | 1+ | mild accentuation of nodularity |
| 3 | 8 | 62 | 27.7 | 14 | 244 | 1+ | 1+ | 2+ | 0 | 0 | 0 | surgical hepatitis |
| 4 | 12 | 32 | 21.1 | 96 | 297 | 1+ | 0 | 0 | 0 | 1 | 0 | |
| 5 | 8 | 124 | 16.8 | 21 | 189 | 2+ | 1+ | 0 | 0 | 1 | 1+ | Rare fat |
| 6 | 16 | 30 | 14.7 | 25 | 119 | 1+ | 1+ | 0 | 0 | 0 | 0 | |
| 7 | 18 | 133 | 17.6 | 55 | 93 | 2+ | 0 | 0 | 1+ | 0 | 1+ | CK7 ectopic pericentral staining 2+ |
| 8 | 9 | 39 | 25.1 | 8 | 199 | 2+ | 2+ | 1+ | 0 | 2 | 1+ | |
| 9 | 3 | 162 | 11.1 | 13 | 171 | 2+ | 2+ | 0 | 0 | 0 | 1+ | |
| 10 | 14 | 72 | 20.8 | 36 | 267 | 2+ | 0 | 2+ | 0 | 0 | 1+ | |
| 11 | 4 | 68 | 8.3 | 11 | 177 | 1+ | 1+ | 0 | 1+ | 0 | 2+ | |
| 12 | 2 | 79 | 8.4 | 10 | 204 | 1+ | 0 | 0 | 0 | 0 | 2+ | CK7 ectopic pericentral staining 1 to 2+ |
| 13 | 13 | 107 | 13.2 | 19 | 158 | 1+ | 0 | 0 | 1+ | 2 | 2+ | CK7 ectopic pericentral staining 2+; few portal macrophages |
| 14 | 20 | 50 | 25.7 | 20 | 68 | 2+ | 0 | 0 | 2+ | 3 | 0 | Prominent veins and lymphatics; collaterals+ |
| 15 | 14 | 170 | 20.8 | 18 | 92 | 2+ | 0 | 0 | 0 | 3 | 0 | Glycogenation of nuclei |
| 16 | 16 | 282 | 22.1 | 30 | 129 | 2+ | 1 | 0 | 2+ | 1 | 1+ | Central ballooning; portal ceroid |
| 17 | 10 | 71 | 15.4 | 38 | 262 | 1+ | 0 | 0 | 2+ | 1 | 0 | Zone 3 steatosis mild |
| 18 | 12 | 59 | 17.9 | 26 | 207 | 2+ | 0 | 0 | 2+ | 2 | 2+ | CK7 ectopic pericentral staining pericentral 2+ |
| 19 | 23 | 34 | 21.7 | 32 | 50 | 2+ | 1+ | 0 | 2+ | 1 | 0 | portal macrophages |
| 20 | 16 | 73 | 24.1 | 90 | 85 | 2+ | 1+ | 3+ | 1+ | 3 | 1+ | CK7 ectopic pericentral staining 1-2+ |
| 21 | 20 | 60 | 19.6 | 42 | 68 | 2+ | 0 | 0 | 2 | 1 | 2+ | Sinusoidal dilatation; CK7 ectopic pericentral staining 2+ |
| 22 | 14 | 39 | 19.4 | 27 | 138 | 2+ | 1+ | 3+ | 3+ | 3 | 2+ | CK7 ectopic pericentral staining 2+ |

Case numbers 14 to 22 represent fibrosis cohort. ALT = alanine transaminase; CK7 = cytokeratin 7.

*Scales 0 to 3.

†Ishak scale 0 to 6.

TABLE 3. Crigler-Najjar type I disease phenotype*

| | All (n = 22) | No fibrosis (n = 13) | Fibrosis (n = 9) | P value** |
|--|--------------|----------------------|------------------|-----------|
| Demographics | | | | |
| Age at transplant, years | 12.8 (5.6) | 10.5 (5.5) | 16.1 (4.3) | 0.02 |
| Percent women | 64 | 54 | 78 | |
| BMI percentile | 51.8 (24.1) | 49.1 (27.5) | 55.8 (19.1) | 0.5 |
| Hepatobiliary markers | | | | |
| Total bilirubin | 18.8 (5.5) | 17.5 (6.4) | 20.7 (3.1) | 0.2 |
| Direct/conjugated bilirubin | 0.9 (1.7) | 1.1 (2) | 0.3 (0.1) | 0.5 |
| ALT | 87.4 (59.4) | 83.4 (41.3) | 93.1 (81.4) | 0.7 |
| AST | 54.6 (23.3) | 57.3 (20.6) | 50.7 (27.5) | 0.5 |
| GGT | 32.3 (24) | 29.8 (26) | 35.9 (21.7) | 0.6 |
| Alkaline phosphatase | 163.1 (72.6) | 191.5 (60.9) | 122.1 (71.1) | 0.02 |
| Synthetic liver function markers | | | | |
| INR | 1.03 (0.07) | 1.05 (0.07) | 1.02 (0.07) | 0.4 |
| Albumin | 4.2 (0.3) | 4.2 (0.3) | 4.2 (0.2) | 0.6 |
| Portal hypertension markers | | | | |
| Platelets | 275.5 (82.7) | 285.2 (78.2) | 261.6 (91.7) | 0.6 |
| WBC | 7.4 (1.8) | 7.9 (2) | 6.6 (1.3) | 0.12 |
| APRI score | 0.4 (0.2) | 0.4 (0.2) | 0.4 (0.2) | 0.8 |
| History of gallstone/cholecystectomy*** | | | | |
| Percent yes | 62 | 50 | 78 | 0.2 |
| Fibrosis-4 (FIB-4) Index | 0.32 (0.2) | 0.27 (0.2) | 0.39 (0.2) | 0.19 |

ALT = alanine transaminase; APRI = AST-to-platelet ratio index; AST = aspartate transaminase.

*Data expressed as mean (standard deviation).

**P values representing differences between 'fibrosis' and 'no fibrosis' cohorts.

***Data missing on one patient in the 'No Fibrosis' group.

DISCUSSION

Here, we report for the first time the findings of significant fibrosis present in hepatic explants of patients with CNI. CNI has been considered a liver-based genetic disorder characterized by a structurally normal liver with preserved synthetic function in which the primary source of morbidity and mortality was extrahepatic bilirubin deposition in the central nervous system with accompanying neurological and behavioral complications. In our cohort of 22 older patients with CNI, however, we found a 41% prevalence of histological fibrosis discovered in the explants at the time of liver transplantation. Fibrosis patterns were both portal, pericentral, and mixed. Individuals with fibrosis were notably older than those without fibrosis suggesting that the injury may be incrementally acquired. All biochemical and clinical indicators of fibrosis that were evaluated, however, were not able to differentiate those with fibrosis portending a clinically silent injury that may go undetected without histological assessment. These findings, along with the inability to noninvasively determine, which individuals may have ongoing structural hepatic damage, is of importance whenever considering future treatment options for CNI such as hepatocyte transplantation (HT) and gene therapy.

HT has for some time been a promising potential alternative to orthotopic liver transplantation (OLT) expanding the therapeutic approach to a collection of liver diseases. HT is suggested to enable the replacement of a critical mass of metabolically normal cells with a functioning gene to support appropriate metabolic processes. CNI has been considered to be an ideal condition for HT, given that the hepatic scaffolding and microenvironment are presumed to be preserved. This is reflected by the fact that CNI has been the most common indication to pursue HT in patients with metabolic liver diseases (18,19). However, only partial metabolic correction has been achieved and HT has not shown to reliably circumvent the need for traditional organ transplant in CNI (12,20,21). Several

barriers have prevented the broader application of HT. Chief among these is sub-optimal engraftment, the process whereby transplanted hepatocytes translocate from the sinusoidal space into the recipient liver plates following disruption of the sinusoidal endothelium and attain integration into the host liver parenchyma (22). Until hepatocytes traverse the endothelium, they remain vulnerable to rapid immunological clearance and any fibrosis will impede this process. HT in animal studies with liver fibrosis demonstrated no benefits in outcomes related to hepatic function, and liver fibrosis did not improve (23). Similarly, poor outcomes have been observed in human-based studies of HT in the setting of established cirrhosis (24). Our findings suggest that the development of fibrosis in CNI patients, may compound the difficulties in achieving effective outcomes following HT. They further suggest that such interventions are more likely to be effective in younger children with CNI, before the development of fibrosis.

Auxiliary liver transplantation has been successfully used in CNI patients on the assumption that the native liver is structurally normal and hence can be utilized if gene therapy becomes available in the future, whereupon immunosuppression could be withdrawn (25,26). Auxiliary liver transplant is a technically demanding procedure, which will be difficult, or impossible, if advanced fibrosis or portal hypertension develops. Our findings suggest that auxiliary transplantation may not be feasible in some older children with CNI and emphasize the importance of obtaining a preoperative liver biopsy wherever auxiliary liver transplantation is being considered in CNI.

Liver-directed viral gene replacement therapy for CNI remains an important goal. Viral vector-mediated gene therapy exploits a virus's proficient genetic delivery machinery in order to transfer identified therapeutic genes to treat monogenetic diseases (27). Several viral vectors, including retrovirus, adenovirus, and adeno-associated virus have demonstrated efficacy in delivering targeted genetic therapy, and clinical trials are currently

investigating the utility of this therapy in several monogenetic inheritable diseases (28–30). In CN, in vivo animal studies have demonstrated success of viral gene therapy in the management of both hyperbilirubinemia (31–33) and bilirubin-induced neurological complications (34). Hepatic fibrosis and cirrhosis, however, have been shown to decrease the efficacy of gene transduction in some models (35). Again, our findings suggest that the severity of hepatic fibrosis needs to be considered whenever new interventions are being evaluated in CNI.

The animal models used to study CN have enabled incredible advancements in the understanding and treatment of CN. The Gunn rat model for CN, originally described in 1934, has been extensively studied leading to a better understanding both pathophysiology and potential therapeutic intervention. However, given the observations that only minimal neurological complications develop in these rats, a recent murine knockout model that produces severe bilirubin-induced neuropathy has also been established to advance the study of CN (34). As is often the case, these animal models likely fail to completely recapitulate human disease. In contrast to patients with CN where the disease process has persisted for decades, animal studies occur over much shorter periods, often only weeks to months. Hepatic architecture in these models are uniformly described as normal at up to 2 years of age (33,34).

The etiopathogenesis of fibrosis in CNI is currently unknown. One potential mechanism includes chronic low-grade biliary obstruction secondary to cholelithiasis. Mutations in the *UGT1A1* gene have been associated with gallstone formation (36,37). The resultant cholangiopathy can cause obstruction with ductular reactivity, which has been linked to fibrogenesis and the development of portal fibrosis (38). Ductular reaction (CK7) was reported in 68% of the patients (15/22), suggesting acute or chronic cholestasis. This factor did not discriminate between the fibrosis and nonfibrosis groups, nor did GGT levels, or a history of gallstone disease. Together with the fact that mainly pericentral instead of portal fibrosis was found suggests that (micro)cholelithiasis is unlikely to play a major role in the formation of fibrosis in this cohort. A second prospective contributor to the development of fibrosis is toxicity-related heme degradation molecules including bilirubin as well as higher order products. Although the major morbidity of unconjugated hyperbilirubinemia is in the central nervous system, extraneural pathology has been reported. Unconjugated bilirubin can trigger apoptosis in erythrocytes, smooth muscle, and murine hepatoma cells, and blocks ATP production in renal tissue (39–43). The potential hepatotoxicity of unique serum bilirubin photoisomerization products found in CNI patients treated with phototherapy (44) has not been investigated. Recent work, however, has shown that higher order heme degradation products, such as Z-BOX A and B, can impair liver function and integrity and lays the foundation for future studies to investigate the effect of such products in patients with CNI (45,46). Alternatively, how hepatic handling of various drugs metabolized by glucuronidation may be affected in patients with CNI with resultant drug-induced liver injury is not known. Specific *UGT1A1* mutations may be more susceptible to injury with the development of fibrosis (47). Ultimately, how these potential causes, or other to be identified factors, contribute to the development of fibrosis in CNI will need to be explored.

Our study was limited by its size and retrospective nature. The clear majority of our cohort came from the Plain communities of Pennsylvania, including the Amish and Mennonite populations. A second confounding genetic predisposition could affect our findings and future studies in other more genetically diverse populations are needed. Additionally, many patients did not receive the whole of their pretransplant care at our institution, restricting the amount of clinical data that was available for review. We are not

able to report on prescribed therapies, such as potential hepatotoxic medications, or neurological complications that had been accrued at the time of transplant because of the retrospective nature of this study. Our population was also limited to those managed in a pediatric center. As the patients with fibrosis were significantly older, this could be a progressive injury that may be even more prominent in an adult population. Lastly, noninvasive determinations of liver fibrosis, such as elastography, were not assessed in our population because of the retrospective nature and prior limited availability of this technology. Prospective evaluations are underway to determine the usefulness of these technologies in patients with CNI. Additionally, future work will investigate the posttransplant courses of these patients, focusing on graft and patient survival, posttransplant complications, neurocognitive, as well as quality of life assessments in this populations. Sub-analyses to distinguish differences between patients with and without fibrosis are planned.

In summary, we report for the first-time hepatic parenchymal distortions in older patients with CNI. Our assessment of explanted livers from patients diagnosed with CNI receiving liver transplant suggests that fibrosis development is common and linked to age. Importantly, the injury appeared clinically silent with no identifiable differences in biochemical markers or physical examination findings to suggest ongoing organ damage. These findings suggest that earlier intervention may be warranted whenever looking to optimize the therapeutic benefit of alternative treatment approaches such as hepatocyte transplant, auxiliary liver transplant, or viral gene therapy.

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