Cystic Fibrosis Liver Disease Network (CFLD-NET)

Prediction by Ultrasound of the Risk of Hepatic Cirrhosis in Cystic Fibrosis (PUSH)

Protocol CFLD Version 004

Amendment #1: 10/09/2009
Amendment #2: 5/30/2011
Amendment #3: 9/24/2013
Amendment #4: 11/11/2014

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Details of Amendments in PUSH protocol

Change	Rationale
Page 1 Indication that this is an amended protocol **Protocol CFLD 002-R1** ***Amendment #4: 11/11/2014***	
Title Page Wikrom Karnsakul is now the PI at Johns Hopkins University; Ed Doo and Averell Sherker are now the Project Official and Project Scientist; Wen Ye replaced Cathie Spino as the lead statistician at the DCC; University of Washington (Seattle) and Anne & Robert H. Lurie Children's Hospital (Chicago) were added as new sites with Karen Murray MD and Estella Alonso MD as site PIs, respectively.	Change of study PI at Johns Hopkins University; Change of project officials at NIDDK; and change of study statistician at the DCC. The network was expanded to 11 sites (Seattle and Chicago) to allow adequate recruitment of subjects with heterogeneous liver in a reasonable time frame.
Section 1 Changed study period from 5 years to 6 years; added text to clarify that study participants are age 3-12 years at the time of enrollment.	To allow for an additional ultrasound (US) visit in year 6; for clarification.
Section 2A #6-7 Eliminated references to five years of study follow up.	To allow for an additional year of follow up, which will include an additional US.
Section 4.1 Changed study period from 5 years to 6 years; added text to clarify that study participants are age 3-12 years at the time of enrollment.	To allow for an additional ultrasound (US) visit in year 6; for clarification.
Section 4.24 Eliminated references to five years of study follow up.	To allow for an additional year of follow up, which will include an additional US.
Section 4.3 Table 1 Changed "follow up visit" column from years 1-5 to years 1-6; changed "US visit" column to reflect the additional US at year 6.	To allow for an additional year of follow up, which will include an additional US in year 6.
Section 4.32 #7 Changed study period from 5 years to 6 years.	To allow for an additional ultrasound (US) visit in year 6.
Section 4.33 #11 Added text to indicate that elastography data will be collected locally as available; #12 Added text to include the ChiLDREN/ChiLDReN Network	Elastography data is being collected locally at sites that have the equipment. This may offer supplemental information. We will simply collect values and method (if performed locally) in addition to other clinical data we are already collecting; for clarification.

Section 5 Changed study period from 5 years to 6 years.	To allow for an additional ultrasound (US) visit in year 6.
Section 6.1 Added text to explain updated sample size calculations and plans for analyses.	We have modified the power and sample size calculations as the variability of US grading was shown to be larger than what has been expected prior to initiation of the study. To achieve a more reliable primary outcome, we extended the study period to 6 years after screening US and introduced an interim analysis based on year 4 US results with a more stringent definition of development of cirrhosis.
Section 10 Changed study period from 5 years to 6 years and changed text to add a fourth US in year 6.	To allow for an additional ultrasound (US) visit in year 6.
Section 10 Added text to address re-consenting of subjects who turn 18 during the course of the study.	To explain that the extension of the study to six years will result in some study subjects turning 18 during the course of their participation in the study. Those subjects will be asked to re-consent for themselves.
Section 12 Added University of Washington (Seattle) and Anne & Robert H. Lurie Children's Hospital (Chicago) to the list of participating centers; corrected the names of existing centers to reflect the names of the institutions that receive grant awards, rather than hospital names.	The network was expanded to 11 sites (Seattle and Chicago) to allow adequate recruitment of subjects with heterogeneous liver in a reasonable time frame; participating centers should be listed by the name of the institution that receives the grant funds for the study.

Change	Rationale
Page 1 Indication that this is an amended protocol **Protocol CFLD 002-R1** ***Amendment #3: 9/24/2013***	
Page 16 Section 4.21 Inclusion Criteria Corrected text for Fecal elastase to Fecal elastase <100 mcg/gm	Correct measurement unit typo for Fecal elastase from mg/gm to mcg/gm

Change	Rationale
Page 1 Indication that this is an amended protocol **Protocol CFLD 002-R1**	This amended protocol includes study followup for subjects found to have cirrhosis or homogeneous liver on US;

Amendment #2: 5/30/2011	these subjects exited study in previous protocols.
Page 1: Alex Weymann is PI at Washington University; Simon Ling is PI at Sick Kids in Toronto; Daniel Leung is PI at Baylor. Emory and Cincinnati have been added with Rene Romero and Joe Palermo, respectively, added as PIs. Cathie Spino was added to the list of Working Group members as the lead statistician.	Change of study PI at Washington University and Hospital for Sick Children, Toronto. The network was expanded to 9 sites (Cincinnati and Emory) to allow adequate recruitment of subjects with heterogeneous liver.
Page 10, Section 1: Objectives: Add text about following children who were not previously followed in this study; added text to clarify that children with known cirrhosis prior to screening are not eligible.	Study is being expanded to follow children who are found to have unsuspected cirrhosis or homogeneous liver on Screening US. Little is known about the progression or impact of early CF liver disease
Pages 11-12, Section 2: Modify hypothesis 4 to include homogeneous or cirrhosis echo pattern of the liver on abdominal US. Add hypothesis 6: Cirrhosis incidentally discovered on screening ultrasound does not progress to clinical evidence of portal hypertension during the 5 year study follow up. Add hypothesis 7: Homogeneous liver on ultrasound does not progress to heterogeneous liver or cirrhosis during the 5 year study follow up.	Add hypotheses regarding impact of cirrhosis or homogeneous liver on US on QOL/pulmonary function/nutrition. Add hypothesis on lack of progression of CF cirrhosis to portal HTN during the study period. Add hypothesis regarding lack of progression of homogeneous liver.
Page 12, Section 2: Broaden specific aim 3 regarding impact of US findings on QOL, pulmonary function, and nutrition to include cirrhosis and homogeneous liver. Add specific aim 4 "To determine if Doppler velocity measurements of hepatic and splenic vessels predict an increased risk for the development of cirrhosis and to characterize these parameters and their progression in children with cirrhosis at screening" Add specific aim 5 to determine if cirrhosis on ultrasound progresses to portal hypertension during the study period Add specific aim 6 to determine if homogeneous liver progresses to either cirrhosis or heterogeneous liver.	Specific aims are expanded to include cirrhosis and homogeneous liver at screening US impact on QOL/pulmonary function/nutrition, study of Doppler, progression of cirrhosis to portal HTN, progression of homogenous liver.
Page 15, Section 4.1: Add to Study Design	Design changed to follow children with

that children who are found to have unsuspected cirrhosis or a homogeneous liver pattern at screening ultrasound will be followed for 5 years to assess for the development of portal hypertension or cirrhosis and its progression.	cirrhotic or homogeneous liver pattern at screening US for the full 5 years of study.
Page 17, Section 4.23: Add text to clarify that we will now follow those children who are found to have cirrhosis or a homogeneous liver at screening US. Changed matching ratio. Heterogeneous subjects will now be matched to two normals for follow-up. Normals who had previously exited the study can be re-consented and followed as part of the new match ratio.	Design changed to follow children with cirrhotic or homogeneous liver pattern at screening US for the full 5 years of study. Matching ratio changed to address current rate of heterogeneous enrollment.
Page 17, Section 4.24: Number of participants: Groups clarified and expanded: A heterogeneous liver, B normal liver, C cirrhosis and D homogeneous. Increase enrollment number to 800 from 580. Decreased number of heterogeneous from 110 to 60 and edited text to reflect 60 matched trios instead of 110 matched pairs.	Subjects with cirrhotic and homogeneous pattern are retained in study (but not matched). Increase enrollment to meet target longitudinal enrollment of matched pairs and new groups to be followed. Updated enrollment totals by group per new matching ratio.
Page 17, Section 4.24: Enrollment procedures: add that subjects with cirrhosis or homogeneous liver will be followed for the study period.	Linked to new study aims.
Pages 17-18, Section 4.24: Re-contact and re-consent of previously exited homogenous and cirrhotic subjects, as well as normals who could now be matched into trios.	Allow these previously exited subjects the opportunity to be followed long term.
Page 18, Section 4.3: Clinical and lab evaluation: clarification that subjects with cirrhosis or homogeneous liver will be followed with yearly visits and biyearly US	Linked to new study aims.
Page 20, Section 4.32: Extend time requirement for completion of Screening US from 4 months to 7 months (also appears in Section 4.42). Added text clarifying that children with homogeneous patterns will be followed as Group D. Added text clarifying that all children with heterogeneous pattern will be followed, whether matched or not. Added text to	Allow for Screening US to be completed at either of next two scheduled CF clinic visits. Linked to new study aims.

clarify that Group B subjects' data will be maintained whether or not they are matched and followed.	
Pages 21-23, Section 4.33: Screening visit and longitudinal visits changed to include cirrhosis (group C) and homogeneous liver (group D)	Linked to new study aims.
Page 22, Section 4.33: Added text to clarify that lab data will typically be collected at the subject's annual CF visit. Labs for the longitudinal follow up visit will be those collected at or after the longitudinal visit. Study specimens can be collected at the time of the annual CF visit.	Labs are not typically drawn at quarterly CF visits. We have made an effort in this study to avoid additional blood draws and instead collect study labs and specimens when they are also being drawn for the annual CF visit.
Pages 25, Section 4.42: Removed text requiring a Grade of 0 or 1 upon entry into the study. Updated number of sites from 7 to 9.	Linked to new study aims. Per addition of two new study sites.
Page 26, Section 4.42: Added text to clarify that new study radiologists will be trained per the process described.	Study personnel may change over time.
Page 28, Section 5: Termination or Withdrawal: modified to state that subjects will be followed for 5 years unless they receive a liver transplant, in which case they are terminated.	Study will follow cirrhotic children until completion unless they develop the endpoint of receiving a liver transplant.
Pages 28-29, Section 6.1: Added text to explain sample size calculation changes and the change in the matching ratio. Updated enrollment targets and statistics to reflect new calculations.	Linked to new study aims.
Pages 29-30, Section 6.2: Added and updated text to correspond to new study aims.	Linked to new study aims.
Page 1 Indication that this is an amended protocol **Protocol CFLD 002-R1** **Amended 5/24/2009**	
Page 2: Table of Contents added	
Page 8: All current and newly diagnosed patients (based on diagnostic and enrollment criteria) with Cystic Fibrosis aged 3 through 12 years of age who are	Toronto is not part of the CF Registry, but has similar data in their registry.

enrolled in the CF Registry Study or the Toronto CF Registry, followed at each CFLD-NET Clinical Site will be offered enrollment into this study.	
Page 9 Inclusion criteria 1. Enrolled in the CF Registry Study or Toronto CF Registry	Addition of the Toronto CF Registry, as Toronto does not participate in the CF Registry (only US centers participate)
Page 9 Exclusion criteria Added: 1. Presence of Burkholderia cepacia	Burkholderia is very rare in the target population and analysis of data suggests that it will be nearly impossible to have an adequate match should a subject be identified with Burkholderia. Subjects who develop Burkholderia during the study will continue to be followed.
Page 10 Matching statement These study participants will be matched (one to one by age, center, gender, ethnicity and *Pseudomonas aeruginosa* status) to subjects from Group B (NL US).	Matching criteria were adjusted to eliminate race (minimal effect by analysis of preliminary data), Burkholderia (see item above) and genotype as all patients will be pancreatic insufficient
Page 10 duration of the study The study will continue to enroll patients until we accrue 110 matched pairs (HTG US and NL US). Thus recruitment of cases and controls will stop when 110 matched pairs are enrolled, our current best estimate for the sample screened is n=580	Based on the recommendation of the DSMB, we have clarified that the study will continue to enroll until we accrue 110 matched pairs. This may not be the case when we recruit the 110^{th} HTG US case.
Page 11: Clarification of follow up Follow Up: The Group A (HTG US) and a portion of Group B (NL US) subjects will be followed annually for 5 years or the development of cirrhosis (whichever comes first) as indicated below.	Once the subject develops cirrhosis, they will be at the endpoint of the study.
Page 11: Table: We have adjusted the Baseline visit name to Longitudinal Follow up visit 1 for clarity. Matching criteria data collection added to the screening visit and Diagnosis removed from the table	Change name of visit for clarity Data on matching is collected at the screening visit. Diagnosis confirmation is part of the eligibility criteria
Page 12: we have clarified the data collected at the screening to meet matching criteria and NIH data collection: a. For Matching: DOB, Center, Gender, Ethnicity, pseudomonas status	This clarifies the data collected for matching and collection of key data elements.

b. Other data: Race, CF Registry patient number, genotype (DeltaF508 homozygous, heterozygous and other).	
Page 12 clarification of the matching strategy Group A (HTG US) subjects will be matched to a Group B subject (within 6 months of enrollment) and followed for up to 5 years. In the event that a match cannot be found for a Group A subject (HTG US) within six months of enrollment, the matching criteria will be relaxed sequentially using the algorithm specified in the MOO. All participants with a HTG US will be followed annually whether matched or not.	We have clarified the timing of the matching algorithm.
Page 13 Change Baseline Visit to Longitudinal Follow Up Visit 1	Visit name change for clarification
Page 13 Clarification of the timing of the enrollment in the Longitudinal FU visit 1 for longitudinal followup This may be obtained any time after consent and after the entry US has been graded. It should occur within 3 months of determination that the subject is eligible for longitudinal follow up (Group 1 HTG US or a matched Control from Group 2 NL US)	This visit is for patients who will be in longitudinal follow up. Matched Controls may be more than 3 months from their US.
Page 14 Addition of the CFQ-R Preschool Version (3-6 years old)	Adding the preschool CFQ tool
Page 15 Numbering change **4.4 Specimens to be Collected**	Correct a numbering issue
Page 16-17 Clarification that the Doppler measurements are a research component . The Doppler US will be the experimental arm of the study. US scans will be obtained after a minimum of a 4-hour fast for flow evaluation. Liver echogenicity and contours will be assessed. Liver echogenicity will be evaluated by comparing it to that of the	Clarification of the Doppler component of the US.

kidney. Irregularity or nodularity of the hepatic parenchyma and liver capsular surface, portal vein diameter, collateral vessel formation, ascites, and splenomegaly will be evaluated. Doppler US will be used to assess the presence and direction of flow in the main portal vein and right, main and left hepatic veins; and flow velocity will be assessed in the portal vein and resistive and pulsitility indices will be sought in the hepatic and splenic arteries.	
Page 17: Clarification that the radiologists will be blinded to results for the training set All 7 center radiologists who will be blinded to the results, will then grade the liver ultrasound images.	Clarification that the radiologists will not know the results of the test sets.
Page 17-18 Clarification of ongoing assessment of radiology readings Center radiologists must meet the accuracy cr outlined in the MOO before the study can sta any center. The same training set used for the radiologists will be used to train the sonographers. In addition, there will be a written guide documenting the required images for the sonographers. There will be ongoing assessment of the quality of the US and the consistency of the US readings. The first five US studies from each institution will be reviewed by the lead radiologist (MS) to ensure uniform quality. A scoring sheet of the image quality for each of the first five US scans will be completed and stored at the data center and also sent to the radiologist at each institution. For each of the US images, there will be three readings of each image and assessment of the image quality (the center radiologist reading and 2 other readings). The additional readings will rotate between	Per the request of the DSMB we have outlined the ongoing quality assessment component for the radiologists.

the radiologists in the study excluding the lead radiologist (MS). There will be ongoing assessment of discordance over time that will be tracked as a group and by individual radiologist as outlined in the MOO. We do anticipate some disagreement within one grade. All discordant readings will be reviewed by the lead radiologist (MS). Criteria for interventions are outlined in the MOO.	
Page 20 Numbering clarification <u>Primary Outcome</u> is the development of cirrhosis • Cirrhosis will be defined by imaging criteria (Grade 3 or 4 as described in 4.4 Section on US Grading)	Number clarification
Page 20: Clarification of endpoints Subjects in the longitudinal follow up will be followed annually for up to 5 years or until they develop cirrhosis.	
Page 22: Addition to the risk statement Medical risks include those related to the identification of abnormalities or incidental findings on the abdominal ultrasound. These results will be discussed by the study investigators with the patient and family and the patient's care team with further management/referral directed by the care team.	Added to clarify the potential to identify other liver diseases.
Page 22 We have removed this statement: There is also a risk that the subject's US staging will be revealed as a result of the transport of US studies to the centers for re-reading. US studies will only contain a study ID with no PHI.	We have validated methods to have only a study ID

Prediction by Ultrasound of the Risk of Hepatic Cirrhosis in Cystic Fibrosis (PUSH)

1. OBJECTIVES

The primary objective of this prospective longitudinal study is to determine the utility of abdominal ultrasound (US) at enrollment to predict the development of cirrhosis in subjects with cystic fibrosis (CF) within approximately a six year period. This is a multi-center prospective longitudinal study of pancreatic insufficient children with CF who are enrolled at age 3 through 12 years. Subjects will be prospectively ascertained, enrolled and followed at yearly intervals for approximately six years, through the completion of the Year 6 US and study closeout procedures. This longitudinal study will involve the collection of clinical and outcome data at annual intervals for six years and standardized US at 2 year intervals, including Year 2, Year 4 and Year 6. The study will test the hypothesis that a heterogeneous echo pattern on ultrasound of the liver of children with CF will predict an increased risk for the development of cirrhosis. The development of the serum and urine repository, and the maintenance of a DNA bank or transformed cell lines for DNA analyses, will be an invaluable tool for current and future ancillary investigations into the pathogenesis of the development of cirrhosis in CF and the development of biomarkers and genetic markers that would be useful in identifying patients at risk of progression to cirrhosis. Data from this study will be stored and analyzed in a secure research database at the Data Coordinating Center (DCC), University of Michigan.

The study population will consist of individuals with cystic fibrosis, who are pancreatic insufficient, 3 through 12 years of age at time of enrollment and without known cirrhosis at the time of screening. In order to study the predictive ability of abdominal ultrasound, subjects will be followed for approximately six years, through the completion of the Year 6 US and study closeout procedures.

This study will:

1. collect detailed clinical and demographic information about each subject at enrollment and during follow up,
2. obtain and store imaging data from the subject at entry and during follow up,
3. obtain and store serum, plasma and urine samples from the subject at entry and during follow up,
4. obtain and store DNA from the subject,
5. obtain and store DNA from the biological parents,
6. obtain and store quality of life data from the subject and parents at enrollment and during follow up

Samples of serum, DNA, urine and imaging data will be stored in repositories for future research. The data and biological specimens may be used for detailed study into the mechanisms and causes of liver problems in subjects with CF and the risk of the development of cirrhosis in order to try to better diagnose and manage this condition. The subject will receive standard-of-care treatment and will not be restricted in type of treatment or from changes in treatment, such as newer treatments as they are developed.

The subjects may not directly benefit from participation in this research, but in the future other children and adults with similar problems may benefit from new information that may lead to better medical care.

Funded by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and the Cystic Fibrosis Foundation (CFF), the Cystic Fibrosis Liver Disease Network (CFLD-NET) is governed by a Steering Committee comprised of the principal investigators from each of the participating clinical sites, the data coordinating center principal investigator, radiology core director, research coordinator representatives, CFF representatives and the NIDDK project scientists.

At the end of the clinical study, all unused biological specimens and data will be stripped of identifiers and will be kept in repositories under contract to NIDDK for future use by investigators who will gain access to them by way of a published review process.

2. HYPOTHESES AND SPECIFIC AIMS

The following hypotheses will be tested in this longitudinal study.

A. Hypotheses:

1. The finding of an isolated heterogeneous echo pattern in the liver on abdominal ultrasound (US) will predict an increased risk for the subsequent development of cirrhosis in children with cystic fibrosis.
2. There will be serum or plasma biomarkers that predict the development of cirrhosis in an individual.
3. There will be genetic modifiers that influence the development of cirrhosis.
4. There will be no effect on the quality of life, pulmonary or nutritional status of subjects by the finding of an isolated heterogeneous, homogeneous or cirrhosis echo pattern of the liver on abdominal US.
5. Doppler analysis of the hepatic and splenic vessels will be a better predictor than gray scale abdominal US alone to predict an increased risk for the subsequent development of cirrhosis
6. Cirrhosis incidentally discovered on screening ultrasound progresses slowly and does not result in complications of portal hypertension or hepatic synthetic failure during study follow up.
7. Homogeneous liver on ultrasound does not progress to heterogeneous liver or cirrhosis during study follow up.

These hypotheses will be tested by addressing the following Specific Aims.

B. Specific Aims:

The specific aims for this study are:

1. To determine if sonographic findings predict the risk of progression of liver disease to cirrhosis by comparing CF subjects with heterogeneous echogenicity pattern on US to those with normal echogenicity pattern on US
2. To develop a data and biorepository of serum, plasma, urine and DNA to aid the investigations in ascertaining the mechanisms, consequences, genetic risk factors and biomarkers for the development of cirrhosis
3. To determine if there are differences in health related quality of life, pulmonary or nutritional status in children with CF who have a heterogeneous, homogeneous or cirrhosis echo pattern on US compared to those who have a normal echo pattern on US
4. To determine if Doppler velocity measurements of hepatic and splenic vessels predict an increased risk for the development of cirrhosis and to characterize these parameters and their progression in children with cirrhosis at screening.
5. To determine if subjects with cirrhosis discovered in this study develop portal hypertension, complications of portal hypertension or worsening hepatic synthetic function during the study.
6. To determine if homogeneous ultrasound progresses to either heterogeneous ultrasound or cirrhosis during the study period.

3. BACKGROUND

Despite the recognition of the involvement of the liver in the pathologic process of CF, there remains a significant issue with the identification and classification of liver disease in CF(1). This has been primarily due to the lack of reliable sensitive and specific diagnostic markers of liver involvement in CF until cirrhosis with portal hypertension is present. In this section the current knowledge about the pathophysiology of cystic fibrosis liver disease (CFLD) will be reviewed as will the few reports in which various biomarkers of CFLD have been studied. The major limitations of these studies are that they were cross-sectional, small-scale and usually limited to one center. There is a major need for prospective, large scale studies of a sensitive and specific biomarker which can identify a subpopulation of CF patients at increased risk for the development of CFLD. This vitally important first step will provide the information necessary to identify patients for interventional therapies that could prevent the progression of CFLD and also to identify modifier genes and other factors that put patients at increased risk for progression of liver disease. In this section we review the limitations of several biomarkers studied as well as the rationale for selecting liver ultrasound as the most promising biomarker of CFLD to date

CF is characterized by an abnormality in the cystic fibrosis transmembrane conductance regulator (CFTR). In the liver CFTR is expressed on the apical membrane of biliary epithelium and not on the hepatocyte (2). The putative role of CFTR in the biliary epithelium is to facilitate water and solute movement via chloride secretion and therefore promote bile flow. The mechanism by which the abnormality in CFTR leads to liver disease in CF is uncertain. The primary end result of liver disease in CF is the development of biliary fibrosis leading to biliary cirrhosis that may progress to multilobular cirrhosis. In autopsy studies that predate significant changes in CF management and outcome, focal biliary cirrhosis/fibrosis was reported in up to 10-20%

of CF subjects by one year of age and up to 80% in adults (3, 4). In some patients portal hypertension and its attendant consequences then develop. Various theories have been proposed for the underlying pathophysiology, but no single mechanism has met with uniform acceptance to explain this progression. Whatever mechanism is involved, it must explain the fact that only a small percentage of patients with CF develop cirrhosis with or without portal hypertension. Despite difficulties in assessing the extent of liver disease in CF, most of the studies of the natural history of CFLD suggest a prevalence of cirrhosis in CF between 5 and 15% (5, 6).

Natural history:

The most readily definable classification of advanced liver disease in CF is with the presence of cirrhosis with or without portal hypertension. Current estimates suggest that the prevalence of this disorder is on the order of 5-8% by 15-20 years of age (6-9). It appears that the development of cirrhosis is likely an early event as the median age of recognition of cirrhosis or portal hypertension is approximately 10 years of age. Unlike many forms of liver disease such as chronic viral hepatitis, the progression from the development of cirrhosis to further complications of portal hypertension is slow in CF and the development of liver failure is a very uncommon event. Nonetheless, the sequelae of portal hypertension cause enhanced morbidity and mortality of CF patients such that liver disease is the third leading cause of death in CF, accounting for 2.8% of deaths in 2006 (10). Thus the available data suggest that the development of cirrhosis is an early event with a low rate of progression to hepatic synthetic failure (7, 11).

A major factor contributing to the lack of clarity regarding the progression of CF liver disease has been the inability to identify patients at high risk for the development of cirrhosis before it develops. As a consequence, it is unclear if the development of cirrhosis in CF is a stepwise progression like other liver diseases. In part, this is a reflection of the lack of consensus for definition of non-cirrhotic stages of fibrosis, in CF making it difficult to define “progression”.

Some risk factors for the development of CF liver disease with cirrhosis have been identified in small studies and include severe *cftr* mutations (Class 1, 2 and 3) conferring pancreatic insufficiency, male sex, and the presence of modifier gene(s) such as Alpha one antitrypsin Z phenotype and transforming growth factor (TGF) β polymorphisms (7, 9, 12, 13).

Thus, there is a pressing need to identify an early marker in patients with CF who are at increased risk for the development of cirrhosis. Timely recognition of these patients would allow for prospective investigations of the pathophysiology of the development of cirrhosis and potentially lead to the development of new treatments. In addition early identification of this subgroup of CF patients allows for close monitoring and prompt assessment of candidates for new treatments as they become available.

3.1 Potential early markers for the risk of progression to cirrhosis

Biochemical abnormalities: Patients with CF frequently have abnormalities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyl transpeptidase (GGTP). In a large study of over 500 patients more than 6 years of age, an elevated AST was found in 11% and an elevated ALT in 16% (14). None of these

subjects had clinical evidence of cirrhosis. By 15 years of age more than 50% of patients with CF will have or have had an abnormal AST or ALT. There was no correlation of abnormal aminotransferases and clinical evidence of cirrhosis or portal hypertension (15). Thus, to date, standard liver enzymes have not been shown to indicate an increased risk for the development of cirrhosis. Other markers such as high–molecular-weight alkaline phosphatase (16), and glutathione-S-transferase β1 (17) have been suggested to be markers of liver disease in CF. In small studies, these been found to be early markers of CF–associated liver disease, but these observations have yet to be confirmed. In another small study, TGF-β (18) correlated with hepatic fibrosis in CF. However the TGF-β data was not replicated in another study (19). Measures of hepatic synthetic function, such as prothrombin time and fasting serum bile acids (20), have not proven to be sensitive indicators of CF–associated liver disease.

Biomarkers of fibrosis: Markers of increased formation of fibrotic tissue are attractive candidates for the identification of early fibrosis. In small studies, hyaluronic acid (21), tissue inhibitor of metalloprotease 1, collagen type IV, prolyl hydroxylase (22) and collagen type VI (23) have been reported to be associated with advanced CF liver disease. There have not been any large studies of any of these markers in CF.

Liver biopsy: In most liver diseases, liver biopsy is generally the gold standard for the diagnosis of the various stages of fibrosis. However the focal nature of CFLD makes liver biopsy less reliable for the diagnosis of fibrosis, even cirrhosis in CF (24). Despite this limitation, liver biopsy has shown significant fibrosis in over 50% of selected patients who have undergone a liver biopsy (25). There may also be a role for the use of liver biopsy in differentiating hepatic steatosis from hepatic fibrosis. However, the use of liver biopsy to screen for CFLD or to identify individuals at risk for CFLD would not be a practical screening test due to the risks associated with liver biopsy.

Abdominal Ultrasound: A heterogeneous echo pattern of the liver on abdominal US has been suggested to be a good marker for patients at risk for cirrhosis (8) and could potentially be used as an outcome measure. In a single center study, 67% of patients with a heterogeneous echo pattern on US progressed to have US features consistent with cirrhosis and 46% progressed to portal hypertension with an average of 10 years of follow up (8). In patients with a normal echo pattern on US, 7-13% developed US findings of cirrhosis and 5-7.5% progressed to portal hypertension. Thus, in this study, patients with a heterogeneous echo pattern of the liver on US had a 5.2 fold increased incidence of cirrhosis and a 6.1 fold increased incidence of portal hypertension compared to children with a normal echo pattern on US. Approximately 25% of subjects will have an abnormal echo pattern on US using the grading system proposed by Williams (8, 26). This study demonstrated an excellent concordance between 2 readers in staging the US findings in CF (26). In addition the combination of an abnormal echo pattern on US and abnormal aminotransferases has been suggested to indicate a risk for advanced CFLD (9). A recent study has suggested that US findings do not correlate with liver biopsy findings of cirrhosis (24) and can be interpreted to show that liver biopsy is a poor test to diagnose cirrhosis in CF due to the large nodules and focal nature of the cirrhotic process in CF.

Transient elastography: Fibroscan (transcutaneous acoustic variability) has shown promise as a possible biomarker in assessing liver fibrosis (27, 28). However, recent data presented at the 2007 AASLD meeting has suggested that while transient elastography is both sensitive and specific for cirrhosis, it is neither sensitive, nor specific for earlier stages of fibrosis and it is not useful differentiating between livers that are normal and those with mild fibrosis (29). There is also concern that it may not be able to detect differences between fibrosis and steatosis which are common differential entities encountered in CF. In addition; the use of transient elastography in children requires modification of the probe and mathematical transformation of the data that would make the generalized use of this technique impractical at present.

In summary, abdominal US appears to be the most promising screening test to predict a risk of developing cirrhosis in CF. Various biochemical markers have also shown promise as screening tools, but their sensitivity and specificity have not been well characterized. To date the only studies using US to screen for liver disease have been single center studies. **Whether US is an effective tool to screen for risk of development of advanced CFLD merits a multicenter study.** Combining a prospective study of abdominal US in CF with prospective collection of serum, plasma, urine and DNA to investigate other potential biomarkers would allow further investigation of multiple biomarkers in parallel with the US study.

4. STUDY DESIGN

4.1 Design

This is a prospective multicenter nested case control study. Children with CF who are pancreatic insufficient with or without evidence of a heterogeneous echo pattern of the liver by abdominal US will be prospectively identified. Because pancreatic sufficient patients have a very low incidence of liver disease, only pancreatic insufficient patients will be studied (30). Patients will be ascertained from a population based sample of subjects aged 3 through 12 years at enrollment, and will be followed for approximately six years, through the completion of the Year 6 US and study closeout procedures, with annual visits and biannual abdominal ultrasound with the primary endpoint being the development of cirrhosis as defined by radiographic criteria. Children who are found to have cirrhosis during the study will be followed for approximately six years, through the completion of the Year 6 US and study closeout procedures to assess for the development of portal hypertension and its complications, or the development of liver synthetic dysfunction. Children found to have homogeneous liver at screening ultrasound will be followed for approximately six years, through the completion of the Year 6 US and study closeout procedures to assess for development of cirrhosis.

The collection of detailed clinical and laboratory data is part of the standard of care of children with cystic fibrosis. This research involves the analysis of clinical, laboratory, pulmonary function and outcome data collected as part of standard care for each subject (Note: please see details about US data and identification below) in this research database of subjects. Samples of blood and urine for research will be obtained, whenever possible,

at the time of clinically indicated blood draws or when there is IV access for a clinical procedure. From the research related abdominal US studies performed in this study, coded data will be entered into this database. The radiology images will be stored at the DCC. The biological specimens and imaging data will be used in investigations into the mechanisms of the liver damage that occur in CF that might lead to cirrhosis. The study will follow-up and record routine standard of care examinations and laboratory tests for approximately six years, through the completion of the Year 6 US and study closeout procedures. All data from this study will be kept in a secure research database at the DCC and then stored in the NIDDK repositories in a de-identified fashion to be used for future studies.

4.2 Enrollment

The study population will consist of males and females 3 through 12 years of age with Cystic Fibrosis and pancreatic insufficiency. All racial and ethnic groups will be included. All current and newly diagnosed patients (based on diagnostic and enrollment criteria) with Cystic Fibrosis 3 through 12 years of age who are enrolled in the CF Registry Study or the Toronto CF Registry and followed at each CFLD-NET Clinical Site will be offered enrollment into this study. Pulmonary and nutritional outcomes will be derived from CF Registry study data, thus the requirement for enrollment in the CF Registry.

4.21 Inclusion Criteria

Inclusion criteria for the study are:

1. Children aged 3 through 12 years of age at time of enrollment diagnosed with Cystic Fibrosis and pancreatic insufficiency
2. Enrolled in the CF Registry Study or Toronto CF Registry
3. CF defined as sweat chloride of >60 mEq/L on one occasion (using the value in the CF registry) or two disease-causing mutations of CFTR with evidence of end organ involvement.
4. Pancreatic insufficient defined as one of the following:
    i. CFTR Mutation associated with pancreatic insufficiency per Castellani et al (31)
    ii. Fecal elastase <100 mcg/gm (at any time)
    iii. 72 hour fecal fat with coefficient of fat absorption <85% (at any time)

4.22 Exclusion Criteria

Exclusion criteria for the study are:

1. Known cirrhosis prior to screening
2. Presence of Burkholderia cepacia
3. Short bowel syndrome defined as not on full enteral feeds by 3 months of age
4. Presence of other serious disease precluding participation in this study (This would include patients with known other causes of chronic liver disease)
5. If in the opinion of the Investigator the study is not in the best interest of the patient
6. Inability to comply with the longitudinal follow-up described below

7. Failure of a family to sign the informed consent document or the HIPAA medical record release form

4.23 Study Enrollment Procedures

All current and newly diagnosed patients with CF at the participating CFLD-NET clinical centers who meet the entry criteria will be offered enrollment into this study. The investigator or clinical research coordinator will recruit the subject and the parent(s) or guardian(s) during or before clinic visits. The investigator will discuss the study protocol, benefits and possible risks with the subject (when age appropriate) and the family. The consent form and printed information about the study will be given to the family. The IRB-approved consent will include the purpose of the trial, the responsible parties and investigators, potential benefits, risks of participation, the right to refuse to be in the study, the right to withdraw from the study without penalty, contact numbers and information about the responsibility for injury and payment for medical care as per the policies of the individual center's IRB. If the family consents to entry into the study, signed informed consent will be obtained from parents or guardians where applicable and assent where applicable and case report forms will be completed. For subjects in Group B (Normal echo pattern), a subset will be matched 2 from Group B to 1 from Group A (heterogeneous echo pattern) and then followed longitudinally. Any child found to have cirrhosis or homogeneous liver at consensus reading of screening ultrasound will be followed longitudinally. Due to the change in the matching ratio in this amendment (1:1 changing to 1 heterogeneous:2 normal) and inclusion of homogeneous and cirrhosis in longitudinal follow-up, subjects previously enrolled who have exited the study can be approached for a repeat consent.

4.24 Number of Participants.

During the duration of this study, the plan is to enroll study participants at all of the Clinical Sites: There will be approximately 800 subjects enrolled. Subjects will be assigned to one of four groups based on their US findings at entry: heterogeneous (HTG) pattern (Group A: HTG US), normal (NL) pattern (Group B: NL US), cirrhosis (Group C) or homogeneous pattern (Group D). Approximately 60 subjects with a heterogeneous echo pattern of the liver (HTG) on abdominal ultrasound (Group A: HTG US) will be enrolled. These study participants will be matched (one to two by age, center, and *Pseudomonas aeruginosa* status) to subjects from Group B (NL US). Subjects from Group B who are not matched to a subject in Group A will only have a single screening visit. The study will continue to enroll patients until we accrue 60 matched trios (HTG US and 2 NL US). Thus recruitment for this study will stop when 60 matched trios are enrolled. Please see Section 6.1 for more information.

- Group A - Approximately 60 subjects with a heterogeneous echo pattern of the liver on abdominal ultrasound (HTG US).
- Group B - Approximately 680 subjects with a normal echo pattern on abdominal ultrasound (NL US). Of these subjects, approximately 120 will be matched 1 from Group A:2 from Group B and followed for the duration of the study. The remaining unmatched subjects will not be followed beyond their initial visit.
- Group C–subjects with cirrhosis found on ultrasound will be followed for the duration of the study with yearly visits and bi-yearly ultrasounds. We estimate that 30 subjects will be in this group.
- Group D- subjects with homogeneous liver on ultrasound will also be followed with yearly visits and biyearly ultrasounds for 5 years. We estimate that 30 subjects will be in this group

Subjects who previously participated in the study and were found to have cirrhosis or homogeous liver and thus exited the study or normal US pattern and are potential matches, can be re-contacted and re-consented for continued participation in the study without repeating the screening ultrasound.

4.3 Clinical and Laboratory Evaluations

The following table (Table 1) indicates the schedule of expected visits and times of data and sample collection.

The types of visits are:

1. Recruitment/Screening: The family is approached for recruitment into the study. At least one parent or guardian as appropriate must sign written consent before data collection can begin. Once consent is obtained, the coordinator may abstract information from the subject's medical chart to determine eligibility and arrange the hepatic ultrasound.
2. Longitudinal Follow Up Visit 1: This visit is for subjects eligible for longitudinal follow up (all Group A, C and D and matched Group B subjects) Once the ultrasound is complete and the subject is matched or eligible for longitudinal follow up, the coordinator will meet with the subject and the parent(s)/guardian(s) to complete the intake and history forms (see below for details).
3. Follow Up: Group A (HTG US), Group C, Group D and a portion of Group B (NL US) subjects (matched to Group A) will be followed annually as indicated below.

Table 1. Schedule of Evaluations

EVALUATION	SCREENING VISIT	LONGITUDINAL FOLLOW UP VISIT 1	YEAR 1-6 FOLLOW UP ±3 MO	US AT YEAR 2 + 4 + 6 FOLLOW UP
Informed Consent/Assent	X	X		
Eligibility	X	X		
Matching Criteria	X			
Intake History/Exam		X		
Interval History/Exam			X	
Quality of Life Questionnaire	X		X	
Anthropometrics		X	X	
Biochemistry		X	X	
Abdominal US	X			X
Urine Sample		X	X	
Serum Sample		X	X	
Plasma Sample		X	X	
Blood for DNA		X		
Parents Medical History		X		
Blood for DNA from Parents		X		

4.31 Data to be Collected

This is an observational longitudinal study which will involve the collection of clinical information, family history, physical findings, laboratory tests, study related radiologic and imaging evaluations, quality of life data, biospecimens and clinically indicated treatments and their outcomes. The data elements are designed to provide data for the development of specific outcome measures that could be used to determine the effectiveness of new strategies to prevent the progression of and/or treat CFLD. In addition to routine care, procedures and tests, several special research procedures will be performed to aid in the development of outcome measures, including abdominal US and quality of life questionnaires. The purpose of collecting serum, plasma and urine is to facilitate the development and testing of biomarkers by providing a link to a large group of patients with consistent definitions of their outcome over time. Blood for DNA/cell lines will be collected to provide the ability to investigate modifier genes for the development of CFLD. Blood for DNA/cell lines will also be collected from both biologic parents (when available).

The study visits for this study are outlined below.

4.32 Screening Visit
This visit would occur in conjunction with a routine clinical visit. Studies performed for this visit would include:

1. Consent/assent procedures
2. Eligibility: Assessment and documentation of the Inclusion and Exclusion Criteria
3. Review of diagnosis of CF (genotype data and sweat chloride data); Confirmation of CF diagnosis
4. Review of the medical record for evidence of pancreatic insufficiency.
   Assessment for pancreatic insufficiency:
   a. Genetic testing: prior genetic testing for CFTR mutations (if performed previously)
   b. Fecal elastase (if previously done)
   c. 72 hour fecal fat (if previously done)
5. Data:
   a. For Matching: DOB, Center, Pseudomonas status
   b. Other data: Race, CF Registry patient number, genotype (DeltaF508 homozygous, heterozygous and other).
6. Quality of Life (All patients at Screening to assure that this is performed before the results of the US are known)
   a. CFQ-R Preschool Version (3-6 years old)
   b. CFQ-R Child Version (6-13 years old)
   c. CFQ-R parent Version (6-13 years old)
   d. CFQ-R Teen Adult (14 and up)
   e. CFQ-R Preschool version (3-5 years old)
   f. PEDS QL Child Report (8-12 years old)
   g. PEDS QL Parent Report (3-12 years old)
7. Abdominal US: Performed per study protocol at the center. [This would be a research study]. This may be obtained at any time in the seven months after consent has been given. Classification of the US findings will be based on the consensus of 3 study radiologists (one of these will be from the center that performs the US). All readings should be completed within approximately 4 weeks. Those with an isolated heterogeneous echogenicity pattern would be assigned to Group A (HTG US) and those with a normal echogenicity pattern would be assigned to Group B (NL US). Those with cirrhosis would be assigned to Group C and those with homogeneous echogenicity to Group D.
   Group A (HTG US) subjects will be matched to 2 Group B subjects (within 6-12 months of enrollment) and followed for approximately six years, through the completion of the Year 6 US and study closeout procedures. In the event that a match cannot be found for a Group A subject (HTG US) within six months of enrollment, the matching criteria will be relaxed sequentially using the algorithm specified in the Manual of Operations (MOO). All participants with a HTG US (whether matched or not), will be followed annually.

Group B (NL US) subjects matched 2 from Group B to one from Group A subject will be followed for approximately six years, through the completion of the Year 6 US and study closeout procedures. Group B subjects who are not utilized as Group A controls, will have an initial visit and no subsequent visits. However, their baseline data will be maintained to allow determination of associations with US findings.

Group C (cirrhosis) and D (homogeneous liver) will be followed annually with visits and bi-yearly with ultrasound.

(Patients not enrolled in the longitudinal follow up component of the study, will not be followed, but we will request permission to re-contact the subject in the consent for this study.)

4.33 Longitudinal Follow Up

At the Longitudinal Follow Up Visit 1, the following data will be collected (data collected at follow up noted). This visit may occur any time after consent and after the entry US has been graded. It should occur within 4 months of determination that the subject is eligible for longitudinal follow up; Group A HTG US, Group B NL US (matched Control), Group C cirrhosis or Group D homogenous).

1. Medical History
    a. Birth history: term, preterm (<36 weeks)
    b. Diagnosis of CF:
        i. Newborn screen (yes, no)
        ii. Prenatal diagnosis (yes, no)
        iii. Family History (yes, no)
        iv. Clinical diagnosis (yes, no)
            1. Pulmonary presentation (acute persistent respiratory symptoms)
            2. GI presentation (liver problems, steatorhea/diarrhea, rectal prolapse pancreatitis)
            3. Meconium ileus/intestinal obstruction
            4. Nutritional presentation (edema, electrolyte imbalance, FTT, hypoalbuminemia)
    c. Nutrition history: Will use the established Cystic Fibrosis Foundation Registry or the Toronto database to get their weight (wt) and height (ht) at annual visits to classify by z score
        i. Growth parameters at 1 and 2 years of age (wt, ht and wt for ht z scores).
        ii. Presence of diabetes
    d. Family History
        i. Maternal (biological mother) family history with an emphasis on liver diseases: whether child's biological mother is living, detailed liver disease history of all first order biological relatives including

the subject's mother, mother's siblings, and subject's maternal grandparents, as well as siblings of the subject.

ii. Paternal (biological father) family history with an emphasis on liver diseases: whether child's biological father is living, detailed liver disease history of all first order biological relatives including the subject's father, father's siblings, and subject's paternal grandparents, as well as siblings of the subject.

e. Surrogate markers for socioeconomic status: zip code of current residence, marriage status of parents, type of medical insurance of parents and subject, annual income of household and education level of parents

2. Current history: (Updated at follow up)
    a. Pulmonary symptoms at baseline for the last week (yes, no)
    b. Gastrointestinal symptoms at baseline using the Gastrointestinal Symptom Scale for Kids (GISSK)(32)
    c. Current medications
        i. Pancreatic enzyme supplementation (yes, no): largest dose of lipase (IU total) for a meal
        ii. Ursodeoxycholic acid – (yes, no) total daily dose (mg)
        iii. Chronic azithromycin (yes, no)
3. Physical examination: (Updated at follow up)
    a. Measurements: weight, length, BMI, triceps and subscapular skinfold thickness, mid-arm circumference
    b. Appearance: jaundice, cyanosis, spider hemangiomas, clubbing, palmar erythema, edema, (yes, no);
    c. Assessment of liver: liver span, texture, ascites, spleen palpable/not palpable
4. Laboratory data, based on standard laboratory studies obtained at least yearly by all participating centers (the study will not plan to pay for missing data): (Updated at follow up). This data will typically be collected at the subject's annual CF visit. Labs for the longitudinal follow up visit will be those collected at or after the longitudinal visit.
    a. total bilirubin, albumin, total protein, alkaline phosphatase, AST, ALT, GGT
    b. HCT, Hbg, WBC, platelet count, ANC (ANC = automated value on the CBC, if not available ANC = WBC times percent neutrophils+bands)
    c. glucose,
    d. calcium,
    e. 25-hydroxy vitamin D, alpha tocopherol, retinol,
5. FVC, FEV1 (values at the most recent routine visit: Percent of predicted using the Wang and Hankinson percent predicted equations). This should not be from a period of pulmonary exacerbation. (Updated at follow up)
6. Presence of *Pseudomonas aeruginosa* and/or *Burkholderia cepacia* complex in the sputum (EPIC definition) (yes, no) (Updated at follow up)
7. Collection of urine, serum and plasma (Updated at follow up) (Biospecimen collection may be timed to coincide with the subject's annual CF labs.
8. Collection of blood for DNA or cell lines (Once)

9. Collection of DNA from parents. (Once)
10. Standardized abdominal US (Updated every other year)
11. Elastography data determined locally
12. Quality of Life (Yearly starting with Longitudinal Follow Up Year 1 visit)
    a. CFQ-R Preschool Version (3-6 years old)
    b. CFQ-R Child Version (6-13 years old)
    c. CFQ-R parent Version (6-13 years old)
    d. CFQ-R Teen Adult (14 and up)
    e. CFQ-R Preschool version (3-5 years old)
    f. PEDS QL Child Report (8-12 years old)
    g. PEDS QL Parent Report (3-12 years old)

CFQ: This is a disease-specific, validated instrument designed to measure impact on overall health, daily life, perceived well-being and symptoms. It was developed specifically for use in patients with a diagnosis of cystic fibrosis. Three versions of the instrument have been developed: one for adults and adolescents 14 years of age and older (CFQ Teen/Adult); two for assessing children ages 6-13 years, one to be completed by the child and one to be completed by parent (CFQ Child and CFQ-Parent respectively). There is also a new tool for younger children (3-6)
PEDS QL is a validated pediatric specific, validated instrument with a parent and child component valid for assessing children >7 years of age. We will use the parent report for children 3-12 years of age and the child report for children >7 years of age. This instrument has been used in PEDS C, BARC, CLiC, and ChiLDREN/ChiLDReN.

Justification: These data fields represent intake data at enrollment. The data will be important for defining baseline characteristics of study participants; evaluating familial, genetic, epidemiologic and demographic factors that may be associated with possible development of cirrhosis; assessing diagnostic accuracy and utility of clinical, laboratory, histology and imaging studies; assessing baseline liver function, pulmonary function, nutritional status, physical findings and imaging findings that may be used in the analysis of factors that predict outcomes.

Cost of study to subjects: Subjects and their third party payers will not be charged for research-related tests. These include abdominal US, questionnaires, anthropometrics, urine collection and transport, DNA collection and transport, and research serum and plasma collection and transport.

4.4 Specimens to be Collected

During this study, blood and urine specimens will be obtained, de-identified and shipped to and stored at the NIDDK repositories for use in future CFLD ancillary studies. This "biobanking" is a critical aspect of this longitudinal study to facilitate the creation of a resource of DNA and other specimens from a meaningful number of patients with CFLD. In addition, obtaining and storing DNA or EBV-transformed leukocytes (from which DNA can be extracted) will allow future studies to investigate genetic causes and influences (modifier genes) in CFLD.

4.41 Research Blood and Urine Specimens

Biological mother and father:

1. 20 ml of whole blood in two 10 ml. EDTA vials to be sent to the NIDDK contract facility for DNA extraction.

Subject at initial enrollment into this study:

1. Draw 9 ml (in subjects <40 kg) or 26 ml (in subjects ≥40 kg) and divide as follows:
    - 2 ml (<40 kg) or 3 ml (≥40 kg) for serum
    - 2 ml (<40 kg) or 3 ml (≥40 kg) for plasma
    - **IF** Subject <40 kg: 5.2 ml of whole blood for lymphoblasts for DNA
    - **IF** Subject ≥40 kg: 20 ml of whole blood for DNA processing and storage.
2. Collect 5 ml of urine

Subject at follow up visits:

1. 2 ml (<40 kg) or 3 ml (≥40 kg) for serum
2. 2 ml (<40 kg) or 3 ml (≥40 kg) l of whole blood for plasma
3. Collect 5 ml of urine

Total Research Blood Drawn: The total volume of blood drawn for research only purposes from children enrolled in this study is outlined in Table 2. This volume should be within acceptable limits of all IRBs at Clinical Sites.

Table 2. Total amount of research blood drawn from subjects and parents

Visit	Amount in ml drawn from subjects <40 kg for research at the visit	Amount in ml drawn from subjects ≥40 kg for research at the visit	Amount in ml drawn from parents for research at the visit
Initial visit	9	26	20
Annual follow up visit	4	6	

Storage of serum and plasma will allow for further investigations of biomarkers. Plasma will be primarily reserved for proteomic development. As such preparation and storage will follow methods designed to optimize the use of plasma for proteomic analysis (33). The serum and plasma would be processed and aliquoted within 1-2 hours and stored at -80°C. Serum is stored as it is the most commonly available sample and should be available at a minimum for validation.

4. 42 Abdominal US

Abdominal US: This may be obtained at any time in the seven months after consent has been given.

The US examination will include a survey examination of the entire abdomen and a detailed examination of the liver and spleen to assess for presence or absence of liver

disease, including fibrosis/cirrhosis. Gray-scale images will be supplemented by Doppler ultrasound in all patients. The gray-scale images will be the determinant of longitudinal follow-up. The Doppler US will be the experimental arm of the study. US scans will be obtained after a minimum of a 4-hour fast for flow evaluation.

Liver echogenicity and contours will be assessed. Liver echogenicity will be evaluated by comparing it to that of the kidney. Irregularity or nodularity of the hepatic parenchyma and liver capsular surface, portal vein diameter, collateral vessel formation, ascites, and splenomegaly will be evaluated. Doppler US will be used to assess the presence and direction of flow in the main portal vein and right, main and left hepatic veins; and flow velocity will be assessed in the portal vein and resistive and pulsatility indices will be sought in the hepatic and splenic arteries.

To aid in statistical analysis, the liver ultrasound findings will be classified as follows:

Grade	**Appearance**
0	Normal (NL US)
1	Heterogeneous echogenicity
2	Diffuse homogeneous increased echogenicity
3	Heterogeneous liver texture with nodular parenchyma and margins, including enlargement of caudate lobe (indicates cirrhosis)(34)
4	Heterogeneous liver with nodular parenchyma and margins and at least 2 of the following: collateral vessels, splenomegaly, thickened omentum, large portal vein, reversed (hepatofugal) flow in the portal vein, enlargement of the hepatic arteries with increase in blood flow resistance (indicates portal hypertension)

There will be an initial training set and validation of consistency in the readings and grading of the US's for the participating radiologists. Center radiologists will undergo web-based training for grading of the liver ultrasounds. Centers will provide de-identified liver ultrasounds for the validation set (one of each grade). All center radiologists who will be blinded to the results, will then grade the liver ultrasound images. Results will be analyzed by the study statistician to determine inter and intra-observer reliability. A sample size of 35 ultrasounds with 7 observations per ultrasound achieves 91% power to detect an intraclass correlation of 0.700 under the alternative hypothesis when the intraclass correlation under the null hypothesis is 0.500 using an F-test with a significance level of 0.05. Center radiologists must meet the accuracy criteria outlined in the MOO before the study can start at any center.

The same training set used for the radiologists will be used to train the sonographers. In addition, there will be a written guide documenting the required images for the sonographers.

There will be ongoing assessment of the quality of the US and the consistency of the US readings. The first five US studies from each institution will be reviewed by the lead radiologist (MS) to ensure uniform quality. A scoring sheet of the image quality for each

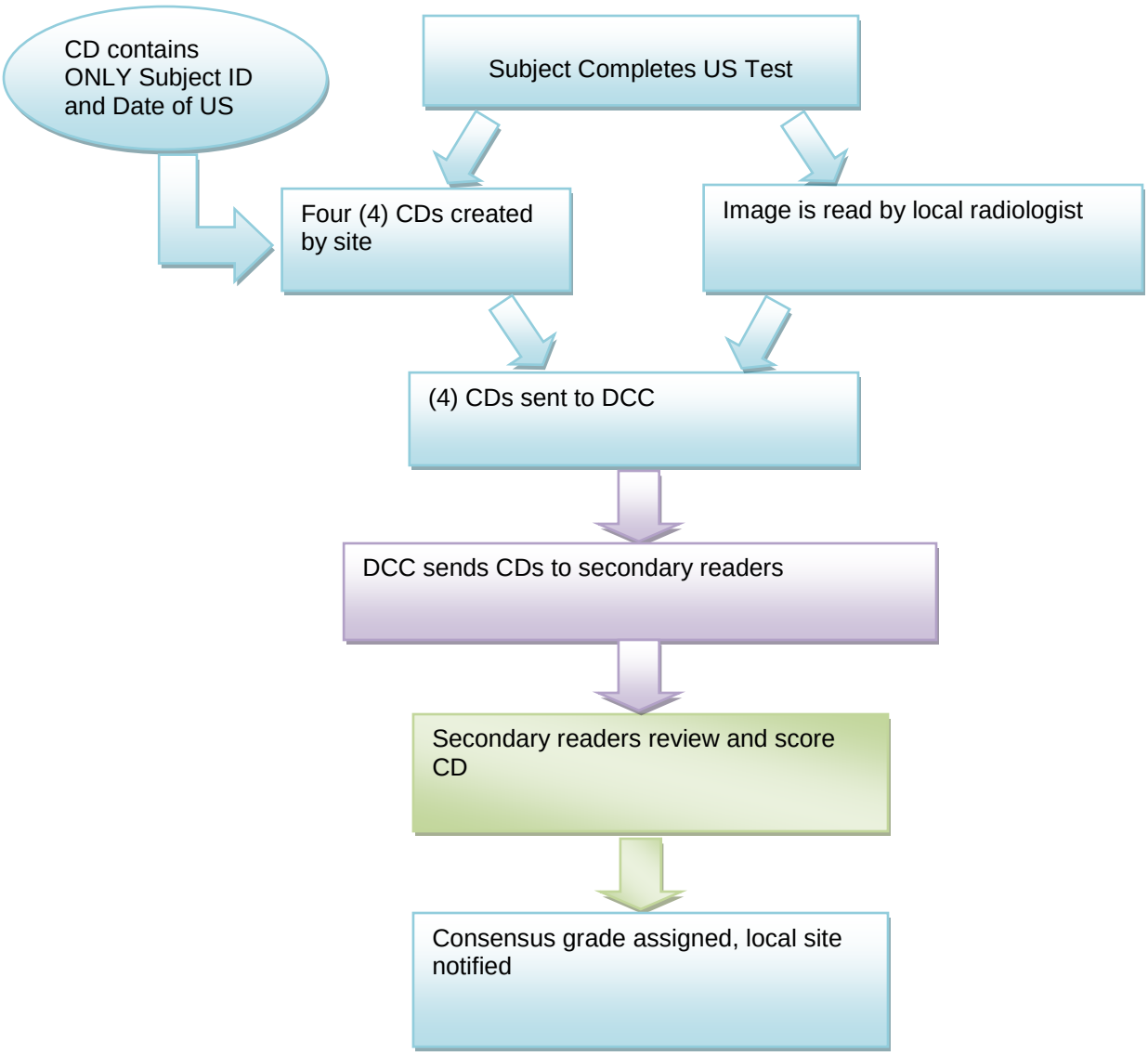
of the first five US scans will be completed and stored at the data center and also sent to the radiologist at each institution.

For each of the US images, there will be three readings of each image and assessment of the image quality (the study site radiologist reading and 2 other radiologists). The additional readings will rotate between the radiologists in the study as outlined in the MOO.

There will be ongoing assessment of discordance over time that will be tracked as a group and by individual radiologist as outlined in the MOO. We do anticipate occasional disagreement within one grade. All discordant readings will be reviewed by the lead radiologist (MS). Criteria for interventions are outlined in the MOO.

If a new radiologist is added to the study, the process for training and verification will be the same as outlined above.

Flow Diagram of Liver US Process



4.43 Specimens Repository

Central repositories have been established by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), a division of the National Institutes of Health, for long-term storage for data, biological specimens and for the establishment and storage of DNA cell lines and DNA extraction. Whole blood for cell lines or DNA extraction will be shipped immediately to the NIDDK genetic repository. Biological samples will be shipped via licensed overnight carrier to the NIDDK biospecimen repository. All specimens will include a research study identifier, but otherwise will be de-identified prior to shipment to either repository. A computer log will record all incoming samples at the central repository, the storage location, and the date, and the type of sample. Receipt of samples will be acknowledged to the originating center.

***NOTE**: Please see the Manual of Operations for detailed instructions on specimen collection and shipping.*

4.44 Specimen Use
Specimen use will be under the policies developed by the CFLD-NET steering committee during the study. Procedures for use of specimens and data after the study has ended are posted on the NIDDK repository website.

5. TERMINATION OR WITHDRAWAL OF SUBJECT PARTICIPATION

Subjects in the longitudinal follow up will be followed annually for approximately six years, through the completion of the Year 6 US and study closeout procedures. The subject or the subject's parents or guardians may request that the subject be removed from the study at any time. In addition, the investigator may withdraw a subject from the study if he/she determines that it is in the subject's best interests. Subjects who receive a liver transplant will end participation in the study.

Note: Upon request of the subject or the subject's parents or guardians, samples and data that have been submitted to the NIDDK repository or to the data coordinating center may be destroyed unless the samples have already been used or the data have been included in reported analyses or unless the linkage between the research identifier and the subject has been destroyed. When the study ends at a clinical site or the subject completes the study, the linkage between the samples and the subject will be destroyed. Once this linkage has been destroyed, it will no longer be possible to withdraw samples and data from the repository and the database in response to a subject request.

6. STATISTICAL CONSIDERATIONS

6.1 Sample Size and Power
The sample size calculations have been modified for Amendment 2 (date 5/30/2011). We have modified the calculations as the prevalence of heterogeneous US pattern in our study has not been as frequent as prior reports have suggested. Previous literature suggested a 15-20% prevalence of heterogeneous US in CF. In contrast, in this study, after 325 US studies, we have found a prevalence of 9%. Thus we have changed from matching heterogeneous US subjects 1:1 to 1:2. The sample size calculations have been further modified for Amendment 4 (11/11/2014). We have modified the calculations as the variability of US grading was shown to be larger than what has been expected prior to initiation of the study. To achieve a more reliable primary outcome, we extended the study period to 6 years after screening US and introduced an interim analysis based on year 4 US results with a more stringent definition of development of cirrhosis.

Characterizing the ability of US abnormalities to identify children at risk for progression to cirrhosis is the primary goal for this study. N =60 patients with heterogeneous livers will be compared with N = 120 patients with normal livers. Based on previous literature, in patients with a heterogeneous liver on US, 67% will progress to have US features consistent with cirrhosis and 46% will progress to portal hypertension (8). In patients

with a normal US, 7-13% will develop cirrhosis 5- 7.5% will develop portal hypertension. Thus, patients with a heterogeneous liver have a 5.2 fold increased incidence of cirrhosis and a 6.1 fold increased incidence of portal hypertension compared to children with a normal US.

Based on the literature and our experience to date, we expect by Year 6 follow-up, 26% of HTGs and 8% of NLs will have converted to CIR. We will conduct an interim analysis when all longitudinal NL and HTG subjects have finished Year 4 US visit. To control for type I error with multiple tests, we consider $p<0.025$ to be statistically significant for both interim analysis and analysis at the end of the study.

At Year 6 follow-up, group sample sizes of 60 and 120 achieve at least 80% power to detect a difference of 18% between the null hypothesis that both group proportions are 14% and the alternative hypothesis that the proportion in the heterogeneous liver (US) group is at least 26% using a two-sided chi-square test without continuity correction and with a significance level of 0.025. This corresponds to a relative risk of developing cirrhosis in the US group of 3.25 and an odds ratio of 3.9.

Preliminary analysis suggests that the US finding of CIR may be slightly more variable than we initially believed. Thus, when conducting the interim analysis, we will use a more stringent definition of development of cirrhosis: only cases graded as CIR with agreement among 3 or more radiologists (3/4 or 4/4) in the follow-up US visits will be considered as an event of converting to CIR in Group A (NL) and B (HTG). If the interim analysis shows significant difference between Group A and Group B, we will terminate the PUSH study.

With approximately 30 subjects in Group C (cirrhosis echo pattern on screening US) and 30 subjects in Group D (homogeneous echo pattern on screening US), the precision to estimate the incidence of portal hypertension or hepatic synthetic failure, and heterogeneous liver or cirrhosis, respectively, is provided in the table below.

Incidence Rate	Precision (Standard Error)
0.05	0.040
0.10	0.055
0.15	0.065
0.20	0.073
0.25	0.079

6.2. Analysis Plan

Outcome Variables

The specific aims of this study are to determine if imaging changes identified by ultrasound (US) predict the risk of progression of liver disease by comparing subjects with heterogeneous livers to those with normal livers.

The Primary Outcome is the development of cirrhosis

- Cirrhosis will be defined by imaging criteria (Grade 3 or 4 as described in 4.4 Section on US Grading) in group A or B

Secondary outcomes relate to effect on associated pulmonary and nutritional issues, signs and symptoms of portal hypertension and markers of hepatic synthetic function:

- Health related quality of life
- Growth (length, weight and BMI Z-score, anthropometrics)
- AST, ALT, GGTP
- FEV1, FVC
- Sputum Culture (Pseudomonas, Burkholderia cepacia)
- Use of IV antibiotics
- Hospitalization for treatment of pulmonary exacerbation
- CBC (WBC, Hbg, ANC, platelet count)
- Fat soluble vitamin levels (Vitamin E, 25 hydroxy vitamin D, Vitamin A)
- Appearance of signs or symptoms of portal hypertension among children with cirrhosis discovered during the study, including ultrasound findings and clinical symptoms
- Development of cirrhosis amongst children with homogeneous echogenicity at screening

Prediction of Cirrhosis from US: Chi square test of independence, logistic regression and multiple logistic regression will be used to assess the odds of developing cirrhosis between those with heterogeneous livers (Group A compared with patients with normal livers (Group B). Since data will be collected longitudinally, Cox Regression with time dependent covariates will also be used to examine time to onset of cirrhosis.

Analysis of Secondary outcomes: Mixed model longitudinal data analysis will be used to compare Group A and Group B over time on health related quality of life, growth (length, weight and BMI Z-score, anthropometrics, body composition), AST, ALT, GGTP, PT, INR, FEV1, rate of change in other PFTs, CBC (WBC, platelet count), spleen size on ultrasound and fat soluble vitamin levels. Logistic regression and general estimating equations will be used to compare results of Sputum Culture (Pseudomonas), use of IV antibiotics, and hospitalization for treatment of pulmonary exacerbation.

For subjects in Groups C (cirrhosis on screening US) and D (homogeneous echogenicity on screening US), the analytic goal is estimation of the incidence of portal hypertension analysis of hepatic synthetic function, and the incidence of progression to heterogeneous liver or cirrhosis. Exact confidence intervals will be calculated.

Descriptive statistics will be provided for each of the secondary outcomes for the subjects in each of the groups defined by screening ultrasound: heterogeneous (Group A), normal (Group B), cirrhosis (Group C) and homogeneous (Group D).

7. DATA MANAGEMENT

7.1 Case Report Forms

The Data Coordinating Center at the University of Michigan is responsible for data management and analysis. It is the data coordinating center for several multi-center trials. Case report forms are developed by the data coordinating center (DCC) and published on the CFLD-NET password-controlled website. The case report forms do not contain any personal subject identifiers, except dates, such as date of birth (which will be converted to age prior to storage in the NIDDK repository), which are necessary for research purposes. As needed, the coordinator prints the forms for each subject. A combination of web-enabled and centralized data entry and management will be used. After the case report forms for a visit are completed, the research coordinator will enter a limited set of data into a web-enabled data management system. Original case report forms will be securely maintained at the clinical sites. Clean copies of the case report forms will be sent monthly by overnight carrier to the DCC where they will be entered into the database using double entry. The forms entered by the coordinator (except for those containing personal identifying information) will also be sent to the DCC and entered a second time for verification. Forms with personal identifiers are not sent to the DCC; they remain at the clinical site.

7.2 Quality Assurance

The Project Manager/Clinical Monitor will review the data submitted to the DCC for accuracy and completeness. The Project Manager/Clinical Monitor will communicate with the study coordinators at each site about queries generated by the DCC and address all questions and concerns regarding the study protocol and problems with data entry or specimen sample shipment. Site visits will be made approximately every 24 months. Interim site visits may be made to centers with low compliance or high error rates. Performance reports will be generated quarterly to investigators and study coordinators at each center, as well as to DSMB.

7.3 Training

The Project Manager will develop a manual of operations to assist study coordinators at each center in following the protocol, entering and transferring data, and collecting, processing and shipping samples. The Project Manager and Clinical Monitor will be responsible for training the study coordinators at each center about the study protocol, the completion of source documents, the use of the web-based data entry system, and proper procedures with shipping samples to the central repository. Test runs of data entry into the web-based data entry system as well as sample shipment will be organized prior to site initiation. The Project Manager/Clinical Monitor will review the study protocol and data entry system, and check all regulatory documents prior to site initiation. A meeting for all investigators and study coordinators will be held in conjunction with the initiation of the study. In-service training for all study coordinators will be held quarterly via conference calls to review frequently encountered questions regarding the protocol, data entry or sample processing.

8. ADVERSE EVENTS

8.1 Risks

There are minimal physical and psychological risks from participating in this study. Medical risks include those related to the identification or failure of identification of abnormalities or incidental findings on the abdominal ultrasound. These results will be discussed by the study investigators with the patient and family and the patient's care team with further management/referral if necessary directed by the care team. Psychological risks to the family and child include gaining additional knowledge about the child's liver disease or the suggestion of the presence of possible (but unproven) liver disease or about possible associations of family or genetic factors with the child having developed liver disease. The risks of venipuncture for the serum bank are pain, bruising or superficial phlebitis. The risks of genetic information being revealed by any future investigations in the Consortium are very slight since the blood samples will be de-identified prior to being deposited in the repository. If there is a loss of confidentiality, the risks include: that knowledge of a genetic risk may be emotionally stressful to a family member, that this might change eligibility for new health, disability or life insurance, that there may be unforeseen paternity issues, and that genetic testing may reveal information regarding health risks to other members of the family who are living or not yet born. Abdominal Ultrasound is a routine, noninvasive procedure. There are no known risks from performing this procedure. Occasionally, patients may experience mild discomfort from lying still on their back during the examination. All possible techniques for relieving patient discomfort will be attempted. Patients unable to tolerate the examination will be excluded from the study.

8.2 Adverse Events

Although mechanisms for reporting serious adverse events have been established, it is anticipated that there will not be any serious adverse events that can be attributed to this study – there will be serious adverse events that are expected clinically in this study population. The only serious adverse events related to the performance of this study are those related to phlebotomy and potential psychological issues related to the uncertainty of the US findings.

An adverse event (AE) is any unfavorable, harmful or pathological change in a research subject as indicated by symptoms, psychological or physical signs and/or clinically significant laboratory abnormalities that occur in association with the study procedures. This definition includes intercurrent illness, injuries, exacerbation of pre-existing conditions. Stable pre-existing conditions and elective procedures to address such conditions are not adverse events. A change in a laboratory variable is considered an adverse event if it was considered by the investigators to be clinically significant (that is, if it institutes a diagnostic evaluation or indicates additional therapy is necessary).
The term serious is based on patient outcome associated with events that could threaten a patient's life or functioning. An event should be considered serious if it results in any of the following:

- Death,
- Life-threatening (patient was at risk of death as a result of the event, does not include hypothetical risk of death if the event had been more severe),
- Inpatient hospitalization or prolongation of existing hospitalization,
- Persistent or significant disability or incapacity,

- Congenital anomaly or birth defect,
- Medical or surgical interventions required to prevent one of the outcomes listed above.

The phrase 'related to study' implies causality or attribution to the study procedures. For purposes of defining as SAE, if a causal relationship cannot be ruled out, then an AE should be considered 'related to the study procedure(s)'. As noted above, it is very unlikely that any adverse events will be attributable to this study.

8.3 Data Safety Monitoring Board

The NIDDK has set up a Data Safety Monitoring Board (DSMB) to oversee this study. The DSMB will act in an advisory capacity to NIDDK to monitor patient safety, data quality and to evaluate the ability of the CFLD-NET to achieve its research goals. Members of the DSMB are independent of the study investigators and represent disciplines related to liver disease, cystic fibrosis, biostatistics, and radiology..

8.4 Reporting of Serious Adverse Events

Each clinical investigator is responsible for reporting serious unexpected adverse events to the IRB at their institution, to the DCC, and to the NIDDK representative in an expedited manner.

9. COSTS AND PAYMENTS TO PARTICIPANTS

In addition to the collection of routinely obtained clinical data and the results of routine laboratory investigations, this research includes the performance of an abdominal US every other year and taking extra blood (7-27 ml depending on the visit), urine, and samples of blood from parents. There will be no costs to the patient or their insurance for any research-related data collection, including the special laboratory investigation. The expenses for the abdominal US, storage and handling of the extra research blood and urine samples are covered by the research. For each scheduled follow up visit the subject or the parents or guardians will receive reimbursement for parking, related to the visit.

10. ETHICAL CONCERNS AND INFORMED CONSENT

There are minimal physical and psychological risks from being in this study. For this study, the risks of venipuncture at the time of the blood draws are pain, bruising or superficial phlebitis. There are no known risks associated with the performance of an abdominal ultrasound except that the diagnosis of abnormal ultrasound may result in psychological stress for the patient and/or family.

The risks of genetic information being revealed by any future investigations in the Consortium are very slight since the blood samples will be de-identified prior to being deposited in the repository; only a research study number will be included in the database and all dates will be converted into ages by the DCC prior to dissemination to the repository or to any laboratory conducting genetic studies, While the study is ongoing, the clinical site will maintain a link between the research study number and the subject's identity. However, this information will not be contained in any data file that is transmitted to the DCC or to the repository. When the study ends (or CFLD ceases to

exist), each clinical site will destroy the linkage between the research study number and the subject's identity.

There is also a risk that the subject's US staging will be revealed as a result of the transport of US studies to the centers for re-reading. US studies will only contain a study ID with no PHI.

If there is a loss of confidentiality, the risks include: that knowledge of a genetic risk may be emotionally stressful to a family member, that this might change eligibility for new health, disability or life insurance, that there may be unforeseen paternity issues, and that genetic testing may reveal information regarding health risks to other members of the family who are living or not yet born.

Methods Taken to Reduce Subject Risks: The study anticipates no excessive risks to the subjects, except the possible pain associated with blood draws and the minimal risks associated with 4 abdominal US examinations over approximately 6 years. EMLA cream may be applied to sites of all blood draws and intravenous lines to minimize pain with these procedures. Whenever possible, blood draws on participants will occur when venipuncture will be performed for clinical indications. They can be performed through indwelling IV lines, placed for clinical purposes. Psychological risks will be minimized by careful explanation of the risks and maintaining complete confidentiality and data security. The results of the US will be communicated to the subject's parents or guardians, and their physician. A standard protocol for the information to be given to subjects regarding the US results will be used.

Recruitment Plan: Participants will be recruited from patients evaluated and followed at the CFLD-NET clinical sites. The investigator or clinical research coordinator will recruit the patients, parent(s) or guardian(s) during clinic visits or, less commonly during an inpatient admission to the hospital. The investigator or his or her designee will discuss the study design, benefits and possible risks with the family. Printed information about the study and the consent/assent form will be given to the family.

Advertising Strategy for Voluntary Enrollments. Since the study will enroll children who are currently followed at their respective centers, we do not anticipate advertising outside of the centers for this study.

Duration of enrollment. We anticipate enrolling all of the participants into this study during years one and two. Thus, we will be enrolling participants in year one and two of the study, to provide at least three year follow-up on all participants.

Drug information: There will be no drug treatment or therapeutic intervention mandated by this study. Participants will be treated by routine local therapy standards, and the treatments given will be recorded in this database.

Supportive care guidelines: Participants will be cared for by the local routine standard of care for children with CF.

Informed Consent: A common template for the informed consent form will be used by all of the centers, modifying the content or format as necessary to meet the requirements of their respective institutional human subjects committees. The subject's family or guardian will retain a signed consent form; one will be retained for the subject's chart; and one will be included in the research records. As extending the study will lead to some of the subjects turning 18, subjects will be reconsented at the next visit after they turn 18 years of age.

All potential participants that are identified by the local PI and/or designee that meet the inclusion/exclusion criteria will be given the opportunity to participate. Parents/guardians/ participants will be given the consent/assent forms to review and ask questions about the study. Parents/guardians/participants will be asked to summarize in their own words what participation in this research study involves and that they are comfortable with the risks and benefits of participating in the research study. Any additional questions they have will also be answered prior to signing the consent/assent. Once the consent/assent form is signed, a copy will be provided to the subject, or parent/guardian. Consent will be obtained for all participants by the Principal Investigator and/or designee with appropriate training regarding human participant protection and HIPAA compliance, as established by the local institutional regulatory requirements. Non-English speaking participants will be able to participate in the study; local IRB requirements will dictate if a consent form in English may be used.

Special Consent/Assent Plan: Spanish only speaking population will not be excluded from this study. Local IRB regulations will be followed regarding the need for translation of the consent form.

Importance of the Knowledge to be Gained from This Research: At present, there is no reliable way to assess for early involvement of the liver in CF and to predict the possibility of progression to cirrhosis. A better understanding of the etiology and pathogenesis of CFLD may lead to improvements in diagnosis, treatment and prevention in the future, resulting in decreased morbidity and mortality. Identification of a group of patients with CF who are at risk for the development of cirrhosis would allow new therapies for the prevention of cirrhosis in this more targeted population. Improved therapy for CFLD could lead to a reduced prevalence of cirrhosis and improved outcomes in children with CF. The detailed anatomical and physiological abnormalities that occur in CFLD are difficult to evaluate with routine clinical or imaging studies. If Doppler US can detect early physiological abnormalities of CFLD, it could change the way patients with this disease are evaluated and also it could help modify treatment options.

Possible Health Benefits to Participants: The participant may not directly benefit from participation in this research, but in the future other children with similar problems may benefit from new information that may lead to better medical care. The abdominal US may reveal abnormalities previously not suspected. In this case, the results will be sent to the patient's primary care physician and primary CF physician who will determine the course of management. The finding of a heterogeneous liver may or may not indicate a risk for the development of more advanced cirrhotic liver disease. However, on occasion

the results may help further explain detailed anatomical and physiological abnormalities that have occurred in an individual patient and help explain the liver damage. This information may alter patient management

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12. APPENDIX

Participating centers:

Johns Hopkins School of Medicine (Baltimore, MD)
Indiana University School of Medicine and Riley Hospital for Children (Indianapolis, IN)
University of Colorado/Children's Hospital Colorado (Aurora, CO)
Baylor School of Medicine (Houston, TX)
Washington University School of Medicine (St Louis, MO)
University of Minnesota Children's Hospital, Fairview (Minneapolis, MN)
The Hospital for Sick Children (Toronto, Canada)
Cincinnati Childrens Hospital Medical Center (Cincinnati, OH)
Emory University School of Medicine (Atlanta, GA)
University of Washington (Seattle, WA)
Ann & Robert H. Lurie Children's Hospital (Chicago, IL)

The ethnicity and racial categories of all of the anticipated participants in the study are outlined in Table 1. The ethnicity and racial categories of the participants in the longitudinal follow up are outlined in Table 2.

Table 1: Enrollment for patients in the study

Denver, Indiana, St Louis, Hopkins, Minnesota, Houston and Toronto Based on 30% of 3-12 year olds

PART A. TOTAL ENROLLMENT REPORT: Number of Subjects Enrolled to Date (Cumulative) by Ethnicity and Race				
	Sex/Gender			
Ethnic Category	**Females**	**Males**	**Unknown or Not Reported**	**Total**
Hispanic or Latino	30	39		69 **
Not Hispanic or Latino	334	303		637
Unknown (individuals not reporting ethnicity)	44	50		94
Ethnic Category: Total of All Subjects*	408	392		800 *
Racial Categories				
American Indian/Alaska Native	3			3
Asian	3			3
Native Hawaiian or Other Pacific Islander				
Black or African American	14	20		34
White	379	368		747
More Than One Race	3			3
Unknown or Not Reported	6	4		10
Racial Categories: Total of All Subjects*	408	392		800 *

PART B. HISPANIC ENROLLMENT REPORT: Number of Hispanics or Latinos Enrolled to Date (Cumulative)				
Racial Categories	**Females**	**Males**	**Unknown or Not Reported**	**Total**
American Indian or Alaska Native				
Asian				
Native Hawaiian or Other Pacific Islander				
Black or African American	2	5		7
White	28	27		55
More Than One Race		7		7
Unknown or Not Reported				
Racial Categories: Total of Hispanics or Latinos**	30	39		69 **

Table 2 Enrollment for patients in the longitudinal component of the study

Denver, Indiana, St Louis, Hopkins, Minnesota, Houston and Toronto Based on 30% of 3-12 year olds

PART A. TOTAL ENROLLMENT REPORT: Number of Subjects Enrolled to Date (Cumulative) by Ethnicity and Race				
	Sex/Gender			
Ethnic Category	**Females**	**Males**	**Unknown or Not Reported**	**Total**
Hispanic or Latino	9	11		20 **
Not Hispanic or Latino	90	83		173
Unknown (individuals not reporting ethnicity)	12	15		27
Ethnic Category: Total of All Subjects*	111	109		220 *
Racial Categories				
American Indian/Alaska Native	1			1
Asian	1			1
Native Hawaiian or Other Pacific Islander				
Black or African American	4	6		10
White	102	102		204
More Than One Race	1			1
Unknown or Not Reported	2	1		3
Racial Categories: Total of All Subjects*	111	109		220 *

PART B. HISPANIC ENROLLMENT REPORT: Number of Hispanics or Latinos Enrolled to Date (Cumulative)				
Racial Categories	**Females**	**Males**	**Unknown or Not Reported**	**Total**
American Indian or Alaska Native				
Asian				
Native Hawaiian or Other Pacific Islander				
Black or African American	1	1		2
White	8	8		16
More Than One Race		2		2
Unknown or Not Reported				
Racial Categories: Total of Hispanics or Latinos**	9	11		20 **