Observational Study of Children with

Acute Liver Failure in North America

(Cohort Study)

Abstract

Aims

- Primary Aim

    o To comprehensively characterize Pediatric Acute Liver Failure (PALF) patients utilizing serially collected traditional clinical, biochemical, diagnostic, and management profiles supplemented by immune, inflammatory, neural injury and liver regeneration markers. We do so to identify the dynamic factors that explain variations in outcomes. Outcomes include survival and liver transplantation (LT) up to 12 months after enrollment with health-related quality of life (HRQOL) and a screening of neurocognitive function at 6 months and a more comprehensive assessment of neurocognitive function, HRQOL, depression, and post-traumatic stress disorder (PTSD) at 12 months after enrollment. A specimen bank comprised of serum, DNA, lymphocytes, and liver tissue obtained from children with PALF to permit the conduct of various ancillary studies will be continued.

- Secondary Aims

    o To develop a more comprehensive risk stratification for children with PALF utilizing not only traditional clinical and biochemical profiles but also dynamic assessment of immune, inflammatory and liver regeneration markers.

    o To develop an integrated, mechanism-based, and data-driven decision analysis strategy that will improve clinical decision-making for individual children, and estimate the impact of improved decision-making on organ allocation for both adults and children.

    o To link the complex PALF phenotype to short-term (8 week), medium-term (6 months) and long-term (12 months) patient outcomes and integrate these analytes into mechanism-based and data-driven models to develop reliable *in silico* analogs that will be used to predict long-term clinical and neurocognitive outcomes in PALF patients.

Type of Study

- Prospective observational

Cohort Study

Approximately 300 patients will be enrolled over 54 months. Children will be followed for up to 12 months following entry into the Cohort Study.

    o **Inclusion Criteria**
      - Written informed consent/assent
      - Birth through 17 years of age
      - Biochemical evidence of acute liver injury at the time of enrollment
      - Coagulopathy not corrected by vitamin K (or other intervention intended to correct coagulopathy)
          o The presence of encephalopathy (ENC) is required if the INR is at least 1.5 and less than 2.0
          o If INR is at least 2.0, the presence of ENC is not required

    o **Exclusion Criteria**
      - Known chronic underlying liver disease
      - Multi-organ system failure following heart surgery or ECMO
      - Solid organ or bone marrow transplantation
      - Acute trauma
      - Previously enrolled in the PALF Cohort Study
      - Other severe illness, condition, or other reason in the opinion of the investigator that would make the patient unsuitable for the study

Children with acute liver failure will be identified within the sites participating in the NIH-funded Pediatric Acute Liver Failure (PALF) network. Patients with appropriately secured consent from a parent or guardian will have results of prior clinical, biochemical and diagnostic studies from the referral institution and the clinical site recorded in a de-identified case report form. To capture the dynamic nature of PALF, daily results of clinical, physiological, and management parameters and treatment interventions during the hospitalization for the episode of PALF will be recorded until discharge from the hospital with native liver, death, or liver transplantation. In addition, serum will be obtained at intervals to assess markers of inflammation, liver regeneration, NK cell function, and central nervous system injury. Surviving patients will be assessed at 8 weeks, 6 months and 12 months following entry into the study for clinical status and age-appropriate neurocognitive testing.

The following outcomes will be noted:

- o Death
- o Liver transplantation
- o Health related quality of life and neurocognitive status at 6 months and 12 months after entry into study
- o Hepatobiliary injury or dysfunction that persists or recurs
- o Medical complications
    - o Renal dysfunction
    - o Cytopenia (low white blood cell count, low hemoglobin, low platelet count)

1. Background and Rationale

1.1. Historical Background

The Pediatric Acute Liver Failure Study Group (PALFSG) began as an ancillary study within the Adult Liver Failure Study Group (ALFSG) from 2000 through 2005 and obtained independent funding for the period from September 2005 through August 2010. The PALFSG created and maintained a registry of 986 patients with extensive data collected during routine clinical care and also created a biosample repository to provide information to enable characterization of the unique features of PALF. The PALFSG also completed a randomized, doubly-masked, placebo controlled trial utilizing intravenous N-acetylcysteine (NAC) for non-acetaminophen PALF that included 184 children.

The PALFSG initially included 24 sites (21 in the United States, 2 in the United Kingdom, and 1 in Canada) with established expertise in pediatric liver disease and transplantation. The current clinical network consists of sites in North America in addition to the Data Coordinating Center at the University of Pittsburgh.

1.2. Scientific Background

1.2.1. Introduction

Acute Liver Failure (ALF) is not a single entity, but rather a clinical syndrome that encompasses a host of conditions many of which are as yet unidentified in children. While hepatic encephalopathy is a required element of adults with ALF, it is now acknowledged that hepatic encephalopathy in children is difficult to assess and may not be clinically apparent until the terminal stages of the disease process (1). Thus, recent reports of PALF have included children without clinical encephalopathy (2-3).

Inclusion criteria for the PALF registry were (1) children with no known evidence of chronic liver disease, (2) biochemical evidence of acute liver injury and (3) coagulopathy not corrected by vitamin K. The presence of encephalopathy (ENC) was required if the prothrombin time (PT) was between 15-19.9 seconds or the INR between 1.5-1.9. If, however, the PT was at least 20 seconds or INR ≥ 2.0, patients were enrolled with or without ENC.

1.2.2. Epidemiology

Acute liver failure in children differs from that observed in adults in many aspects including presentation, etiology and outcome (4). Etiologies vary by age with metabolic and infectious diseases prominent in the first year of life and acetaminophen overdose and Wilson's disease occurring in adolescents. Overall, 50% of the pediatric cases are of indeterminate etiology, with 61% of children between 1-10 years of age having an indeterminate diagnosis. Short term outcomes are better in children than adults, but are dependent upon the degree of encephalopathy and diagnosis. Acetaminophen (APAP) causes hepatotoxic injury either by acute intentional ingestion or inadvertent therapeutic misadventures. Occult APAP toxicity may occur more commonly than expected and may exacerbate liver injury of other etiologies, especially in younger children (5-6). Autoimmune hepatitis, a potentially treatable cause of ALF in children of all ages, should be considered early in the evaluation process to enable prompt initiation of treatment (7-8). ALF may be the initial symptom associated with metabolic defects related to carbohydrate, fatty acid and protein metabolism as well as enzyme defects within mitochondria and peroxisomes with these defects more commonly identified in infancy (9-12). Medication induced ALF, not associated with APAP, is difficult to identify in some cases, or may be falsely implicated in patients with occult metabolic defects (13-16). For instance, investigators now recognize that some children with ALF related to valproate toxicity actually have an underlying mitochondrial disorder which is the primary cause of their liver injury (17-18). Of significance, recognition of unrecognized underlying mechanisms of liver injury such as immune dysregulation, metabolic disorders, and chronic APAP exposure will identify patients who may be amenable to targeted treatment strategies.

1.2.3. PALF as a Dynamic Clinical Condition

As yet there are no tools to reliably predict whether children with ALF will survive or not. Biochemical tests (lactate, total bilirubin, phosphorous, INR, prothrombin time, ammonia, Gc-globulin), clinical features (encephalopathy, cerebral edema), diagnosis (acetaminophen) or combinations of the three have been tried without much success (19-29). Liver transplantation decisions including the risks associated with living organ donation are made difficult given the uncertainty of patient outcome. Without good markers of survival, considerable clinical judgment must be used to make these important decisions. Early identification of children who will survive without transplantation and those who will likely die without it would be a tremendous advance to a field strained by an insufficient number of organs to satisfy patient needs.

1.2.4. Sequelae

Historically, various outcomes have been measured in children with PALF. Survival and liver transplantation are the more commonly determined, but patient physical, hematologic, renal, mental function, and quality of life are also critical measures.

1.2.4.1. Clinical and Neurocognitive Outcomes

1.2.4.1.1. Markers of Liver and Clinical Disease Severity in the Setting of Acute Liver Failure

The initial hepatic insult frequently extends to multi-system organ dysfunction and failure (30). As true measures of disease severity are dynamic, serial measurements of biomarkers that assess hepatocellular function, cellular injury, and vascular integrity may more reliably predict outcome. Metabolic (19, 23), infectious (31-32), hematologic (33-36), and neurological (37) complications impact outcome as well. All but one of the disease severity scores in the setting of PALF have not been validated (19-24, 31, 38-45), The Liver Injury Unit (LIU) score was validated with a population from the same institution (46), and validation within the larger PALF cohort is currently in progress. Components of these various severity scores could be used as components of a predictive model.

1.2.4.1.2. Neurocognitive Outcomes and Health Related Quality of Life

Patients that develop acute liver failure experience varying levels of hepatic encephalopathy and cerebral edema as an important aspect of their disease progression. Signs of encephalopathy typically resolve following liver transplantation or spontaneous resolution of the hepatic injury. However, there may be residual sub-clinical neurological injury that compromises long-term neurocognitive function in this group of children. Detailed neurocognitive testing has never been performed in a cohort of children that survive acute liver failure and this study seeks to close that information gap by defining the spectrum of neurocognitive outcomes in this population. Outcomes research in pediatric LT recognizes that "excellent outcomes" must extend beyond mortality and morbidity (47). PALF patients who undergo LT have cognitive impairment that interferes with normal day-to-day function, though it remains unknown whether this impairment results from pre- or post-LT events or medications (48). Survivors without LT are likely at increased risk for similar outcomes, but medical follow-up after discharge is inconsistent. Thus, cognitive deficits and emotional disturbances may be largely unrecognized and under-reported. Likewise, health related quality of life may be lower than expected as compared to healthy children or survivors of liver transplantation with other primary diagnoses. Detailed assessment of neurocognitive function and HRQOL in survivors is an important aspect of understanding the full impact of acute liver failure in children.

1.2.5. Biomarkers that Could Be Used to Predict Clinical Outcomes

Our over-arching hypothesis is that the interaction of cellular injury, the inflammatory response, and recovery drives outcome in children with acute liver failure, particularly those with an indeterminate cause. We believe that further investigation will provide an opportunity to not only identify populations of patients who may respond to immunomodulatory therapy (i.e., prednisone), but also provide insight into the outcomes of children who undergo liver transplantation and intense immunosuppression when they are transplanted within either a "hyper- or hypo-inflammatory" state.

1.2.5.1. Markers of Liver Regeneration

All cases of acute and chronic liver disease are dependent at least in part on the ability of the liver to regenerate if survival and recovery is to occur (49). Moreover, impairment of the hepatic regenerative response likely contributes to complications of liver disease such as cirrhosis, chronic failure, cancer and perhaps primary graft failure following liver transplantation. Children with PALF are no exception. Indeed, low rates of spontaneous recovery in adults with idiopathic and drug-induced ALF have been attributed, in part, to diminished capacity for regeneration (50). Biological markers of liver regeneration would be particularly useful in the evaluation and management of patients with ALF.

1.2.5.2. Markers of Brain Injury

Non-invasive measurements of neurological function that may predict long-term neurological outcome have been a goal for many years. Detection of brain-specific proteins in serum, urine or cerebrospinal fluid can suggest that a brain injury has occurred. The theoretical concept behind this argument is that cell damage within the central nervous system (CNS) causes widespread release of these structural proteins, ultimately being metabolized within the body due to leakage of the blood-brain barrier or some other mechanism. Of the putative neurological markers, S100β, neuron specific enolase (NSE), and glial fibrillary acidic protein (GFAP)

are the most commonly used. S100β is increased after cardiac arrest either unprovoked (51) or during pacemaker placement (52), traumatic brain injury inflicted (53-54) or unintentional (54-57), cardiac bypass (58-59) and birth asphyxia (60-61). NSE, a glycolytic enzyme localized within neurons, is another marker of neurological injury in trauma (55-56), cardiac arrest (62-63) and other neurological disorders (54). The evidence for linking these markers to ultimate neurological outcome is increasing in children with traumatic brain injury (64) and after cardiac arrest (65). An alteration in GFAP is associated with central nervous system injury associated with septic shock in children and experimental hepatic encephalopathy (66-68).

1.2.5.3. Inflammatory Biomarkers in PALF

We hypothesize that a detailed characterization of the dynamic inflammatory response in PALF could help with clinical decision-making and may refine inclusion/exclusion criteria for future clinical trials. Principal components of Luminex™ data on 25 cytokines obtained in serum samples have been performed in human traumatic brain injury (TBI) patients, identifying sub-groups of patients based on the principal components analysis (PCA) of cytokine data associated with different degrees of severity (69). Our preliminary results in PALF patients suggest that we may be able to develop a biomarker profile for outcomes in PALF. Moreover, we may be able to utilize data-based analyses to develop patient-specific biomarker profiles of PALF.

1.2.5.4. Markers of Immune Activation

We conducted a prospective cross-sectional study in 62 PALF patients utilizing markers of NK cell function and immune activation that include IL2R levels, Natural Killer (NK) lytic activity, Perforin **+** NK cells, Perforin **+** CD8 cells, Perforin **+** NKT cells, Granzyme **+** NK cells, Granzyme **+** CD8 cells, Granzyme **+** NKT cells, and % CD16/56 cells. We used Classification and Regression Trees (CART) to identify NK characteristics that discriminated between participants who survived without LT and those who died without LT and identified a possible patient phenotype that predicted survival (normal sIL2R) and one in which 50% of patients died (elevated sIL2R, decreased NKT perforin cells, normal NK perforin, and without decreased %CD16/56 cells). These data may also afford us the ability to identify a patient cohort, or cluster, which might respond to immunomodulation (e.g., steroids) and design a Phase 2 open label protocol to study their safety in this patient cohort. Delineating patient clusters will also serve as the foundation for physiologic modeling.

1.2.6. Decision Analysis

1.2.6.1. Liver Transplantation Decisions

Developing models that integrate the effect of individual decisions on allocation is crucial to making optimal decisions in individual children because the decisions are dependent upon one another. Therefore, a model that aggregates multiple transplantation-related decisions into a representation of the entire US organ allocation system can estimate the overall effects of changing individual treatment decisions on overall transplantation outcomes. This is exactly the reason that the Organ Procurement and Transplantation Network (OPTN) has moved to using this type of simulation for the evaluation of transplant allocation policy changes (70). We (Roberts) have participated in the development and evaluation of those policy decision models as a member of the Technical Advisory committee to the Scientific Registry of Transplant Recipients (SRTR).

1.3. Summary

We propose trans-disciplinary collaboration to ensure a structured diagnostic and management strategy, to monitor clinical, biochemical, and physiological parameters, and to collect markers of the immune and inflammatory milieu, neurological injury and liver regeneration. We will associate these parameters with survival and neurocognitive outcomes. To accomplish these tasks requires a strong infrastructure for multi-center, patient-based research, clinical expertise and translational expertise related to our emerging understanding of the pathobiology of PALF. If successful, this approach will change the paradigm of research and patient management in PALF by: 1) improving mechanistic understanding of etiology, progression, and prognosis; and 2) identifying sub-cohorts amenable to directed therapy.

2. Aims

- Primary Aim
    - o To comprehensively characterize PALF patients utilizing serially collected traditional clinical, biochemical, diagnostic, and management profiles supplemented by immune, inflammatory, neural injury and liver regeneration markers. We do so to identify the dynamic factors that

explain variations in outcomes. Outcomes include survival and liver transplantation (LT) at 8 weeks, 6 months, and 12 months after enrollment with health-related quality of life (HRQOL) and a screening of neurocognitive function at 6 months and a more comprehensive assessment of neurocognitive function, HRQOL, depression, and post-traumatic stress disorder (PTSD) at 12 months after enrollment. A specimen bank comprised of serum, DNA, lymphocytes, and liver tissue obtained from children with PALF to permit the conduct of various ancillary studies will be continued.

- Secondary Aims

  o To develop a more comprehensive risk stratification for children with PALF utilizing not only traditional clinical and biochemical profiles but also dynamic assessment of immune, inflammatory and liver regeneration markers.

  o To develop an integrated, mechanism-based, and data-driven decision analysis strategy that will improve clinical decision-making for individual children and estimate the impact of improved decision-making on organ allocation for both adults and children.

  o To link the complex PALF phenotype to short-term (8 week), medium-term (6 months) and long-term (12 months) patient outcomes and integrate these analytes into mechanism-based and data-driven models to develop reliable *in silico* analogs that will be used to predict long-term clinical, health-related quality of life and neurocognitive outcomes in PALF patients.

To meet these aims, we will collect information about all children who are enrolled concerning:

1. Demographics
2. Diagnosis
3. Liver disease onset and severity
4. Management and treatment interventions including LT
5. Other organ dysfunction
6. Serial biomarkers
7. Quality of Life measures
8. Neurocognitive measures
9. Outcome

3. Inclusion and Exclusion Criteria

Inclusion Criteria:

1. Written informed consent/assent
2. Birth through 17 years of age
3. Biochemical evidence of acute liver injury
4. Coagulopathy not corrected by vitamin K (or other intervention intended to correct coagulopathy)
   - o The presence of encephalopathy (ENC) is required if the INR is at least 1.5 and less than 2.0
   - o If INR is at least 2.0, the presence of ENC is not required

Exclusion Criteria:

1. Known chronic underlying liver disease
2. Multi-organ system failure following heart surgery or ECMO
3. Solid organ or bone marrow transplantation
4. Acute trauma
5. Previously enrolled in the PALF Cohort Study
6. Other severe illness, condition, or other reason in the opinion of the investigator that would make the patient unsuitable for the study

4. Data Collection

Components of validated clinical and liver disease severity scores as well as markers of central nervous system injury, inflammatory milieu, end-organ damage, immune activation, and potential for liver regeneration will be included. As the clinical course is dynamic over time, these parameters will be measured serially. These data will be used to generate both data-driven and mechanistic computational models.

These data will be included in the research database when the information is available through clinical care. The standard of clinical care in management of children with PALF involves, at a minimum, daily assessment of clinical and biochemical status. We recognize that there may be circumstances in which specific elements may not be able to be obtained each and every day. In a research database, each data element would be required by protocol and a missing data element would be a protocol violation. However, this approach would also put significant restrictions upon blood volumes drawn for research purposes in a dynamic clinical condition such as PALF. Therefore, the investigators have agreed to capture a minimal daily monitoring dataset that reflects best clinical practice. Missing data elements will be recorded for data quality purposes. However, the absence of an expected data element will not be considered a protocol violation. The minimal daily monitoring assessments considered to reflect best practice are identified in the manual of operations.

Data may be used by secondary investigators if sponsored by a member of the PALF Study Group and if the investigators have a protocol approved by the PALF Steering Committee.

4.1. Screening

A Screening Log will be kept at each site for each potentially eligible participant. The screening log will capture basic demographic information (age, sex, race, and ethnicity) and initial diagnosis. An enrollment criteria form will also be completed to determine inclusion/exclusion criteria and the date that consent was obtained or refused. If a parent or guardian was approached for consent but consent was not obtained, the date offered and reason not obtained will be recorded. De-identified data will be entered into the study database so that a summary of those screened can be generated to monitor enrollment.

4.2. Laboratory and Diagnostic Tests Prior to Enrollment

Results of laboratory and diagnostic tests obtained prior to study entry will be recorded on enrolled patients. These results may impact the prioritization of subsequent testing at the PALF site as the PALF investigator may not repeat certain diagnostic tests found to be negative or positive prior to study enrollment.

4.3. Data Collection after PALF Enrollment, During the Initial Hospitalization

4.3.1. Demographics

The date of birth, sex, race, and ethnicity will be collected.

4.3.2. History

Presence of systemic features that include fever, vomiting, diarrhea, altered mental status, seizures, jaundice, the date jaundice was first noted, and exposures to potentially hepatotoxic substances such as mushrooms. A list of medications taken by the child within one month prior to study entry will be recorded.

4.3.3. Physical Examination

Neurological assessment and mental status features at entry into the study and then daily during the hospitalization for ALF until the INR falls below 2.0 in a patient without ENC or below 1.5 in a patient with ENC, discharge from the hospital with their native liver, death, or liver transplantation. An increase in the INR to ≥ 1.5 coupled with ENC or an increase in the INR to $\geq$ 2.0 without ENC during the original hospitalization for ALF will prompt re-institution of daily exam. The exam on study entry will confirm presence or absence of digital clubbing or spider angioma. The examination will include the distance (cm) the spleen extends below the left costal margin at study entry and then weekly. Heart rate, respiratory rate, blood pressure and maximum temperature will be recorded daily.

4.3.4. Laboratory Data

The frequency of specific laboratory tests used to monitor the child's clinical status will be performed according to local best practice. Available laboratory results will be recorded once daily during the hospitalization for ALF until the INR falls below 2.0 in a patient without ENC or below 1.5 in a patient with ENC, discharge from the hospital with native liver, death, or liver transplantation. An increase in the INR to ≥ 1.5 coupled with ENC or an increase in the INR to $\geq$ 2.0 without ENC during the original hospitalization for ALF will prompt re-institution of daily lab monitoring. Results from a daily routine draw will be captured according to the guidelines outlined in the manual of operations.

4.3.5. Diagnosis

We have developed a diagnostic prioritization strategy. Within specific diagnoses, we have identified diagnostic tests that impart levels of certainty that the final diagnosis is sound. These definitions should serve to increase the likelihood of identifying the likely cause for PALF in an individual patient, minimize unnecessary diagnostic

testing, and identify those patients whose diagnosis is unable to be determined based upon our current knowledge. The latter group will provide us with the best opportunity to identify causes and mechanisms of liver injury that might be amenable to medical therapy. The diagnostic prioritization strategy guidelines are documented in the manual of operations.

4.3.6. Liver Disease Severity

We will record data elements that are collected as a component of clinical care that will allow us to sequentially calculate validated disease severity scores such as Kings College Criteria (44), the Liver Injury Unit Score (41), the Glasgow Coma Score, the Pediatric Risk Mortality Score (PRISM) (23, 71), the Model for End Stage Liver Disease (MELD) (40), and the Pediatric End Stage Liver Disease (PELD). The components of these scores are listed in the manual of operations.

4.3.7. Management and Treatment Interventions

General principles of PALF management are dynamic, may differ among sites, and may change over time. Time of initiation and discontinuation of interventions, such as platelets or other blood products, plasmapheresis, and renal support therapy will be recorded to assess their impact on the dynamic features of PALF, disease severity scores, and patient outcomes. Specific therapies to prevent and treat infection and to modulate the immune system will be captured**.** If diagnostic procedures such as liver biopsy and radiological studies are obtained for clinical purposes the date will be recorded.

4.3.8. Discharge

Date of discharge and a final diagnosis will be recorded as determined by the principal investigator.

4.4. Follow-up Visits

All surviving participants, with or without LT, will have three study visits at 8 weeks, 6 months, and 12 months after study enrollment, with allowable windows for these timepoints specified in the manual of operations. Clinical and laboratory data will be recorded and research labs collected. Clinical data will include vital signs, weight, height or length, and physical evidence of chronic liver disease (e.g. splenomegaly). Laboratory data will include results of: diagnostic tests related to the cause of the episode of ALF, liver biopsy results, and new diagnoses. A list of current medications will be recorded.

In a patient who undergoes liver transplantation, additional data obtained at each study visit will include transplant re-listing, indication for re-transplant listing, solid organ (other than liver) or bone marrow transplantation, lymphoproliferative disease, or cancer. Research labs will be obtained in post-transplantation patients.

5. Biospecimen Collection

The biospecimens collected for this study are blood and liver tissue. At the time of routine blood draw during the hospitalization for acute liver failure, additional blood samples will be collected for research purposes. Samples will be processed and stored locally according to guidelines outlined in the manual of operations and then batch shipped directly to a designated testing laboratory or a NIDDK-funded repository. The biological samples will be stored in the repository indefinitely to be used for future research studies conducted by the PALF investigators or secondary investigators sponsored by a PALF investigator.

The amount of blood taken for research purposes is dependent upon the weight of the participant and the amount of blood taken for clinical purposes. Center specific IRB guidelines will be followed in regard to the maximum amount of whole blood taken during a single draw and the cumulative amount taken over the study period. When possible, the weight specific whole blood volumes identified in **Appendix A** will be obtained each day during the hospitalization for ALF along with a routine draw (as outlined in the manual of operations), until the INR falls below 2.0 in a patient without ENC or below 1.5 in a patient with ENC, discharge from the hospital with their native liver, death, or liver transplantation. An increase in the INR to ≥ 1.5 coupled with ENC or an increase in the INR to $\geq$ 2.0 without ENC during the original hospitalization for ALF will prompt re-institution of daily sample for storage. A blood sample will also be obtained for research purposes at the 8 week, 6 month, and 12 month visits on all participants.

5.1. Serum

Blood will be collected at the time of a routine blood draw during the hospitalization for acute liver failure and processed according to study guidelines. Blood obtained for research purposes at the 8 week, 6 month, and 12 month visits will be used to assess recovery from the episode of liver failure and include studies that assess liver

function, liver inflammation, renal function, and cytopenias. Serum aliquots will be frozen and stored locally and then batch shipped to a designated testing laboratory or a NIDDK-funded repository. A number of the serum aliquots are earmarked for the following research tests already integrated into the aims of this protocol: Luminex™ markers of inflammation and regeneration, markers of neural injury, and liver regenerative markers. Aliquots not needed for these specific tests will be stored indefinitely at the NIDDK repository to be used for future studies.

5.2. DNA

If consent has been provided for collecting a DNA specimen, a whole blood sample will be collected and processed according to study guidelines and sent directly to a NIDDK supported or other established laboratory where the DNA will be extracted from the blood sample. If a patient is discharged before the genetics sample is obtained the sample will be collected at the earliest possible follow-up visit.

5.3. Liver Tissue

If a liver biopsy is performed for clinical purposes, or in the event of liver transplantation or autopsy, a liver tissue sample will be obtained when available. The sample will be processed according to study guidelines, stored locally and then batch shipped to a NIDDK sponsored repository to be used for future research studies.

Samples collected at the time of autopsy will require a separate consent and a liver tissue sample will be collected only when the parent or guardian has provided consent.

5.4. Guthrie Spot for Blood

A Guthrie card will be collected at enrollment and will coincide with the collection of another blood draw. Blood will be placed on a Guthrie card according to study guidelines, stored locally, and then batch shipped to a NIDDK-funded repository to be used for future research studies.

5.5. NK Cell Analysis

Blood samples for this study will be collected within the first three days of enrollment or as soon as possible following enrollment into the PALF cohort study. A second blood sample for NK cell analysis will be obtained at least once at a follow-up visit, for those who survive without a liver transplantation and who also had NK cell function studies following study entry. The samples will be processed according to study guidelines and then sent to Cincinnati Children's Hospital Clinical Immunology Laboratory for evaluation of NK cell function. Blood for NK cell function collected during the hospitalization for ALF should be obtained prior to liver transplantation or starting immunomodulatory therapy (e.g. corticosteroid therapy).

6. Outcomes

The following outcomes will be noted:

6.1. Death

The date and cause of death will be recorded. We will identify those who underwent autopsy.

6.2. Liver Transplantation

6.2.1. Initial Listing

At the time the participant is listed for liver transplantation, the following will be recorded: first day of evaluation, initial list status, and subsequent changes to listing status. For those participants who have the option of receiving a living donor transplant, the following additional data will be recorded: first day of evaluation for each volunteer and the date the volunteer was deemed to be an acceptable donor. If the participant is removed from the LT list, date and reason(s) for removal will be recorded.

6.2.2. Liver Transplant

Data elements will include the type of transplantation, cold ischemia time, as well as donor characteristics (date and time of offer, age of donor, turn down code, turn down date, acceptance date and time, blood type of donor and recipient). We will only collect data on those donor offers which are actually made and not provisional offers.

6.3. Neurocognitive Functioning, HRQOL, PTSD, and Depression

Measures of neurocognitive function and mood will be limited to those children who are between the ages of 2 years and 16 years, 0 months, 0 days at PALF study enrollment. At the 12 month timepoint, participants must be at least 3 years but less than 17 years of age. Surveys tapping HRQOL and executive function will be completed by parents and teachers as well as participants, depending on age, at 6 months and again at 12 months. Allowable windows around these timepoints are specified in the manual of operations. Participants will also undergo neurocognitive testing at 12 months to assess IQ, visuo-motor function, attention, and executive function. In addition, participants and parents will complete depression and PTSD surveys at the 12 month visit.

> Inclusion
>   a. Subject's parent(s)/guardian(s) is fluent in English
>   b. Subject is fluent in English
>   c. Subject is at least 2 yrs, 0 months at PALF study enrollment and not more than 16 yrs, 11 months, 29 days at the 12 month timepoint (see table below).

Age at Neurocognitive Testing	PALF study enrollment	12 month follow-up
Age range - lower limit	2 yrs, 0 months, 0 days	3 yrs, 0 months, 0 days
Age range - upper limit	16 yrs, 0 months, 0 days	16 yrs, 11 months, 29 days

> Exclusion
>   a) Subject is awaiting liver transplantation
>   b) Subject has a cancer diagnosis.
>   c) Subject has been hospitalized within the past 4 weeks prior to testing.
>   d) Subject with weakness or abnormality of muscle tone or coordination, such as cerebral palsy, sufficiently severe that it impairs their ability to perform physical tasks required for testing
>   e) Subject with no speech (no intelligible words) and/or those unable to follow simple commands
>   f) Subject with uncontrolled seizures

6.4. Hepatobiliary Injury or Dysfunction that Persists or Recurs

If a subject who was previously enrolled in the PALF Cohort Study is admitted to the hospital for a subsequent episode(s) of liver failure at a PALF site during the 12 month follow-up period, and meets entry criteria for the Cohort Study, a minimal amount of clinical information will be obtained from the medical record. If the same conditions are met for a patient who is admitted to the hospital for liver failure after the 12 month follow-up period, the patient will not be approached for enrollment in the PALF Cohort Study again.

6.5. Medical Complications

6.5.1. Renal Dysfunction

Renal function will be assessed by a calculated creatinine clearance using the participant's height (cm), and a proportionality constant using the Schwartz method (72-73). Subjects whose calculated creatinine clearance decreases to <50 ml/min should have this value confirmed within 72 hours. If confirmed, the individual would be considered to have renal dysfunction and will be stratified using the National Kidney Foundation Classification of Chronic Kidney Disease (CKD): Stage 2 CKD = GFR 60-89 ml/min, Stage 3 CKD = GFR 30-59 ml/min, Stage 4-5 CDK = GFR < 30 ml/min.

6.5.2. Cytopenias (affecting ≥ 2 of 3 lineages in the peripheral blood)
     o Hemoglobin < 9 g/dL (in infants < 4 weeks of age: Hgb < 10 g/dL)
     o Platelets < 100 x 10^6/ml
     o Neutrophils < 1.0 x 10^6/ml

7. Data Management

Data will be submitted to the Data Coordinating Center (DCC) via a distributed web-based data entry system. Clinical center coordinators and other project personnel will be trained and certified to collect and enter data using established systems. Clinical centers will have the option to use any front end device (ex. Tablet PC, laptop, desktop) that provides access to the Internet.

DCC personnel will closely monitor clinical center adherence to study protocol and data collection practices for complete and accurate research data. Monitoring will be performed via established data management procedures with on-site monitoring visits conducted at designated intervals, or as needed, to facilitate the smooth conduct of the study. At the time of the on-site visit, DCC personnel will have access to all study and participant documents and to clinical center personnel. All participant and study documents will be kept confidential. Identifiers such as participant name and address will not be included on any data sent to the DCC.

DCC and CCC personnel meet at least once weekly to discuss study status, recruitment, compliance, review data issues, clinical center participation, and other issues that arise during the course of the study.

8. Statistical and Design Considerations

8.1 Longitudinal Cohort Study

We will identify patient characteristics associated with 1 year outcome. More data will be collected on comparatively fewer cases than currently for the PALFSG registry so we will use data reduction techniques (e.g., principal components analysis [PCA], summary measures). To identify factors significantly associated with outcome, models, including logistic regression for short-term (e.g., 21 day outcome) and proportional or non-proportional hazards incorporating censored data for longer-term (e.g., 1 year outcome) will be used. For transplantation as an outcome, methods for competing risks analysis will be used to account for censoring due to death. When examining factors associated with neurocognitive function and quality of life in survivors, generalized linear regression models will be used when outcomes are continuous (e.g., IQ, PedsQL) and polychotomous regression models for ordinal outcomes (e.g., number of cognitive deficits). PALF descriptives (e.g. underlying cause, severity of disease, participant characteristics) will be used as independent measures, with time-varying values enabling models of the dynamic nature of the condition.

Models can be either hypothesis testing or hypothesis generating. For the latter, we will utilize stepwise regression modeling to identify variables that are independently associated with outcome. An advantage of having an ongoing registry is that results of such an exploratory analysis can be confirmed by testing whether the models identified in this manner also fit newly diagnosed cases. Hence, subsequent to hypothesis generation, hypothesis testing can be performed using the PALFSG cohort. Another approach to these analyses that will be employed to address this aim is an empirical approach for classification, such as Classification and Regression Trees (CART). Rather than looking for significant associations, these methods identify factors, and values of those factors, which optimize classification into outcome categories.

Prior to undertaking inferential analyses, the DCC will perform sample size calculations to assure adequate statistical power to address the research question(s) with acceptable error. Because the database will continue to grow, it is important to consider when particular questions could, or should, be addressed. Consequently, the DCC will estimate how many cases would be required and estimate when that number would be reached, given the historical accrual rates. The other rationale for sample size calculations is to assure that clinically meaningful associations will be included in the model. Hence, unlike standard power calculations, this calculation will assure sample size is not "over-powered", i.e., a coefficient has an associated p-value smaller than 0.05 because the sample size is large enough to identify small associations as statistically significant.

8.2 Decision Analysis

The data collected from the cohort study will be used to calibrate several components of the decision model describing the treatment strategies in PALF, including quantitative pre-transplantation disease progression as well as post-transplantation survival. To construct quantitative disease progression, the longitudinal course of each laboratory value in each child will be predicted using penalized cubic splines (70). These splines will be sampled at regular intervals and used to predict the clinical change in laboratory values of simulated children in the model time period by time period. The simulation model also requires predictors of pre and post-transplantation death: there will likely be insufficient deaths in the 300 children pre-transplantation to estimate survival functions, so national data sets from the OPTN will be used to estimate pre-transplantation death.

Predictors of 1-year survival based on clinical characteristics at the time of transplantation will be estimated using Cox proportional hazard models, provided there are sufficient deaths during the first year of follow-up. Survival past one-year will be estimated using Cox proportional Hazard models from OPTN data.

8.3 Inflammatory and Immune Marker Analysis

In addition, common patterns of inflammatory and immune mediators will be assessed by hierarchical clustering analysis (HCA). To identify principal inflammatory drivers and interconnected networks of the inflammatory response in PALF, Principal Component Analysis (PCA) as well as Dynamic Network Analysis (DyNA) will be carried out. PCA will be employed as a tool for discerning principal drivers of inflammation in both patient cohorts and individual patients, and DyNA will be utilized to define the networks and connectivity that characterize patient cohorts.

Data-driven modeling: Hierarchical clustering analysis [HCA] of inflammatory/immune data in PALF

Following assays of inflammatory and immune mediators as described in Section 1.2.5, hierarchical clustering will be performed over all observations. The goal of this analysis is to highlight the natural variability, as well as any overlap, in inflammatory mediators in PALF patients. Hierarchical clustering is a simple and unbiased clustering method which aims to build a hierarchy of clusters. The limitation is the cluster must be built pairwise. Each observation is taken to be a point in a multi-dimensional feature space (one dimension for each analyte measured). The closest points are clustered together and then the closest clusters are merged iteratively until all points will be grouped into a single cluster. The most similar observations will be clustered most closely together. If there is some natural segregation in the raw data, distinct subgroups of patients will emerge. If not, further processing of the data will be necessary.

Each row of the data matrix will correspond to a sample from a single patient at a single time point, and each column will correspond to an inflammatory or immune analyte. The magnitudes of these values will be log-transformed. A dendrogram (a branching diagram used to show relationships between members of a group) on the y-axis will show the similarities among samples according to their correlation measures (the correlation between the inflammatory mediators profiles) across all analyte values. The calculation will be performed using the Bioinformatics Toolbox in Matlab® 7.6.0.

Data-driven modeling: Principal Component Analysis [PCA] of inflammatory/immune data in PALF

Often, the raw cytokine data are uninformative, highlighting the large variability observed in the plasma compartment in this disease. To test the hypothesis that despite this variability certain key inflammatory drivers or bio-signatures (i.e., a patient-specific "inflammation barcode") could be observed, a novel technique developed by the Vodovotz group (Patient-specific PCA) will be performed. The goal of this analysis is to identify the subsets of mediators (in the form of orthogonal normalized linear combinations of the original mediator variables, called principal components) that are most strongly correlated with the inflammatory response trajectory of a given PALF patient. PCA is a non-parametric statistical method of reducing a multidimensional dataset to a few principal components (87). These are the components that account for the most variability in the dataset. The underlying hypothesis is that a mediator that changes during a specific process is important to that process. If the mediators change more than other mediators, then they are more important. This method allows us to identify the mediators that account for the most change, or variance, in the dataset. To discover this patient-specific "inflammation barcode," each patient's measurements of each cytokine will be normalized so that all cytokine levels are converted into the same scale (from 0 to 1). This normalization will be performed on a per-patient basis.

Next, the PCA will be computed for each individual over the time course of cytokine measurements. This procedure will allow for the identification of the mediators that contribute the most to the overall variance in the dataset. A PCA score will be calculated for each cytokine that summarizes its contribution to the overall variance. This is calculated by scaling each principal component's eigenvector by its respective eigenvalue and summing together the coefficients that correspond to a given cytokine over all eigenvectors (sufficient to capture at least 95% of the variance in the data). Thus, each patient will have a PCA score for each cytokine, corresponding to how much that cytokine accounts for the total variance in that patient's data. These PCA scores taken together make up a patient's "inflammatory barcode." This barcode will be used to group patients, again using HCA as described above. Using a correlation distance metric and average linkage protocol, this

method is expected to better define patient sub-groups; these sub-groups will be correlated with the clinical outcomes defined in Section 6.

Data-driven modeling: Dynamic Network Analysis [DyNA]

The goal of this analysis is to gain insights into dynamic changes in network connectivity of the inflammatory response over time, in individual PALF patients as well as in defined patient cohorts. The mathematical formation of this method is essentially to calculate the correlation among the variables by which we can examine their dependence. To do so, cytokine networks will be created in adjacent time periods (see blood sampling protocol) using Matlab® and Inkscape® software. In order to be included in the DyNA, a given mediator will need to be statistically significantly different from its baseline value ($p < 0.05$ by Student's t-test). Connections in the network will be created if the correlation between two nodes (inflammatory mediators) were greater or equal to a threshold of at least 0.7. In the network density calculation, in order to account for network sizes (number of significantly altered nodes) in the adjacent sampling time periods detailed above, we will utilize the following formula (a minor revision of the one reported in 88)

$$\frac{\text{Total number of edges} * (\text{Number of significantly altered nodes})}{(\text{Maximum possible edges among significantly altered nodes})}$$

8.4. Participant and Demographic Data

8.4.1. Study Completion

Summary descriptive statistics (counts, percents) will be presented to describe the numbers of participants completing the study and those not completing the study for various reasons.

8.4.2. Baseline Characteristics and Demographics

Summary descriptive statistics for baseline and demographic characteristics will be provided for all enrolled participants. Demographic data will include age, race, sex, body weight, and height; these data will be presented in the following manner:
- Continuous data (i.e., age, body weight, and height) will be summarized descriptively by mean standard deviation, median, and range.
- Categorical data (i.e., sex and race) will be presented as enumerations and percentages.

8.4.3. Medical History

Medical history will be collected, including the existence of current signs and symptoms and clinical significance for each body system and summarized by study group.

8.4.4. Use of Medications

The number and percentage of participants receiving concomitant medications or therapies will be presented. Statistical presentation of concomitant medications or therapies may be further summarized by subsets of cohort participants as appropriate.

8.5. Interim Analyses

No interim analyses are planned for this study. Unspecified interim analyses may be conducted if they will not adversely affect the integrity of the study or for safety reasons.

9. Human Subjects Issues

9.1. Overview

The study protocol and consent forms will be submitted to each clinical center's IRB, the CCC IRB, and to the DCC's IRB. Additionally, each clinical center will submit to their IRB any recruitment materials to be used at their site and the data collection forms if required. A site may not initiate any patient contact about the PALF study until the site has IRB approval for the studies and the DCC has certified the site for initiation of patient activities. All study personnel will have completed training in the Protection of Human Subjects per NIH guidelines. Given the epidemiology of PALF, subjects included in this study will include racial/ethnic minorities (Asian, American Indian/Alaska Native, Native Hawaiian or other Pacific Islander, Black or African American, Hispanics or Latino) as well as non-Hispanic white subjects. We anticipate that the patients recruited from diverse sources, including community and tertiary referral populations, will capture the entire spectrum of PALF.

9.2. Standard of Care

All subjects enrolled in the PALF study will receive standard of care for PALF and identified associated medical problems. This will include provision of health care, laboratory testing, counseling and education at enrollment and on an ongoing basis during follow-up.

9.3. Enrollment in PALF Treatment Study

The PALFSG is planning to conduct a clinical trial for PALF. All participants to be enrolled in the treatment trial will have been enrolled in the Cohort study. Those found to be potentially eligible for enrollment in a treatment study will be offered participation and asked to sign a separate consent form.

9.4. Institutional Review Board (IRB) Approval

A site may not initiate patient activities in the PALF study until the site has IRB approval. Consent forms must have IRB/REB approval. Sites must provide the DCC with copies of the initial IRB/REB approval notice and subsequent renewals, as well as copies of the IRB approved consent statements.

9.5. Consent Forms

Prototype consents and assents will be prepared for the study. Individual sites may add material but may not delete material thought to be necessary for informed consent. Sites may reformat and reword information to conform to their local requirements. A copy of the approved site-specific consent form must be submitted to the DCC for review and archive. A signed consent form will be obtained from the parent or legal guardian. The subject's assent must also be obtained if he or she is able to understand the nature, significance, and risks associated with the study and is over the age of 12 years. The consent form will describe the purpose of the study, the procedures to be followed, and the risks and benefits of participation. A copy of the consent form will be given to the subject or legal representative, and this fact will be documented in the subject's record.

9.6. Subject Confidentiality

Clinical sites are responsible for the confidentiality of the data associated with participants in the PALF study in the same manner they are responsible for the confidentiality of any patient information within their spheres of responsibility. All forms used for the study data will be identified only by coded identifiers to maintain subject confidentiality. All records will be kept in locked file cabinets at the clinical centers with access limited to PALF study staff. All study staff will identify patients by the patient identifier number generated for the study. Clinical information will not be released without written permission of the participant, except as necessary for monitoring by the IRB/REB or Data and Safety Monitoring Board (DSMB). Clinical information may be reviewed during site visits by the DCC and the NIDDK representative. Participants grant permission to share research data with these entities in the consent document. Federal regulations govern the protection of participants' rights relative to data confidentiality and use of research data.

Consent procedures and forms, and the communication, transmission and stoppage of participant data will comply with individual site IRB/REB and NIH requirements for compliance with The Health Insurance Portability and Accountability Act (HIPAA in the U.S.) or the Personal Information Protection and Electronic Documents Act (PIPEDA in Canada). The DCC will require that clinical centers provide documentation from the site IRB/REB with the appropriate authorization or consent form.

9.7. Data and Safety Monitoring Plan

Data and safety will be monitored by the NIDDK in conjunction with a NIDDK-appointed Data and Safety Monitoring Board (DSMB). This board serves in a consultative capacity to inform the NIDDK decisions regarding conduct of the study. The description of DSMB activities is included in the DSMB Charter.

9.8. Risk/Benefit Ratio

The only risk associated with the collection of clinical data is the unlikely risk of a breach of confidentiality. To protect participants' confidentiality, participants' names will be used only for the informed consent form and medical chart reviews. Participants will be given unique study identifiers, which will be written on all data collection forms. In addition, documents that link the study IDs with participant names will be kept in secured, locked files at the clinical center.

Serum samples collected involve removal of small quantities of blood at the time of a routine blood draw, for basic clinical care, and thus do not require special phlebotomy procedures. The risks of venipuncture at the time of the blood draws are pain, bruising, and superficial phlebitis. The total quantity of blood withdrawn per day for the purposes of the study is based upon the participant's weight and amount of blood drawn for clinical purposes. The combined volume drawn for clinical and research purposes will not exceed the volume specified by the clinical center IRB/REB for daily volume or total volume over the study course.

Liver tissue samples taken at the time of a liver biopsy performed for clinical purposes, or at the time of liver transplantation or autopsy do not require special study procedures.

Parents and children who are asked to participate in neurocognitive testing may experience some discomfort by a feeling of loss of privacy or unexpected emotions due to the personal nature of some of the questions. Site personnel will facilitate contact with social work or psychological support services as needed.

Participants are unlikely to receive any personal benefit from being in this study. However, a study to delineate the nature of acute liver failure in the United States has not been performed previously due to the logistical difficulties of coordinating many centers. This study has the potential to bring to light new information concerning this serious and usually fatal condition, which often affects young people. In addition, parents of children who participate in neurocognitive testing will be provided with a brief, written, non-clinical summary of their child's standardized test performance from his or her participation. Although the neurobehavioral assessment is not a substitute for a complete clinical psychological evaluation, the summary will provide parents with information pertinent to their child's well-being which would otherwise be unavailable except through formal school testing or private testing.

9.9. Participant Withdrawal from Study

If a participant or his/her legal representative chooses to withdraw from the PALF study, all data collected up to the point of withdrawal will remain in the study database, but no further data may be collected. The participant or legal representative must submit a written request to withdraw to the clinical center personnel. This is consistent with HIPAA guidelines and regulations. A participant or his/her legal representative may also withdraw consent for use of data or stored specimens – in this case, any specimens collected from this subject will be destroyed and data deleted.

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Appendix A

Table 1. Blood volume for research studies based upon weight

ITEM	Weight (kg)								
	Study Entry			Daily until stopping rule			Out-Patient Follow-up		
	<10	10-15	>15-20	<10	10-15	>15-20	8 wk	6 mo	12 mo
Volume in mL (clinical labs)									
Research (# = Priority)									
NK cell (Filipovich-Cincinnati)	NA	7.0	7.0					7.0*	
LFTs							2	2	2
DNA^	5.2^								
Max Research volume	3.0	3.0	3.0	2.5	4.0	7.0	5.0	5.0	5.0
Luminex Testing									
Liver regenerative markers									
Markers of CNS injury									
Storage									
Blood on Guthrie Card	X	X	X						
TOTAL VOLUME FOR RESEARCH	3	10	10	2.5	4	7	7	7	7

ITEM	Weight (kg)								
	Study Entry			Daily until stopping rule			Out-Patient Follow-up		
	>20-50	>50-70	>70	>20-50	>50-70	>70	8 wk	6 mo	12 mo
Volume in mL (clinical labs)									
Research (# = Priority)									
NK cell (Filipovich-Cincinnati)	10	10	10					10*	
LFTs							5	5	5
DNA^									
<50 kg	5.2^								
≥ 50 kg	20^								
Max Research volume	9.8	10	10	8	10	15	10	10	10
Studies prioritized from repository									
Luminex Testing									
Liver regenerative markers									
Markers of CNS injury									
Storage									
Blood on Guthrie Card	X	X	X						
TOTAL VOLUME FOR RESEARCH	**19.8**	**20**	**20**	**8**	**10**	**15**	**15**	**15**	**15**

* for children > 10 kg who had previous NK cell testing and have their native liver, sample will be obtained at least once at a follow-up visit when blood volume allows.

^ DNA can be obtained at any time during hospitalization or at a follow-up visit when blood volume allows

Note: Total volume for research does not include the DNA sample or the NK Cell sample at a follow-up visit because those samples will be collected when blood volume allows.

Table 2. Whole blood volumes in the following table will be used as a guide.

Patient Weight	Estimated Blood Volume (ml) 80 ml x body weight	Estimated 5% of Blood Volume (ml)	Estimated 10% of Blood Volume (ml)
5 kgs	399 ml	19.5 ml	39 ml
≥5 and <10 kgs	400 ml – 799 ml	20 ml – 39.9 ml	40 ml – 79.9 ml
≥10 and <20 kgs	800 ml – 1592 ml	40 ml – 79.5 ml	80 ml – 159 ml
≥20 and< 50kgs	1600 ml – 3992 ml	80 ml – 199.5 ml	160 ml – 399 ml
50 kgs and over	4000 ml	200 ml	400 ml