NASH CRN

Nonalcoholic Steatohepatitis
Clinical Research Network

Cysteamine Bitartrate Delayed-Release for the Treatment of Nonalcoholic Fatty Liver Disease (NAFLD) in Children (CyNCh) Trial

IND # 114,924

Protocol

CONFIDENTIAL

06 September 2013

Contents

Design Synopsis

Title

- Cysteamine Bitartrate Delayed-Release for the Treatment of Nonalcoholic Fatty Liver Disease (NAFLD) in Children (CyNCh) Trial

Sponsor

- NIDDK

Objective

- Primary Objective: To evaluate whether 52 weeks of treatment with cysteamine bitartrate delayed release (DR) in children improves NAFLD as measured by changes in histology, compared to treatment with placebo.

Population

- Pediatric participants ages 8-17 years of age with histologically confirmed NAFLD

Type of trial

- Phase IIb randomized clinical trial
- Multicenter, double-masked, placebo-controlled, parallel treatment groups consisting of children with histologically-confirmed NAFLD with a 52 week histological change as the primary outcome.

Treatment groups

- Group 1: Cysteamine bitartrate delayed-release (DR) capsules (300 mg, 375 mg or 450 mg orally twice a day)
- Group 2: Placebo

Inclusion criteria:

1. Children age 8-17 years
2. Liver biopsy within 90 days of screening visit and not more than 120 days before randomization
3. Clinical history consistent with NAFLD
4. Definite NAFLD based upon liver histology
5. No evidence of any other liver disease by clinical history or histological evaluation
6. A histological severity of: NAFLD Activity Score (NAS) ≥ 4.
7. Sexually active female participants of childbearing potential (i.e., not surgically sterile [defined as tubal ligation, hysterectomy, or bilateral oophorectomy]) must agree to utilize the same two acceptable forms of contraception from screening through completion of the study and to complete a pregnancy test at each study visit. The acceptable forms of contraception for this study include hormonal contraceptives (oral, implant, transdermal patch, or injection) at a stable dose for at least 3 months prior to screening, and barrier (condom with spermicide, diaphragm with spermicide). Sexual activity will be ascertained at each study visit for post-menarchal females and if sexually active, subject must verify use of the same 2 acceptable forms of contraception. For pre-pubescent children, a documented attestation of abstinence from their parent or guardian will be acceptable.
8. Participants must be able to swallow cysteamine bitartrate DR capsules
9. Written informed consent from parent or legal guardian

10. Written informed assent from the child

Exclusion criteria:

1. There will be no exclusion criteria based on race, ethnicity or gender.
2. Participants with a current history of the following conditions or any other health issues that make it unsafe for them to participate in the opinion of the Investigators:
   - Inflammatory bowel disease (if currently active) or prior resection of small intestine
   - Heart disease (e.g., myocardial infarction, heart failure, unstable arrhythmias)
   - Seizure disorder
   - Active coagulopathy
   - Gastrointestinal ulcers/bleeding
   - Renal dysfunction with a creatinine clearance < 90 mL/min/m^2
   - History of active malignant disease requiring chemotherapy or radiation within the past 12 months prior to randomization
   - History of significant alcohol intake (AUDIT questionnaire) or inability to quantify alcohol consumption
   - Chronic use (more than 2 consecutive weeks) of medications known to cause hepatic steatosis or steatohepatitis (systemic glucocorticoids, tetracycline, anabolic steroids, valproic acid, salicylates, tamoxifen) in the past year.
   - The use of other known hepatotoxins within 90 days of liver biopsy or within 120 days of randomization
   - Initiation of medications with the intent to treat NAFLD/NASH in the time period following liver biopsy and prior to randomization
   - History of total parenteral nutrition (TPN) use in year prior to screening
   - History of bariatric surgery or planning to undergo bariatric surgery during study duration
   - Clinically significant depression (patients hospitalized for suicidal ideations or suicide attempts within the past 12 months)
   - Any female nursing, planning a pregnancy, known or suspected to be pregnant, or who has a positive pregnancy screen.
3. Non-compensated liver disease with any one of the following hematologic, biochemical, and serological criteria on entry into protocol:
   - Hemoglobin < 10 g/dL;
   - White blood cell (WBC) < 3,500 cells/mm^3 of blood;
   - Neutrophil count < 1,500 cells/mm^3 of blood;
   - Platelets < 130,000 cells/mm^3 of blood;
   - Direct bilirubin > 1.0 mg/dL
   - Total bilirubin >3 mg/dL
   - Albumin < 3.2 g/dL
   - International normalized ratio (INR) > 1.4
4. Poorly controlled diabetes mellitus (hemoglobin A1c (HbA1c) > 9%)
5. Evidence of other chronic liver disease:
   - Biopsy consistent with histological evidence of autoimmune hepatitis
   - Serum hepatitis B surface antigen (HBsAg) positive.
   - Serum hepatitis C antibody (anti-HCV) positive.
   - Iron/total iron binding capacity (TIBC) ratio (transferrin saturation) > 45% with histological evidence of iron overload
   - Alpha-1-antitrypsin (A1AT) phenotype/genotype ZZ or SZ
   - Wilson's disease

6. Children who are currently enrolled in a clinical trial or who received an investigational study drug within 180 days of screening or liver biopsy.
7. Subjects who are not able or willing to comply with the protocol or have any other condition that would impede compliance or hinder completion of the study, in the opinion of the investigator.
8. Failure to give informed consent

Outcome measures:

- **Primary:**

  Centrally scored and masked assessment of histologic improvement in NAFLD between the baseline liver biopsy and follow-up biopsy after 52 weeks of treatment, where improvement is defined as: (1) decrease in NAS of 2 or more and (2) no worsening of fibrosis.

- **Secondary:**
  - Reduction in serum aminotransferase and gamma-glutamyl transpeptidase.
  - Reduction in MRI-determined hepatic fat fraction.
  - Changes to markers of oxidation and anti-oxidant status: malondialdehyde, F2 alpha-isoprostane, total antioxidant capacity, oxidized LDL
  - Changes in fasting insulin and glucose
  - Changes in weight, height, BMI, and waist circumference
  - Changes in the Peds QL score
  - Changes to any symptoms the patient may have experienced
  - Proportion with a change from a histological diagnosis of definite NASH or indeterminate for NASH to not NASH at end of treatment
  - Individual histological characteristics at end of treatment compared to baseline such as steatosis, lobular inflammation, portal chronic inflammation, ballooning, fibrosis score and stage 1a vs. 1b fibrosis
  - Change in mean NAS

Study duration (per participant)

- Screening must occur within 90 days of liver biopsy and randomization within 120 days of liver biopsy.
- 52 week treatment period
- 24 week post–treatment follow-up

Study duration (calendar time):

- Recruitment phase**: 1**9 months
- Follow-up phase: 37 months
- Expected rate of recruitment is 16 per clinical center; approximately 2 per month

Number of clinical centers

- 10

Randomization: Centrally administered randomization stratified by clinical center

Visit schedule:

- Screening visit must occur within 90 days of liver biopsy and randomization within 120 days of liver biopsy.
- Randomization: Final pre-treatment interview followed by web-based randomization into one of 2 groups to receive either cysteamine bitartrate DR or placebo. The randomization design will be stratified by clinical center and baseline body weight into one of three categories (less than or equal to 65 kg, greater than 65 kg up to 80 kg, or greater than 80 kg) with assignments in permuted blocks of random length within each stratum to achieve a target dose of 9 to 12 mg/kg per day up to a maximum total dose of 600-900 mg per day and dispensing of study drug
- Follow up visits (N=6):
  - 4, 12, 24, 36, and 52 treatment weeks after randomization
  - 76 weeks after randomization (24 weeks after treatment ends)
- Both groups will be administered current standard of care nutrition and exercise recommendations, as a series of one page hand-outs given to participants at randomization and each follow-up study visit (one hand-out per visit). These one-page handouts will include serial strategies to limit screen time, reduce saturated fat, simple carbohydrate and fructose intake, and increase physical activity, as well as fruit and vegetable intake.

Liver biopsy schedule:

- Standard of care biopsy prior to screening for the trial
- 52 treatment weeks after randomization

MRI schedule:

- Prior to treatment initiation
- 52 treatment weeks after randomization (to coincide with liver biopsy when possible)

Statistical Analysis:

- The primary analysis is an intention-to-treat analysis in which the proportions of subjects in the active treatment group (cysteamine bitartrate DR orally twice daily) with histological improvement in NAFLD (primary outcome, defined above) is compared with the proportion of subjects in the placebo group in whom there is improvement. The comparison is made using a stratified (by clinical site) Mantel-Haenszel chi-square test; a P-value of 0.05 will be considered statistically significant. Subjects who do not undergo an end-of-treatment biopsy will be counted as not improved.

Sample size and assumptions:

- Total of **160** participants in 2 groups of equal size (**80** per group)
- Primary comparison: cysteamine bitartrate DR vs. placebo
- Primary outcome measure: Histological improvement in NAFLD
- Error protection
  - Type I = 0.05
  - Type II = 0.10 (90% power)
- Uncorrected chi-square test using Dupont and Plummer, Power and Sample size software (1998)

- Missing data: 10% will not have 52 week biopsy and will be considered not improved
- Minimum clinically important difference = 33% relative reduction in percent without clinically important improvement in NAFLD in the active treatment group compared to placebo group.
- Allocation ratio of active treatment to placebo groups = 1:1
- Assumed response rates: Expected percent with no clinically important improvement in NAFLD in the placebo group: 75% (based on TONIC data and assumed background use of medications that may influence histology = 33%)
- Expected percent with lack of clinically important improvement in NAFLD in the cysteamine DR group: 50%

Safety Monitoring

- NIDDK-appointed DSMB will monitor the data for safety and efficacy for outcomes such as hepatotoxicity, pregnancy, and any other outcomes or events identified as safety related.

1. Objectives

We hypothesize that administration of cysteamine bitartrate DR for 52 weeks in children with nonalcoholic fatty liver disease (NAFLD) will result in improvement in liver disease severity as measured by changes in the NAS.

The principal objective of this randomized, multicenter, double-masked placebo-controlled trial is to evaluate whether 52 weeks of treatment with cysteamine DR in children improves NAFLD as measured by changes in histology, compared to treatment with placebo.

Secondary objectives include:

- To assess the safety and tolerability of cysteamine DR in children with NAFLD when given for 52 weeks.
- To assess the effects of cysteamine DR on:
  - Traditional hepatic biochemistry--serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST) and serum γ-glutamyl transferase (GGT).
  - Liver fat fraction, as measured by magnetic resonance (MR)
  - Markers of oxidation and anti-oxidant status: malondialdehyde, F2 alpha-isoprostane, total antioxidant capacity, 4-hydroxynonenol
  - Insulin resistance
  - Anthropometrics
  - Quality of life
  - Liver related symptoms
  - Proportion with a change from a histological diagnosis of definite NASH or indeterminate for NASH to not NASH at end of treatment
  - Individual histological characteristics at end of treatment compared to baseline such as steatosis, lobular inflammation, portal chronic inflammation, ballooning, fibrosis score and stage 1a vs. 1b fibrosis
  - Change in mean NAS

2. Background and significance

2.1 Introduction

NAFLD has become the most common form of chronic liver disease in the developed world. It commonly occurs in the setting of obesity, insulin resistance and a sedentary lifestyle and it is often considered the liver manifestation of the metabolic syndrome. The primary form of treatment is optimization of lifestyle including nutrition and exercise. In the real world, success is limited by the difficulty of diet and exercise for many children, as well as the fact that NAFLD may not always respond to these lifestyle interventions even when fully implemented. Therefore, pharmacologic treatments have been sought but none has proved universally efficacious. This may be related to the fact that the histopathological changes seen on liver biopsy currently described as NASH may be the result of multiple pathogenetic mechanisms acting in concert to varying degrees. Based on the prevalence and risk of progression of NAFLD to cirrhosis and cancer, the burden of significant disease is large, and drug therapy to prevent or treat NAFLD is needed.

While the pathologist's approach to liver histology is not age-specific, histologic features differ in adults and children, therefore data from adult studies cannot be extrapolated to the pediatric population.[1]

2.2 Definitions

Nonalcoholic fatty liver disease (NAFLD) is defined by the presence of greater than "normal" amounts of fat in the liver. The pathologists' definition is based on observed steatotic droplets (triglyceride) exceeding 5% of surface area. This figure evolved from older studies showing that the normal liver was 5% lipid. In the largest population study of adults using MR spectroscopy, the threshold value for abnormal liver fat fraction was similar to these other assessments.[2]

Nonalcoholic steatohepatitis (NASH) is the name applied to a constellation of biopsy abnormalities occurring in the presence of NAFLD that typically includes hepatocyte ballooning with or without Mallory-Denk bodies, a mixed polymorphonuclear leukocyte and mononuclear inflammatory cell infiltrate in the lobules, chronic inflammation in the portal tracts and sometimes zone 3 perisinusoidal fibrosis.[3]

A name for ***NAFLD that is not NASH*** has not been universally established. Terms such as nonalcoholic fatty liver (NAFL), simple steatosis, benign steatosis, bland steatosis, and isolated steatosis have been used, but each has limitations that preclude general acceptance.[4]

2.3 Significance of NAFLD

Prevalence

In the Study of Child and Adolescent Liver Epidemiology (SCALE) for children ages 2-19 the standardized prevalence of NAFLD was 9.6% after adjusting for age, gender, race, and ethnicity.[5] Studies evaluating the prevalence of NASH in children vary greatly by setting. In hepatology clinics in San Diego 84% of children, and in Italy 86% with biopsy proven NAFLD were reported as having NASH.[6,7] However, in the San Diego based SCALE study, only 23% of children with NAFLD showed evidence of NASH.[5] In the NASH CRN study, which included American children from various geographic locations, 38% had borderline steatohepatitis and 39% were found to have definite NASH.[8] Among morbidly obese American adolescents undergoing bariatric surgery, intra-operative biopsy revealed 83% had NAFLD while only 20% had NASH.[9]

Progression to cirrhosis

In studies of children undergoing liver biopsy for suspected NAFLD, rates of cirrhosis have been reported to range from 2 to 10%.[1,7,10] Of those patients without evidence of cirrhosis on their initial biopsy, the risk for developing cirrhosis may vary by histology and subtype. In adults with steatohepatitis, Matteoni et al reported that one in four patients went on to develop cirrhosis.[11] Longitudinal data are needed to further elucidate the risk of disease progression in pediatric NAFLD.

Development of hepatocellular carcinoma

Multiple cross-sectional studies suggest that NAFLD is a significant risk factor for the development of hepatocellular carcinoma (HCC).[12-14] It is estimated that roughly 1,000 cases of HCC in the United States each year can be attributed to NAFLD.[15] One prospective study of adult patients with NASH cirrhosis found HCC to develop in roughly 7% of patients over a 10 year period.[16] Little is known about the risk for HCC in children. However, the long duration of NASH may impact the development HCC for those who have NAFLD as children.

Comorbidities

Current data suggest that NAFLD confers an increased risk for the development of cardiovascular disease, particularly through its association with metabolic syndrome. The key components of metabolic syndrome include central obesity, impaired glucose tolerance, elevated blood pressure, and dyslipidemia.[17] A study demonstrated NAFLD to be more frequent among children with metabolic syndrome compared to children without metabolic syndrome.[18] Of the 300 children evaluated, those with biopsy-proven NAFLD had a significantly greater cardiovascular risk profile. Higher values for fasting glucose, insulin, low-density lipoprotein cholesterol, triglycerides, and systolic blood pressure were observed in the NAFLD group. In a Swedish cohort study, patients with a history of steatohepatitis had significantly higher rates of cardiovascular disease and mortality when compared to patients with isolated steatosis.[19] Identification of NAFLD should prompt consideration of cardiovascular health and relative risk reduction through lifestyle changes.

Children with NAFLD may have an increased risk for developing type 2 diabetes. In large studies of children with biopsy-proven NAFLD, 5 to 10% of children have type 2 diabetes at the time of diagnosis.[7,20] Additionally, nearly 50% of children diagnosed with type 2 diabetes have suspected fatty liver based on ALT elevation.[21] Progression to diabetes is important to understand from both preventive and therapeutic standpoints. In adults, the risk for development of diabetes may be as high as 20 to 25% over 5 years.[22,23]

2.4 Pathogenesis of NASH

The majority of studies on the pathogenesis of NAFLD have been in the adult population. While pediatric and adult NAFLD share many characteristics, known differences between the two, including histological differences, indicate variation in the development of pediatric versus adult NAFLD.[24,25] While our understanding of the pathogenesis is in its infancy, obesity, central adiposity, and insulin resistance are strongly associated with pediatric NAFLD and inflammation with progression to NASH.

Insulin resistance may be defined as the state in which a given concentration of insulin is associated with a lower than normal uptake of glucose by tissues such as muscle and liver. Insulin resistance is associated with multiple metabolic abnormalities including metabolic syndrome, abnormal glucose metabolism, reproductive abnormalities in women, and cutaneous abnormalities including acanthosis nigricans and skin tags.[26-28] Systemic insulin resistance is thought to be critical to the pathogenesis of pediatric NAFLD and has been demonstrated to be present in a majority of children with biopsy-proven NAFLD.[7] Studies in animals provide insight to the physiologic link between insulin resistance and fatty liver; hyperinsulinemia and insulin resistance result in increased

adipose tissue lipogenesis and very low density lipoproteins (VLDL) uptake resulting in increased adipocyte fat sequestration and obesity.[29] Insulin resistance in both adipose and liver tissue may be integral to the development of NAFLD.

Mechanisms of hepatocellular injury

To the extent that mitochondrial dysfunction, ATP depletion, endoplasmic reticulum stress, and oxidant stress play a role in hepatocellular injury in NASH, putative mechanisms have been proposed to tie these processes to subsequent cell death.[30,31] Because the fatty acid metabolites responsible for initiating lipotoxic injury are not fully known, how they promote cell injury and death has yet to be resolved.[32] A major mechanism of hepatocyte death in NASH is apoptosis.[33]

Fibrosis and progression to cirrhosis

Liver fibrosis results from hepatocyte injury as demonstrated by studies showing that the production of cytokines and lipid peroxidation species from stressed or dying hepatocytes promote proliferation and activation of hepatic stellate cells. The balance of extracellular matrix deposition versus degradation is thus disrupted in favor of net accumulation of fibrosis. This pathway has been challenged by recent data indicating that epithelial progenitor cells in the liver can undergo epithelial-mesenchymal transition (EMT) when stressed.[34] The relative roles of EMT versus activation of existing stellate cells in progression of NASH fibrosis to cirrhosis has yet to be established.

2.5 Treatment of NAFLD

2.5.1 Lifestyle modification

Because obesity, poor dietary habits, and a sedentary lifestyle predispose to the development of NAFLD, the primary therapeutic intervention is to address these factors through a combination of gradual and sustained weight reduction through a balanced, calorically appropriate diet composed of healthy food choices coupled with increased physical activity.[35] Although the direct benefit of lifestyle modification has not been proven for NAFLD, there are enough data to support this recommendation[36] for the typical NAFLD phenotype which includes conditions such as obesity and metabolic syndrome. However, better data are needed on the histological response of children with NAFLD to standardized nutritional lifestyle interventions as recommended by the American Academy of Pediatrics.

2.5.2 Metformin

Insulin resistance is believed to be central to the development of NAFLD. Therefore, several studies have evaluated metformin as a potential treatment for NAFLD in children. In the first study to use magnetic resonance spectroscopy as a measure of hepatic steatosis, 10 non-diabetic children with biopsy-proven NASH received 500 mg of metformin by mouth twice daily for 6 months in an open-label pilot trial.[37] At the completion of the study, ALT normalized in 40% and AST normalized in 50%. Hepatic fat fraction was significantly reduced in 9 of 10 subjects, decreasing from a baseline mean of 30 ± 11% to 23 ± 9% after 24 weeks of treatment.

An open-label, 2-year observational pilot study from Rome evaluated the effect of metformin on NAFLD in children.[38] Thirty children with biopsy-proven NAFLD and mildly elevated ALT were enrolled and treated with 1,500 mg of metformin daily. All subjects also received lifestyle advice, including an individually tailored hypocaloric or isocaloric diet, physical activity recommendations, and monthly 1-hour sessions with a dietitian. Of those enrolled, 40% had a follow-up biopsy. In this subset, several histologic features, including steatosis, ballooning, and lobular inflammation were noted to have improved. However, there was no change in fibrosis.

The Treatment of NAFLD in Children (TONIC) trial was a multicenter, randomized, double-

masked, placebo-controlled trial conducted by the NASH CRN.[39] A total of 173 children ages 7 to 17 years with biopsy-proven NAFLD were randomized to receive either metformin, vitamin E, or placebo for 96 weeks to assess the change in serum ALT and histology. The outcomes from the trial were published in March 2011.[40]

2.5.3 Thiazolidinediones

The thiazolidinediones (TZDs, glitazones) are a class of drugs developed to treat type 2 diabetes because of their insulin sensitizing effect in states of insulin resistance. Studies have shown that the benefits of TZDs are at least partly explained by their ability to improve insulin responsiveness in adipose tissue and reduce inappropriate peripheral lipolysis.[41,42] As ligands for the nuclear transcription factor peroxisome proliferator-activated receptor gamma (PPARγ), this class of drugs has multiple complex effects. Improved insulin signaling has been attributed to the ability of TZDs to induce adipocyte differentiation and also prevent the inhibitory effect of c-Jun N-terminal kinases (JNKs) on post-receptor insulin signaling. Pilot studies indicated that the TZDs rosiglitazone and pioglitazone might improve the histology of NASH. Placebo controlled trials have had somewhat mixed results with the French FLIRT trial showing primarily improvement in steatosis[43,44] and other trials showing improvement in inflammation as well.[45-47] The pioglitazone treated patients in the PIVENS trial did not achieve the pre-defined histological endpoint or demonstrate improved fibrosis, but did have significant improvements in steatosis, inflammation, and the presence of steatohepatitis.[48] Similar to findings in other trials of TZDs, the improvement in ALT occurred over 3-6 months and was not sustained when the drug was discontinued. Whether the histological improvement occurs in parallel with the ALT decrease is unknown since no trial has examined serial liver biopsies in TZD treated patients. The primary side effect of using TZDs over the typical 1-2 year time course of most trials is significant weight gain in some subjects. Exacerbation of congestive heart failure, osteoporosis with distal limb fractures, and rare idiosyncratic hepatotoxicity are additional side effects known to occur with the use of TZDs, but in general they have not been observed in the relatively small NASH trials. This class of medication has not yet been tested and proven safe in children.

2.5.4 Vitamin E

Trials of antioxidant agents for the treatment of NASH have been undertaken because of the proposed role of oxidant stress in the pathogenesis of steatohepatitis.[49] Vitamin E (RRR-alpha-tocopherol) has been of particular interest. Pilot studies were inconclusive but the PIVENS trial demonstrated that 43% of patients treated for two years reached the desired histological endpoint compared to 19% in the placebo group ($P<0.01$).[48] Improvement in fibrosis was not observed. Similar to the pioglitazone-treated patients, the ALT improved over a time period of 3-6 months. Unlike treatment with pioglitazone, weight gain was not observed, but neither was any improvement in insulin sensitivity.

An open-label pilot study of vitamin E in 11 children with suspected NAFLD based on ultrasound showed improvement in serum aminotransferases in all subjects, without concomitant weight loss. Subsequent studies have shown conflicting results. In a 1-year trial of 88 children with biopsy-proven NAFLD, Nobili et al evaluated the effect of 600 IU/day of vitamin E and 500 IU/day of vitamin C or placebo on serum aminotransferases and prevalence of NAFLD by ultrasound.[50] Both the treatment group and the control group also received nutritional and physical activity counseling, with monthly 1-hour sessions with a dietitian. Serum ALT decreased in both the treatment and placebo group, but was not significantly different between groups.

2.5.5 Cysteamine

The final common pathway by which steatosis develops into steatohepatitis is likely to be mediated through oxidative stress due to reactive oxygen species (ROS) and decreased anti-oxidant

defense.[51] ROS can be generated in the liver through multiple mechanisms including cytochrome P450, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and lipooxygenase.[52] Insulin resistance and hyperinsulinism have been shown to increase hepatic oxidative stress and lipid peroxidation through increased hepatic CYPE21 activity. Glutathione (gamma-glutamyl-cysteinyl-glycine; GSH) is a major endogenous antioxidant and its depletion is implicated in the development of hepatocellular injury. One example of acute glutathione deficiency is acetaminophen poisoning. After a toxic dose of acetaminophen, excess metabolite (N-acetyl-benzoquinoneimine) covalently binds to hepatic proteins and enzymes resulting in liver damage.[53,54] Restoring glutathione levels can have a protective effect through the reduction of ROS. Glutathione itself does not enter easily into cells, even when given in large amounts. However, glutathione precursors do enter into cells and have been shown to be effective in the treatment of conditions such as acetaminophen toxicity by preventing significant GSH depletion. Examples of GSH precursors include cysteine, N-acetylcysteine, methionine, and other sulphur-containing compounds such as cysteamine. Studies in mice and humans showed oral and intravenous cysteamine to be effective in preventing acetaminophen-induced hepatocellular injury. However, because cysteamine has been associated with gastrointestinal symptoms and because of its previously poor commercial availability, drugs such as N-acetylcysteine have become the mainstay for treatment of acetaminophen poisoning. In fact, N-acetylcysteine has also recently been used in the treatment of NASH. In reports from Turkey, obese individuals with NASH treated with N-acetylcysteine for 4-12 weeks did have an improvement in aminotransferase levels and GGT even though there was no reported change in subject BMI.[55] This study suggests that increasing GSH levels may be a therapeutic strategy for the treatment of NAFLD.

Recent studies have suggested that the conditionally essential amino acid cysteine is a major limiting factor for GSH synthesis, and the factors (e.g., insulin and growth factors) that stimulate cysteine uptake by cells generally result in increased intracellular GSH levels.[56,57] Cysteamine, a GSH precursor, is a small molecule ($HS\text{-}CH_2\text{-}CH_2\text{-}NH_2$) which is able to cross cell membranes easily. Cysteamine is believed to react with extracellular cystine to form cysteine which then is readily taken up into the cell and transformed into GSH. When ingested, much of the cysteamine is thought to be taken up by the liver, and may therefore deliver optimal “anti-oxidant effect” to this organ. Cysteamine is currently available and is used in the treatment of cystinosis, an intra-lysosomal cystine storage disorder.[58]

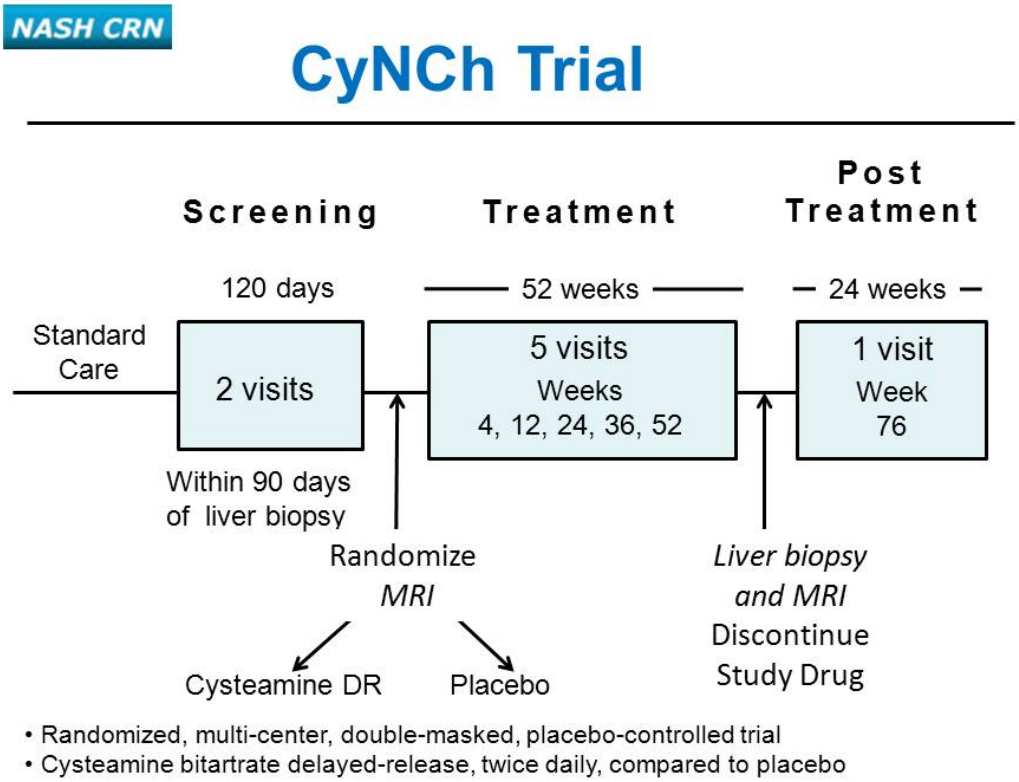
In cystinosis, cysteamine acts by converting cystine to cysteine and cysteine-cysteamine mixed disulfide which are then both able to leave the lysosome through the cysteine and lysine transporters respectively.[58] Within the cytosol, the mixed disulfide can be reduced by its reaction with glutathione and the cysteine released can be used for further GSH synthesis. The synthesis of GSH from cysteine is catalyzed by two enzymes, gamma-glutamylcysteine synthetase and GSH synthetase. This pathway occurs in almost all cell types, with the liver being the major producer and exporter for GSH. The reduced cysteine-cysteamine mixed disulfide will also release cysteamine, which, in theory is then able to re-enter the lysosome, bind more cystine and repeat the process.[59] In a recent study in children with cystinosis, enteral administration of cysteamine resulted in increased plasma cysteamine levels, which subsequently caused prolonged efficacy in the lowering of leukocyte cystine levels.[59] This may have been due to “re-cycling” of cysteamine when adequate amounts of drug reached the lysosome. If cysteamine does act in this fashion, then GSH production may also be significantly enhanced. Hepatic fat accumulation and oxidative stress contribute to the pathogenesis of NAFLD. Recently, an open-label pilot trial of the thiol agent cysteamine bitartrate (Cystagon®) for biopsy-proven pediatric NAFLD and elevated ALT was completed to evaluate the safety and potential efficacy of enteric-coated cysteamine therapy for NAFLD in children.[60] Cysteamine bitartrate readily crosses cellular membranes and is FDA approved for the treatment of the lysosomal storage disorder cystinosis. Eleven of thirteen children were enrolled and completed EC-Cysteamine therapy. For these 11

children with NAFLD, mean ALT decreased significantly from a mean of 120 U/L at baseline to 55 U/L after 6 months of treatment. There was a similar significant reduction in AST.

3. Study design

3.1 Design overview

CyNCh is a multicenter, double-masked, randomized, placebo-controlled, phase IIb clinical trial of treatment with either cysteamine bitartrate DR capsules (300 mg orally twice daily for patients ≤ 65 kg, 375 mg orally twice daily for patients >65-80 kg or 450 mg orally twice daily for patients >80 kg) or placebo for children with histologically-confirmed NAFLD. Eligible patients will be randomized to receive either cysteamine bitartrate DR or placebo capsules orally twice a day for 52 weeks. Patients ≤ 65 kg will have a starting dose of 150 mg per day (one 75 mg capsule twice daily) with dose escalation to 600 mg per day (four 75 mg capsules twice daily); patients weighing >65-80 kg at randomization will start with a dose of 300 mg per day (two 75 mg capsules twice daily) with a dose escalation to 750 mg per day (five 75 mg capsules twice daily) and patients >80 kg will start with a dose of 450 mg per day (three 75 mg capsules twice daily) with dose escalation to 900 mg per day (six 75 mg capsules twice daily). The patients will return for a follow-up visit at week 4 to ensure that they have reached the full dose as planned and that any side effects are identified and managed. The patients will return for follow-up visits approximately every 12 weeks after randomization (weeks 12, 24, 36, and 52). There will be a 24 week post-treatment follow-up period at the end of the treatment phase to assess the durability of effects, if any, and to ensure patient safety following the end of treatment (week 76). The study design can be schematically shown as below:



3.2 Treatment groups

Participants who have signed an informed assent statement along with their parent signing an informed consent statement and who meet the eligibility criteria will be randomly assigned to one of

two groups for 52 weeks of treatment. The dose of cysteamine bitartrate DR assigned will be either 600 mg/day (four 75 mg capsules twice daily) for patients ≤ 65 kg; 750 mg/day (five 75 mg capsules twice daily) for patients >65 - 80 kg, or 900 mg/day (six 75 mg capsules twice daily) for patients >80 kg. Patients in the placebo group will be assigned corresponding numbers of capsules based on their baseline body weight. Patients ≤ 65 kg at baseline will take four 75 mg capsules twice daily; patients >65 kg – 80 kg will take five 75 mg capsules twice daily and those patients greater than 80 kg at baseline will take six 75 mg capsules twice daily. The number of capsules taken will be increased gradually during weeks 1-4 to the assigned number and remain fixed thereafter regardless of weight changes after randomization according to the following schemes:

- **Group 1: cysteamine bitartrate DR:**
  - 600 mg/day (four 75 mg capsules twice daily) for patients ≤ 65 kg at baseline
  - 750 mg/day (five 75 mg capsules twice daily) for patients >65 - 80 kg at baseline
  - 900 mg/day (six 75 mg capsules twice daily) for patients >80 kg at baseline

- **Group 2: cysteamine bitartrate DR placebo** (as identical capsules to active drug)
  - 600 mg/day (four 75 mg capsules twice daily) for patients ≤ 65 kg at baseline
  - 750 mg/day (five 75 mg capsules twice daily) for patients >65 - 80 kg at baseline
  - 900 mg/day (six 75 mg capsules twice daily) for patients >80 kg at baseline

The randomization scheme will assign patients into two groups to receive either cysteamine bitartrate DR or placebo. The randomization design will be stratified by clinical center and baseline body weight into one of three categories (less than or equal to 65 kg, greater than 65 kg up to 80 kg, or greater than 80 kg; with assignments in permuted blocks of random length within each stratum to achieve a target dose of 9 to 12 mg/kg per day up to a maximum total dose of 600-900 mg per day (children with weights exceeding 100 kg will receive a dose of 900 mg per day). This scheme will ensure that the two groups will be balanced by calendar time of enrollment (to minimize secular effects) and by clinic (to minimize clinic-specific effects of differences in patient populations and management).

The randomization plan will be prepared and administered centrally by the Data Coordinating Center (DCC) but will not require real time interaction with a DCC staff member. Requests for randomizations will be made by the clinics using a web-based application. An assignment will be issued only if the database shows that the patient is eligible, has signed the consent/assent statement, and has had all required baseline data keyed into the database.

3.3 Study drug dosing schedule

Study drugs will be shipped to each clinical center's pharmacy. The research pharmacy staff will then provide the study staff with masked study drug bottles based on the DCC randomization schedule. Patients will be dispensed bottles labeled “Cysteamine bitartrate delayed-release 75 mg capsules or placebo”. Study drug should be taken 30 minutes prior to a meal.

Patients will be on a dose escalation regimen, the number of capsules taken will be increased gradually during weeks 1-4 to the assigned number and remain fixed thereafter regardless of weight changes according to the following schemes:

For patients with a baseline weight of 65 kg or less:

Week 1: One 75 mg capsules orally twice a day (1 in the morning and 1 in the evening) (150 mg/day),
Week 2: Two 75 mg capsules orally twice a day (2 in the morning and 2 in the evening (300 mg /day)
Week 3: Three 75 mg capsules orally twice a day (3 in the morning and 3 in the evening) (450 mg/day)
Weeks 4-52: Four 75 mg capsules orally twice a day (4 in the morning and 4 in the evening) (600 mg/day)

For patients with a baseline weight greater than 65 kg up to 80 kg:

Week 1: Two 75 mg capsules orally twice a day (2 in the morning and 2 in the evening) (300 mg /day)
Week 2: Three 75 mg capsules orally twice a day (3 in the morning and 3 in the evening) (450 mg/day)
Week 3: Four 75 mg capsules orally twice a day (4 in the morning and 4 in the evening) (600 mg/day)
Weeks 4-52: Five 75 mg capsules orally twice a day (5 in the morning and 5 in the evening) (750 mg/day)

For patients with a baseline weight greater than 80 kg:

Week 1: Three 75 mg capsules orally twice a day (3 in the morning and 3 in the evening) (450 mg/day)
Week 2: Four 75 mg capsules orally twice a day (4 in the morning and 4 in the evening) (600 mg/day)
Week 3: Five 75 mg capsules orally twice a day (5 in the morning and 5 in the evening) (750 mg/day)
Weeks 4-52: Six 75 mg capsules orally twice a day (6 in the morning and 6 in the evening) (900 mg/day)

3.3.1 Cysteamine DR

Cysteamine DR will be taken orally twice daily 30 minutes prior to meals (morning/breakfast and evening/dinner) for weeks 1-52. The rationale for the 9 mg/kg to 12 mg/kg daily dose range of cysteamine DR is that this target dose was the best tolerated in the pilot study and had the greatest benefit [60].

3.3.2 Rationale for a placebo group

Lifestyle interventions are considered the standard treatment for NAFLD, but are often not available to children with NAFLD due to both the lack of available care and limitations of insurance coverage for lifestyle interventions. In this study we will provide all participants, including those receiving placebo with a standardized nutrition and exercise intervention consistent with the recommendations of the American Academy of Pediatrics. Thus all children will receive treatment.

Currently, there are no FDA approved therapies for NAFLD in children. Medications that are being investigated for the treatment of NAFLD cannot be compared with an active alternative treatment arm. In order to assess the efficacy of an agent in NAFLD, a placebo-arm is needed to determine its relative efficacy in improving liver histology beyond that achieved with a placebo. Previous non-randomized and pilot studies have shown the efficacy of several agents such as ursodiol and betaine in the treatment of NAFLD, but follow-up randomized-placebo-controlled studies failed

to show improvement in liver histology beyond that observed in placebo groups.[61,62] In order to have the highest quality of evidence to test our hypothesis, the CyNCh trial utilizes a randomized, double-masked, placebo-controlled study design (http://www.ahrq.gov/clinic/uspstf/grades.htm). As there is no proven pharmacologic therapy for NAFLD in children, using a placebo for comparative purposes is justified.

Rationale for a 90 day maximum duration between liver biopsy and registration

Liver histology, especially steatosis, may change over time. A 90-day window provides for adequate time to screen eligible patients but minimizes the likelihood of changes in liver histology before the start of treatment.

3.4 Standard treatment recommendations

The use of prescription or non-prescription medicines or herbal remedies or dietary supplements, consumption of alcohol, and management of various co-morbid illnesses will be discussed with the patients. These recommendations have been prepared by the NASH CRN Pediatric Standard of Care Committee and are approved by the NASH CRN Steering Committee to be applied across all study sites. This will help ensure that the patients in both groups receive the same standard of care treatment for NAFLD.

Enhanced Lifestyle Intervention

All children, including those in the placebo group will receive an intervention in the form of standardized lifestyle intervention recommendations consistent with the latest recommendations from the American Academy of Pediatrics (AAP). Assessing the impact of such an intervention on NAFLD will be important. Attaining a healthy weight is the cornerstone of current treatment of pediatric NAFLD, given the lack of proven pharmaceutical therapy in this age group and the strong association of NAFLD with excess adiposity, in particular central adiposity.[63] Weight loss has been associated with improvements in liver enzymes and histology in adults with NAFLD, while weight loss in children with NAFLD has been shown to improve serum aminotransferase levels in small pilot studies.[36,64,65] Therefore, lifestyle intervention through changes in diet and exercise will be encouraged for participants in both the placebo and active study drug treatment group of the CyNCh trial, as this represents the current standard of care for children.

The standard of care lifestyle intervention designed for this trial will incorporate components of the AAP's 2007 Expert Committee Recommendations Regarding the Prevention, Assessment, and Treatment of Child and Adolescent Overweight and Obesity that can be reproduced across the study sites.[66] CyNCh Trial participants will be given written materials that will include evidence-based strategies to achieve a healthier diet and increase physical activity, as endorsed by the American Academy of Pediatrics as well as the CDC and NIH.[64-68] A family-based, patient-centered and stepped approach to making lifestyle changes will be employed as recommended by the AAP.[66] Accordingly, the lifestyle materials will be reviewed with trial participants and their family members at each study visit and they will be encouraged to select 1-2 goals which they consider personally obtainable and that they can commit to pursuing in the interval until their next study visit.

Topics to be covered in the materials will include:

1) Reduce fat intake and sugar intake to 0 servings per day
2) Reduce screen time to 2 hours or less per day
3) Increase physical activity to 1 hour or more per day
4) Increase fruits and vegetable intake to 5 or more servings per day
5) Reduce fast food intake and make healthier choices when eating out

These topics and the specific strategies to be included in the lifestyle intervention materials are in accordance with healthy weight strategies currently recommended by the Centers for Disease Control and Prevention and the National Institutes of Health National Heart Lung and Blood Institute's "We Can" program.[67-70] Therefore, we will also include references in the study's lifestyle intervention materials to these freely accessible, federal government sponsored websites so that participants and their families can easily access additional information on these lifestyle changes and strategies to achieve them.

Treatment duration-rationale for 52 weeks

An ideal duration of a treatment should only expose participants to a study drug long enough to show meaningful improvement in liver histology if it is going to occur. This enables any positive findings to be reported as soon as possible.

3.5 Primary outcome measure

Centrally scored and masked assessment of histologic improvement in NAFLD between the baseline liver biopsy and follow-up biopsy after 52 weeks of treatment, where improvement is defined as: (1) decrease in NAS of 2 or more and (2) no worsening of fibrosis.

Justification for performing an end-of-treatment liver biopsy:

NAFLD is a clinicopathologic entity and liver biopsy characteristics are required for the diagnosis of NAFLD, the determination of NASH, and the staging of fibrosis. Currently, there are no non-invasive markers to diagnose or grade NASH. Therefore, a liver biopsy is needed to document improvement or worsening. Several of the histologic features of NAFLD including ballooning degeneration and fibrosis have prognostic significance and predict liver-related deaths in patients with NAFLD. Therefore, documentation of improvement of the pathologic lesions of NASH in liver biopsy is needed to show efficacy in a clinical trial.

Rationale for using the NAS:

The primary outcome measure requires improvement in disease activity after 52 weeks of treatment as determined by liver biopsies pre- and post-treatment. The measure is derived from changes from baseline to the end of treatment in the NAFLD activity score (NAS).[70] The NAS ranges from 0 to 8 (highest activity) and is calculated as the sum of three components of the standardized histologic feature scoring system for liver biopsies:

NAS = Steatosis (0-3)

+ Lobular inflammation (0-3)

+ Hepatocyte ballooning (0-2)

The NAS provides a semi-quantitative tool to assess treatment response in a clinical trial. It has been validated and this measure is considered a gold standard in the field. It was utilized to assess the efficacy in the NIDDK PIVENS (IND 69,751) and TONIC (IND 71,217) trials conducted by the NASH CRN.[48] This would also provide a tool to compare the findings across various treatment trials within and outside the NASH CRN. Therefore, we believe that the NAS provides the most objective and reproducible measurement that is available to assess efficacy of treatment in NAFLD.

3.6 Secondary outcome measures *(see section 7.2 for details)*

Individual histological characteristics

- Change in NAS after 52 weeks of treatment compared to baseline NAS.

Changes in fibrosis, steatosis, lobular inflammation, ballooning, and other specific features from the histologic scoring system after 52 weeks of treatment compared to baseline. Improvement in specific features will be defined as any improvement in the NAS.

- Presence or absence of a diagnosis of NASH in the follow-up biopsy

Laboratory

- Aminotransferases
- Normalization of ALT and AST after the end of treatment
- Time-dependent decline in serum mean ALT and AST
- Change in insulin sensitivity
- Markers of oxidation and anti-oxidant status before and after treatment will be compared between groups.

Imaging

- Change in MRI-determined liver fat fraction from baseline to end of treatment

Symptoms and exam

- Changes in Pediatric Quality of Life
- Changes in anthropometric measures

4. Patient selection

4.1 Recruitment

Approximately 160 participants in 2 groups of equal size (80 per group) will be recruited at the clinical centers of the NASH CRN (averaging 16 patients per center) over a 19 month period.

Eligible patients will be identified and recruited at the participating clinical centers subject to the inclusion and exclusion criteria. Clinics will be expected to recruit sufficient overall numbers of minorities and females so that results can be generalized to these populations. Each clinic will develop a recruitment plan. These plans will vary from clinic to clinic depending on the available pools of patients and local recruitment resources and referral patterns.

4.2 Inclusion criteria

Patients must satisfy all of the following criteria to be eligible for enrollment:

- Children age 8-17 years inclusive.
- Liver biopsy within 90 days of screening visit and not more than 120 days before randomization.
- Clinical history consistent with NAFLD.
- Definite NAFLD based upon liver histology.
- No evidence of any other liver disease by clinical history or histological evaluation
- A histological severity of NAFLD Activity Score (NAS) ≥ 4.
- Sexually active female participants of childbearing potential (i.e., not surgically sterile [defined as tubal ligation, hysterectomy, or bilateral oophorectomy) must agree to utilize the same two acceptable forms of contraception from screening through completion of the study and to complete a pregnancy test at each study visit. The acceptable forms of contraception for this study include hormonal contraceptives (oral, implant, transdermal patch, or injection) at a stable dose for at least 1 month prior to screening, and barrier (condom with spermicide, diaphragm with spermicide). Sexual activity will be ascertained at each study visit for post-menarchal females and if sexually active, subject must verify use of the same 2 acceptable forms of contraception.
- Participants must be able to swallow cysteamine bitartrate DR capsules.
- Written informed consent from parent or legal guardian.
- Written informed assent from the child.

4.3 Exclusion criteria

Exclusions will not be based upon gender, race, or ethnicity. Participants with a current history of the following conditions or any other health issues that make it unsafe for them to participate in the opinion of the investigators:

- Inflammatory bowel disease (if currently active) or prior resection of small intestine
- Heart disease (e.g., myocardial infarction, heart failure, unstable arrhythmias)
- Seizure disorders
- Active coagulopathy
- Gastrointestinal ulcers/bleeding
- Renal dysfunction with a creatinine clearance < 90 mL/min/m^2
- History of active malignant disease requiring chemotherapy or radiation within the past 12 months prior to randomization

- History of significant alcohol intake (AUDIT questionnaire) or inability to quantify alcohol consumption
- Chronic use (defined as more than 2 consecutive weeks in the past year) of medications known to cause hepatic steatosis or steatohepatitis including:
    - systemic glucocorticoids
    - tetracycline
    - anabolic steroids
    - valproic acid
    - salicylates
    - tamoxifen,
- The use of other known hepatotoxins within 90 days of liver biopsy or within 120 days of randomization
- Initiation of medications with the intent to treat NAFLD/NASH in the time period following liver biopsy and prior to randomization
- History of total parenteral nutrition (TPN) use in the year prior to screening
- History of bariatric surgery or planning to undergo bariatric surgery during study duration
- Clinically significant depression (patients hospitalized for suicidal ideations or suicide attempts within the past 12 months)
- Any female who is nursing, planning a pregnancy, known or suspected to be pregnant, or who has a positive pregnancy screen
- Non-compensated liver disease with any one of the following hematologic, biochemical, and serological criteria on entry into protocol:
    - Hemoglobin < 10 g/dL
    - White blood cell (WBC) < 3,500 cells/mm3 of blood
    - Neutrophil count < 1,500 cells/mm3 of blood
    - Platelets < 130,000 cells/mm3 of blood
    - Direct bilirubin > 1.0 mg/dL
    - Total bilirubin >3 mg/dL
    - Albumin < 3.2 g/dL
    - International normalized ratio (INR) > 1.4
- Poorly controlled diabetes mellitus (hemoglobin A1c (HbA1c) > 9%)
- Evidence of other chronic liver disease:
    - Biopsy consistent with histological evidence of autoimmune hepatitis
    - Serum hepatitis B surface antigen (HBsAg) positive
    - Serum hepatitis C antibody (anti-HCV) positive
    - Iron/total iron binding capacity (TIBC) ratio (transferrin saturation) > 45% with histological evidence of iron overload
    - Alpha-1-antitrypsin (A1AT) phenotype/genotype ZZ or SZ
    - Wilson's disease
- Children who are currently enrolled in a clinical trial or who have received an investigational study drug within 180 days of screening or liver biopsy
- Subjects who are not able or willing to comply with the protocol or have any other condition that would impede compliance or hinder completion of the study; in the opinion of the investigator
- Failure to give informed consent

5. Trial protocol

5.1 Visit schedule overview

The patient-related activities of the CyNCh trial can be divided into 4 phases:

- Screening for eligibility for enrollment
- Randomization to treatment (1 visit)
- Treatment phase (5 visits over 52 weeks)
- Post-treatment washout phase (1 visit at 76 weeks)

The visit and data collection schedule described below in detail is summarized in Appendix 10.2

5.2 Screening and baseline data collection

Patients who appear to be eligible after chart review will be invited to undergo screening. Recording of screening data on NASH CRN forms may not start until the patient has signed the consent statement. Screening and baseline data collection procedures will include questionnaires, physical examination, measurement of fasting serum glucose and insulin, standard of care liver biopsy, lipid and metabolic tests, etiologic tests, urine analysis, and blood collection for DNA and serum and plasma banking. Prior therapy for NAFLD will be reviewed as outlined in the inclusion and exclusion criteria. Patient charts will be reviewed for historical information and previous liver biopsy findings.

All participants who sign the consent/assent documents will be registered in the trial database. Each participant who starts screening will be accounted for at the end of screening, as either a screening success (enrolling in the trial) or a screening failure. A screening failure is defined as a participant who signed the consent form, but is found to be ineligible prior to randomization. Screening failures include patients who meet medical eligibility criteria but who refuse enrollment in the trial. Reasons for screening failure will be recorded in the trial database. Screening and baseline data collection will be conducted over two clinic visits usually completed on separate calendar days. The goal of the first screening visit is to obtain consent and record data regarding the trial's inclusion and exclusion criteria; the goal of the second screening visit is to complete collection of baseline data on patients who appear eligible. This separation of procedures between two visits is provided as a practical guideline. Screening procedures and data collection can be organized as appropriate at each clinical center. The procedures completed during screening are described below.

Screening visits

Determination of eligibility will be based mostly on chart review of standard of care tests and procedures that were completed before the first screening visit. The parent and child will sign the consent and assent at the first screening visit (or before) to obtain any tests and procedures needed to finalize eligibility after chart review and will undergo a history and physical examination including anthropometric assessments (body weight [kg], body height [m], body mass index [BMI], waist circumference [cm], and hip circumference [cm]) to identify other illnesses and contraindications for participation, including hepatosplenomegaly, peripheral manifestations of liver disease, ascites, wasting or fetor. History of prior liver biopsies and use of anti-NASH, anti-diabetic, statin, and fibrate medications in the three months prior to the biopsy will be obtained and recorded. Laboratory test results that need to be recorded from chart review or obtained as part of the screening visits include: tests for hepatitis B (HBsAg) and hepatitis C, anti-nuclear antibody (ANA), anti-mitochondrial antibody (AMA), anti-smooth muscle antibody (ASMA), ceruloplasmin, A1AT concentration, iron overload (iron, TIBC, and ferritin), fasting serum glucose, insulin, HgbA1C,

complete blood count (CBC) with white blood cell differential, hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase, albumin, total protein, GGT, PT, INR), metabolic panel (sodium, potassium, chloride, carbon dioxide, calcium, BUN, creatinine, uric acid), and lipid panel (total cholesterol, triglyceride, LDL, HDL).

Nutritional information will be obtained using the Dietician administered 24-hour food recall (NDS-R). Three separate 24 hour food recalls will be obtained during screening. Women of childbearing potential must have a negative pregnancy test. Frequency and amount of alcohol intake will be obtained using the Alcohol Use Disorders Identification Test (AUDIT). Patients will complete the health-related quality of life questionnaire (PedsQL) and a liver symptoms questionnaire. A blood sample will be drawn to obtain DNA for banking and serum/plasma for banking for future analysis of markers of oxidant status. Participants may also undergo an MRI scan to estimate liver fat fraction. The screening MRI will be performed after registration in the CyNCh trial and before treatment initiation as contemporaneously as possible to pre-treatment biopsy (see section **5.6** Magnetic Resonance (MR) examinations).

Baseline liver biopsy

Eligibility requires a liver biopsy within 90 days of registration. The baseline liver biopsy is not performed as a procedure in this study but as standard of care done for clinical care. Usually, this will have been done prior to consideration for this study. If a child's hepatologist believes that the child has NAFLD, has scheduled him or her for a clinical liver biopsy, and they meet all other inclusion and exclusion criteria, then the child may be registered prior to the liver biopsy. Regardless of whether the biopsy is done before or after registration, the liver histology must be determined to be NAFLD with a NAS of at least 4. The NASH CRN study physician should check if tissue blocks and/or additional slides can be obtained from the original biopsy.

Clinic staff should note that the date of the biopsy establishes a hard window for completion of screening procedures prior to randomization – randomization must take place within 120 days of the date of biopsy. Clinic staff will have to monitor completion of screening procedures in order to assure adherence to the allowable time window.

The NASH CRN clinic data system will include software to check patient eligibility based on keyed data forms. The eligibility check task may be run at any time, and there is no limit on the number of times it may be run. The output from the task will list the eligibility checks that the patient has failed and a summary finding that the patient is eligible or ineligible for the trial. Clinic staff can use this task to identify the items that still need to be completed, keyed, or verified after data from the screening visit are keyed. The randomization visit should not take place until the eligibility check indicates that the patient is eligible except for the items that can be completed only at the randomization visit.

5.3 Randomization visit

The randomization visit is the visit at which randomization takes place and the patient is issued the study medication randomly assigned to the patient. Randomization is the act of generating the random study medication assignment and is the procedure which defines a patient's enrollment into the trial. Randomization can only occur after eligibility has been fully checked and all data collected at the screening visits have been keyed to the trial database. Since these processes take time, randomization cannot be done at a screening visit, and since study medication needs to be issued to the patient, the randomization visit must be completed in person with the patient. Therefore a visit separate from the screening visit is necessary. Since this will be a visit on a different calendar day and medication will be started at this visit, good clinical practice requires that a few basic checks of the patient's well-being be completed at the randomization visit.

The procedures completed at the randomization visit are: pregnancy test for females of child bearing potential; verification that the patient is feeling well; affirmation of consent; review of concomitant drugs and vital signs (systolic and diastolic blood pressure, heart rate, respiratory rate, body temperature). All patients will be given information on a healthy life style and diet appropriate for their weight and other factors.

Generation of the random treatment assignment will occur at this visit. The randomization process includes the same electronic check on eligibility that the staff may run prior to the randomization visit. The medication assignment will not be generated unless the check finds that the patient is eligible, and the clinic staff indicates that they want to randomize the patient. The random treatment assignment will consist of medication bottle numbers; these numbers will be unique and will be specific to the particular patient and visit they were generated for. They will correspond to numbered bottles of medications which have been sent to the clinical center's research pharmacy (or clinical coordinator if not using a pharmacy) by the NASH CRN Drug Distribution Center. The research pharmacy (or clinical coordinator) will issue the specific numbered bottles to the patient. Each patient's random treatment assignment will be generated for that specific patient and will not be transferable to another patient. Once the assignment has been generated, the patient should be issued the assigned study drugs (in person) and instructed about starting the drugs and monitoring for adverse effects. The date of randomization is the 0 time for reckoning all follow-up visits (i.e., all follow-up visits are scheduled at specific times measured from the date of randomization). The randomization computer program will generate a personalized appointment schedule for the patient; this schedule will indicate the ideal date for each follow-up visit, as well as the time window around the ideal date during which the follow-up visit may be done. This will ensure that the data collected at the follow-up visit may be used in the trial.

5.4 Follow-up visits

Patients will return for follow-up visits at 4, 12, 24, 36, 52, and 76 weeks after randomization. Patients will be seen for one visit at four weeks after randomization and then, starting at 12 weeks after randomization, will be seen at 12 week (3 month approximately) intervals with an end of treatment visit and liver biopsy at 52 weeks. A post-treatment washout phase visit will occur at week 76. Each visit will have an interval of time surrounding the ideal date for the visit during which the visit may be done and the data included in the trial database. The ideal date for a visit is the exact anniversary date from randomization. Visit windows will be constructed to be contiguous, so that at any point in time, some visit window is open, subject to a check on the minimum separation required between consecutive visits. The specific procedures to be completed at each of the follow-up visits are:

- **Week 4 visit**: Review of medications, adverse effects, study drug adherence, pregnancy test, blood draw for CBC, hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase, albumin, total protein, GGT, PT, INR); focused physical examination including height, weight, waist and hip measurements, vital signs (temperature, heart rate, respiratory rate, blood pressure), and liver signs; and standardized nutrition and exercise prescription and counseling. Review study drug adherence with patient and dispense study drug.
- **Week 12 visit**: Follow-up medical history including review of medications, adverse effects, and interim drinking history; liver symptoms questionnaire; focused physical examination, including height, weight, waist and hip measurements, vital signs (temperature, heart rate, respiratory rate, blood pressure), and liver signs; blood draw for CBC, hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase, albumin, total protein, GGT, PT, INR); serum and plasma for banking at a central repository;

pregnancy test (for women of child-bearing potential); standardized nutrition and exercise prescription. Review study drug adherence with patient and dispense study drug.

- **Week 24 visit**: Follow-up medical history including review of medications, adverse effects, and interim drinking history; liver symptoms questionnaire; detailed physical examination, including height, weight, waist and hip measurements, vital signs (temperature, heart rate, respiratory rate, blood pressure), and liver signs; blood draw for CBC, comprehensive metabolic panel (sodium, potassium, chloride, carbon dioxide, calcium, BUN, creatinine, uric acid), hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase, albumin, total protein, GGT, PT, INR), fasting glucose, insulin, HbA1c, lipid profile; pregnancy test (for women of child-bearing potential); blood draw for plasma and serum banking at a central repository; standardized nutrition and exercise prescription and counseling. Review study drug adherence with patient and dispense study drug.
- **Week 36 visit**: Follow-up medical history including review of medications, adverse effects, and interim drinking history; liver symptoms questionnaire; focused physical examination, including height, weight, waist and hip measurements, vital signs (temperature, heart rate, respiratory rate, blood pressure), and liver signs; blood draw for CBC, hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase, albumin, total protein, GGT, PT, INR); serum and plasma for banking at a central repository; pregnancy test (for women of child-bearing potential); standardized nutrition and exercise prescription. Review study drug adherence with patient and dispense study drug.
- **Week 52 visit**: Follow-up medical history including review of medications, adverse effects, and interim drinking history; liver symptoms questionnaire, a health-related quality of life questionnaire (PedsQL); three separate dietician administered 24-hour food recalls (NDS-R); detailed physical examination, including height, weight, waist and hip measurements, vital signs (temperature, heart rate, respiratory rate, blood pressure), and liver signs; blood draw for CBC, comprehensive metabolic panel (sodium, potassium, chloride, carbon dioxide, calcium, BUN, creatinine, uric acid), hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase, albumin, total protein, GGT, PT, INR), fasting glucose, insulin, HbA1c, lipid profile; pregnancy test (for women of child-bearing potential); blood draw for plasma and serum banking at a central repository. A follow-up liver biopsy will be done as well as a follow-up MRI of the liver. Review study drug adherence and collect all unused medication bottles from the patient.
- **Week 76 visit**: Follow-up visit 24 weeks after discontinuation of study drug. Follow-up medical history including review of medications, adverse effects, and interim drinking history; liver symptoms questionnaire, a health-related quality of life questionnaire (PedsQL); detailed physical examination, including height, weight, waist and hip measurements, vital signs (temperature, heart rate, respiratory rate, blood pressure), and liver symptoms; blood draw for CBC, comprehensive metabolic panel (sodium, potassium, chloride, carbon dioxide, calcium, BUN, creatinine, uric acid,), hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase, albumin, total protein , GGT, PT, INR), fasting glucose, insulin, HbA1c, lipid profile, and for plasma and serum banking at a central repository.

5.5 Standardized questionnaires

Several standardized questionnaires will be administered to patients enrolled in the CyNCh trial. Questionnaires will be administered at screening (prior to randomization) and during follow-up at specified intervals (see Appendix 10.2 for the data collection schedule). The purpose of the questionnaires is to obtain important information regarding alcohol intake, nutrition, health-related quality of life, and liver-related symptoms.

Alcohol Use Disorders Identification Test (AUDIT) is a 10-item questionnaire with a simple scoring scale that will be administered during screening. A 3-item interim drinking history (AUDIT-C) measuring consumption in the past 90 days will be obtained during follow-up visits as part of the follow-up medical history. The purpose of these questionnaires is to ascertain that there is no significant alcohol consumption prior to enrollment or during the study period.

Health-related quality of life: The Pediatric Quality of Life (PedsQL) questionnaire will be administered during screening, after 52 weeks of treatment, and at the week 76 visit.

Liver symptoms Questionnaire: A questionnaire on liver symptoms for children with NAFLD has been developed by the NASH CRN will be administered during screening and after 12, 24, 36, and 52 weeks of treatment, and at the 76 week visit (24 weeks after withdrawal of study medication).

Dietician administered 24-hour food recall (NDS-R): The Nutrition Data System for Research (NDSR) food recall is a Windows-based dietary analysis program designed for the collection and analyses of 24-hour dietary recalls and the analysis of food records, menus, and recipes. Calculation of nutrients occur immediately providing data per ingredient, food, meal, and day in report and analysis file formats. The software includes a dietary supplement assessment module so that nutrient intake from both food and supplemental sources may be captured and quantified. Three separate 24 hour NDSR food recalls will be obtained during screening and again after 52 weeks of treatment.

5.6 Magnetic Resonance (MR) examinations

Magnetic Resonance Imaging (MRI) exam purpose and rationale

The purpose of the MRI exam is to quantify the hepatic fat fraction noninvasively in participants of the CyNCh Trial. The fat fraction is the proportion of mobile protons in liver tissue attributable to fat and thus is a non-invasive MR-based biomarker of liver triglyceride concentration.

To quantify the fat fraction, the MRI exam will use a fast spoiled gradient recalled echo (FSPGR) sequence that uses a low flip angle to reduce T1 bias, acquires multiple echoes after a single excitation to measure and correct for T2*decay, and uses spectral modeling to address fat-water and fat-fat signal interference effects. Using MR spectroscopy as the reference standard, the proposed MRI technique measures hepatic fat fraction accurately in children and adults at 1.5T (Tesla) or 3T, and across different vendors. The technique provides high within-examination and between-examination precision. Linearity is maintained across the entire relevant biological range from <1% to >40% hepatic fat fraction. The technique is robust to minor variations in acquisition parameters, including those that may be encountered during usage in a clinical trial.

The FSPGR sequence proposed for imaging-based hepatic fat fraction quantification can be implemented on any up-to-date clinical scanner and thus can be used at all NASH CRN clinical or satellite centers with access to such a scanner. Moreover, the technique is imaging based and covers the whole liver, thus providing information on both the quantity and distribution of hepatic fat fraction. These characteristics represent critical advantages over the alternative technique of single-voxel proton MR spectroscopy to measure hepatic fat fraction in a multicenter clinical trial. While MR spectroscopy is currently considered the most accurate noninvasive technique to quantify hepatic proton density fat-fraction (PDFF), [73-76] it is not available at most NASH CRN clinical or satellite centers, and it is restricted in spatial coverage. The latter limitation may be problematic for reliable assessment of longitudinal changes in hepatic fat content,[77,78] as is required by the CyNCh trial.

Target population

The target population for the MRI exams will consist of eligible participants in the CyNCh trial. Eligibility criteria are listed below:

- Inclusion: Willing and able to complete MRI examination procedures
- Exclusion:
  - A contraindication to MRI examinations
  - Extreme claustrophobia
  - Pregnant or trying to become pregnant
  - Weight or girth exceeds the scanner capabilities
  - Any condition or circumstance that, in the opinion of the site investigator, would interfere with completion of MR examinations

MRI safety screening

Clinical sites will screen subjects for MRI safety per institutional standard.

MRI exam scheduling

Subjects will undergo two MRI examinations. The screening MRI will be performed after registration in the CyNCh trial and before treatment initiation as contemporaneously as possible to pre-treatment biopsy. The follow-up MRI exam will be performed as contemporaneously as possible to the end-of-treatment biopsy at 52 weeks.

Pre-MRI exam instructions

Subjects will be instructed to fast for four or more hours prior to the scheduled MRI exam but will be allowed to take necessary medications and small quantities of water.

MRI exam performance

Subjects will be positioned supine with a phased-array coil centered over the liver. After localizing sequences, an axial multi-echo-echo 2D FSPGR sequence will be performed through the liver. Imaging parameters will be selected as appropriate for 1.5T or 3T scanners. After completion of the exam, clinical centers will transfer MR images to the Radiology Reading Center (RRC) at UC San Diego.

MRI exam analysis

Image analyst(s) at the RRC will review and analyze images. The analysts will place regions of interest in each hepatic segment. The fat fraction in each region of interest will be calculated using custom software developed by the RCC and validated in pediatric clinical patients and research subjects with liver fat fraction values ranging from <1% to >40%.The fat fraction in each region of interest will be recorded. The average fat fraction across segments will be calculated. The average hepatic fat fraction value calculated in each patient on each of the screening and follow-up MRI exams will be recorded by the RCC on a data form to the DCC.

5.7 Liver biopsy

Liver biopsies will be performed 52 weeks after enrollment (window: 44-64 weeks) by the NASH CRN principal investigator or designee. Liver biopsies will generally be obtained from the right lobe of the liver using a 16G or larger biopsy instrument (if the initial biopsy was obtained from the left lobe, then the 52 week biopsy must be obtained from the left lobe). Biopsies should be at least 1.5 cm in length.

5.8 Specimen repositories

Specimens will be collected and stored in the NIDDK central repositories for use as approved by the Steering Committee of the NASH CRN (see Appendix 10.3 for blood collection schedule). Specimens include serum, plasma, DNA, and liver tissue. The blood collected during screening, and at the 12, 24, 36, 52, and 76 week follow-up visits will be separated into plasma and serum, and divided into 0.5 mL aliquots. Aliquots will be kept in a storage facility at -70 degrees C until they are

shipped to the NIDDK Biosample Repository on dry ice. If the patient provided additional consent, blood will be collected during screening and shipped to the NIDDK Genetics Repository for extraction of DNA and banking. When possible, a portion of the liver biopsy specimen will also be collected in RNA*later*® and refrigerated at 4° C overnight to allow thorough penetration of the liver tissue and then transfer to -70° C freezer for storage.at -70 degrees C until shipping to the NIDDK Biosample Repository on dry ice.

5.9 Adherence and retention

Two important goals of this protocol are to optimize adherence to the pharmacological regimen and to maximize the retention of participants in the study. Assessment of adherence to the assigned study drug will provide clinic staff a means to identify participants having problems with adherence. Adherence will be assessed by:

- Counts of capsules in the patient's returned study drug bottles
- Conducting a brief, structured interview, in which the study coordinator will assist the patients to identify problems in taking the study drug and to estimate adherence to the prescribed medicine since their previous visit.

These assessments will guide the consideration of strategies to improve adherence. Resources will be provided to remove barriers to participation such as child or elder care, transportation, and parking expenses. These resources can be provided as cash, transportation vouchers, or parking passes. An honorarium may be paid to participants in recognition of their time and effort when scheduled visits and procedures are completed successfully. Certificates of appreciation may be given at enrollment and at conclusion of the CyNCh trial as an incentive.

5.10 Management of concomitant conditions

Hypertension, hyperlipidemia, and diabetes will be managed in conjunction with the patient's primary care physician according to the protocols described in the Standard of Care document prepared by the Pediatric Standard of Care Committee of NASH CRN.

Pregnancy will be managed according to the guidelines shown in section 6.10. In the event of major dermatological reactions such as generalized urticaria, bullous rashes, exfoliative dermatitis, or Stevens-Johnson Syndrome, study drug will be discontinued immediately and not restarted. For local skin reactions, study drug may be discontinued if the skin reactions are potentially drug related. If the rashes clear, the study drug may be restarted.

If local skin reactions recur with restarting the study drug, study drug should be discontinued. In cases where the study medication has been discontinued, the study drug will be unmasked and the participant, investigator, and the primary care provider will be notified in order to prevent future exposures.

6. Safety monitoring

6.1 Historical safety issues in children with nephropathic cystinosis taking immediate release cysteamine bitartrate (Cystagon®) capsules

Cystinosis is a rare genetic disorder that causes an accumulation of the amino acid cystine within cells which causes a myriad of problems including damage to the skin, eyes, kidneys, liver, endocrine system, hematologic system, and the central nervous system. The treatment for cystinosis has been immediate release cysteamine 4 times per day since Cystagon® was approved by the FDA in 1994. The dosing of Cystagon® for children with nephropathic cystinosis is between 1.30 grams/m^2/day and 1.95 grams/m^2/day. At this dose, immediate release cysteamine is known to have common gastrointestinal side effects including vomiting, anorexia, and diarrhea. In clinical trials of Cystagon®, withdrawals due to intolerance, vomiting associated with medication, anorexia, lethargy, and fever appeared dose related, occurring more frequently in children receiving 1.95 grams/m^2/day as compared to 1.30 grams/m^2/day. Immediate release cysteamine is also associated with side effects that may be attributable to the underlying disease, cystinosis, rather than the medication itself. These include symptoms such as reversible leucopenia, abnormal liver function studies, skin lesions, headache, tinnitus, diplopia, blurry vision, loss of vision, pain behind the eye or pain with eye movement. Moreover, in children with cystinosis treated with doses > 1.95 grams/ m^2/day there were additional side effects. Children treated with high doses of cysteamine may develop skin lesions. These include skin striae (which are like stretch marks), bone injuries (such as fractures), bone deformities, and joint problems. Benign intracranial hypertension (or pseudotumor cerebri) with papilledema; skin lesions, molluscoid pseudo tumors, skin striae, skin fragility; joint hyperextension, leg pain, genu valgum, osteopenia, compression fracture and scoliosis have been reported in patients taking immediate-release cysteamine.

Although these historical data are important background information, it is important to note:

1. The CyNCh trial will NOT enroll any children with nephropathic cystinosis
2. The CyNCh trial will NOT utilize immediate-release cysteamine
3. The CyNCh trial will use a MUCH lower dose of cysteamine than even the lowest dose ever used for nephropathic cystinosis

6.2 Safety issues in children with NAFLD taking delayed release cysteamine bitartrate (RP103) capsules

For the reasons above, efforts over the past decade were focused on improving the side effect profile of cysteamine therapy by changing the location and rate of drug release. To this end an enteric coated cysteamine (EC-cysteamine) capsule was developed[60]. In patients with cystinosis, at the high doses required, EC-cysteamine was shown to have better GI tolerability than immediate release cysteamine. EC-cysteamine was then used in a pilot study as a therapy for children with NAFLD. At the high dose of 1000 mg orally twice a day, abdominal pain, nausea, and/or vomiting was noted in 5 of 13 children (38%) [60]. Notably, however, a clinical response to EC-cysteamine was seen at the much lower dose of 300 mg taken orally twice a day. At this dose, which was selected for patients weighing ≤ 65 kg in the CyNCh Trial, gastrointestinal tolerance was satisfactory. Nausea and/or abdominal pain were noted in only 2 of 13 children (15%). Moreover, these symptoms resolved completely in less than 2 weeks with tolerance of this dose thereafter in all children. In order to take advantage of the improved pharmacokinetics resulting from enteric-coating, Raptor Therapeutics, Inc. has developed delayed-release capsules (RP103), with cysteamine bitartrate formulated in microspheronized core beads that are subsequently enteric-coated. The enteric-coated core beads are then encapsulated in gelatin capsules (Raptor Therapeutics Inc. Cysteamine Bitartrate Delayed-release 75 mg capsules, Investigator's Brochure, 1st Edition-January 13, 2012). This new

formulation, RP103 75 mg capsules is being used in the CyNCh Trial. RP103 was approved by the FDA in April 2013 for the treatment of nephropathic cystinosis (Raptor Therapeutics Inc., PROCYSBI (cysteamine bitartrate delayed release capsules, Investigator's Brochure, April 2013). The dosing in CyNCh will be based upon weight not body surface area as done for cystinosis, but the average dose of RP103 will be 0.470 grams/m^2/day, which is much lower than the dose of cysteamine used in the treatment of children with cystinosis (1.30 – 1.95 grams/m^2/day). There have been no deaths, serious adverse events or severe adverse events reported in subjects who received single doses of RP103, nor in patients receiving 600 mg twice a day for the treatment of Huntington's disease (Raptor Therapeutics Inc. Cysteamine Bitartrate Delayed-release 75 mg capsules, Investigator's Brochure, 1st Edition -January 13, 2012).

6.3 Safety issues related to placebo

Currently, there are no FDA approved therapies for NAFLD. Therefore, use of a placebo is justified for comparison of efficacy with cysteamine DR. There are no known safety issues with the placebo capsules.

6.4 Management of adverse effects attributed to cysteamine bitartrate DR

During the trial, if a participant develops side effects thought to be due to the study medication and of a severity that requires cessation of study medication, the medication will be stopped for 4 weeks. If the side effects disappear, an attempt will be made to reintroduce the study medication after 4 weeks. If the symptoms reappear, study medication will be once again stopped and the patient will no longer receive the study medication, but will continue to be followed in the study according to the protocol, in keeping with the "intention-to-treat" paradigm.

6.5 Safety issues related to MRI

Standard safety precautions: screening; exclusion of potential participants with contraindications to MR. MRI is a minimal risk procedure if standard precautions and practice are exercised. Standard safety issues for MRI procedures include: claustrophobia, anxiety, discomfort from lying supine for 30 minutes, hearing loss, and heating of metal in the body. Patients will be screened by questionnaire and excluded when the presence of metal in the body is known or suspected. Sources include the presence of a cardiac pacemaker or defibrillator; metal fragments in eyes, skin, body; heart valve replacement, brain clips, venous umbrella; being a sheet-metal worker or welder; aneurysm surgery, intracranial bypass, renal, aortic clips; prosthetic devices such as middle ear, eye, joint or penile implants, joint replacements; hearing aid, neurostimulator, insulin pump; intrauterine device; shunts/stents, metal mesh/coil implants; metal plate/pin/screws/wires, or any other metal implants such as permanent eyeliner or eyebrows.

6.6 Safety issues related to end of treatment liver biopsy

Participants will have one liver biopsy done for research purposes after 52 weeks of treatment. About 20% of people who have a liver biopsy have some degree of pain over the liver that may last a few minutes up to several hours. This occasionally requires pain medication and usually disappears completely within a day or two. A rare complication of liver biopsy is severe bleeding such that a blood transfusion or even radiological/surgical interventions are required to stop the bleeding (between 1 in 1,000 and 1 in 5,000). Very rarely (less than 1 in 10,000 cases) death has occurred from bleeding after a biopsy. We intend to minimize the risks associated with liver biopsy (a) by requiring that each of the physicians who will obtain liver biopsies in the NASH CRN be very experienced in safely obtaining the liver biopsy specimens, (b) by not enrolling subjects with clinical cirrhosis or subjects with coagulopathy, and (c) by adhering to good clinical practice in performing the liver biopsy. In the NASH CRN TONIC trial (IND 71,217), 147 children had a similar research biopsy which was well tolerated and there were no serious adverse events[40].

6.7 Concerns related to participant privacy

It is the investigator's responsibility to conduct the protocol under the current versions of the Declaration of Helsinki, Good Clinical Practice, and rules of local IRBs. The investigator must ensure that the child's and parent's anonymity be maintained in their data submission to the Data Coordinating Center and IRB. Participants will be identified only by an identification code but not by their name, SSN, or hospital medical record number. Investigators will maintain a separate confidential enrollment log which matches identifying codes with the patients' names and addresses (i.e., available only to local clinic staff). All study material will be maintained in strict confidence.

6.8 Concerns related to specimen repository

It is anticipated that serum, plasma, DNA, and liver tissue from the participants will be stored for future studies related to NAFLD and possibly other liver/metabolic diseases. These samples will be stored in the NIDDK Genetics Repository and the NIDDK Biosample Repository. The NASH CRN Steering Committee will follow the NIDDK standards for (a) obtaining a separate informed consent, (b) storage, (c) transportation of the material, (d) who will have access to the material, and (e) the NIDDK and NASH CRN will follow their policies addressing what investigations will be conducted.

6.9 Food and Drug Administration

The CyNCh trial will be conducted under an Investigational New Drug application (IND # 114,924) held by the NIDDK. The investigators will complete a Statement of Investigator (FDA Form 1572) and must obtain IRB approval per the Code of Federal Regulations before the initiation of the CyNCh trial. The trial will not begin until the IND application is in effect. The safety data required to meet IND regulatory requirements will be collected through adverse event reporting by the clinic investigators and will be provided by the Data Coordinating Center to the NIDDK for transmission to the FDA, the Steering Committee, and the DSMB.

6.10 Adverse event reporting

The CyNCh trial investigators and staff will monitor and report adverse events to ensure patient safety. There are two separate sets of government regulations that apply to unanticipated or adverse events in research studies: (1) 45 CFR Part 46, Subpart A; the "Common Rule"[79], shared by 17 Departments and Agencies and (2) 21 CFR 312[80], the FDA regulation for adverse events. The Common Rule requires written procedures and policies for ensuring reporting of "unanticipated problems" involving risks to participants to IRBs, appropriate institutional officials, and the Department or Agency Head. The FDA regulation (21 CFR 312.66) requires reporting of "unanticipated problems" involving risks to participants to IRBs and notification of the FDA and participating investigators of any adverse event associated with the use of a test article that is "both serious and unexpected." Since the definitions and reporting requirements for unanticipated events differ between the two sets of Federal regulations, the CyNCh trial definitions and procedures for adverse events are designed to satisfy both sets of requirements.

6.10.1 Definitions

Adverse event means any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug related.

Life-threatening adverse event or life-threatening suspected adverse reaction: An adverse event or suspected adverse reaction is considered "life-threatening" if, in the view of either the investigator or sponsor, its occurrence places the patient or subject at immediate risk of death. It does not include an adverse event or suspected adverse reaction that, had it occurred in a more severe form, might have caused death.

Serious adverse event or serious suspected adverse reaction: An adverse event or suspected adverse reaction is considered "serious" if, in the view of either the investigator or sponsor, it results in any of the following outcomes: Death, a life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions, or a congenital anomaly/birth defect. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse.

Suspected adverse reaction means any adverse event for which there is a reasonable possibility that the drug caused the adverse event. For the purposes of IND safety reporting, "reasonable possibility" means there is evidence to suggest a causal relationship between the drug and the adverse event. Suspected adverse reaction implies a lesser degree of certainty about causality than adverse reaction, which means any adverse event caused by a drug.

Unexpected adverse event or unexpected suspected adverse reaction: An adverse event or suspected adverse reaction is considered "unexpected" if it is not listed in the investigator brochure or is not listed at the specificity or severity that has been observed; or, if an investigator brochure is not required or available, is not consistent with the risk information described in the general investigational plan or elsewhere in the current application, as amended. For example, under this definition, hepatic necrosis would be unexpected (by virtue of greater severity) if the investigator brochure referred only to elevated hepatic enzymes or hepatitis. Similarly, cerebral thromboembolism and cerebral vasculitis would be unexpected (by virtue of greater specificity) if the investigator brochure listed only cerebral vascular accidents. “Unexpected”, as used in this definition, also refers to adverse events or suspected adverse reactions that are mentioned in the investigator brochure as occurring with a class of drugs or as anticipated from the pharmacological properties of the drug, but are not specifically mentioned as occurring with the particular drug under investigation.

6.10.2 Monitoring for adverse events

Adverse events will be recorded on study data forms whether or not they are thought to be associated with the study or with the study drug. Adverse events may be discovered during regularly scheduled visits or through unscheduled patient contacts between visits.
Summary data on adverse events will be monitored by the DSMB quarterly and at its semiannual meetings or more frequently, as needed. These summaries will include analyses comparing rates of adverse events by treatment group, by clinic, or in other subgroups requested by the DSMB. Where applicable, signs and symptoms associated with the adverse event will be graded as to severity by the clinical site staff as mild, moderate, or severe using Version 3.0 of the National Cancer Institute’s Common Terminology Criteria for Adverse Events (CTCAE).[81]

After each DSMB meeting, the NIDDK will issue a written summary of the review of the study data, including adverse events, for transmission to the IRBs at each of the study centers. Analyses or listings of adverse events will not be provided to the IRBs; however, adverse events involving unanticipated problems involving risks to participants, or breaches of protocol which might entail risk to participants must be reported to local IRBs as soon as possible after they are discovered. Each participating center is responsible for ensuring that all local IRB requirements for reporting adverse events are met.

A summary of adverse events will be reported to the FDA as part of the IND annual report.

6.10.3 Reporting serious adverse events

Serious adverse events (SAE) must be reported upon discovery at the clinical center. This will involve completing a data form describing the severity and details of the event, which must be submitted to the Data Coordinating Center within one business day for review by the Safety Officer.

If the SAE is **unexpected AND there is a reasonable possibility that the study drug caused the SAE**, then the clinical center must complete a data form for an IND Safety Report and submit it along with a narrative and a copy of the IRB report to the DCC. The DCC will submit a preliminary report to the NIDDK for review within three business days of receiving the SAE data form. The pharmaceutical manufacturer will also be notified within 7 days of the serious adverse event, if applicable. If NIDDK determines that the SAE requires an expedited IND Safety Report, the NIDDK program official or the NIDDK Regulatory Affairs Specialist will notify the FDA no more than 15 calendar days from the initial receipt of the SAE by the DCC (no later than 7 calendar days if the SAE is fatal or life threatening), if applicable. The clinical center investigator may also be responsible for completing an FDA MedWatch 3500 form and additional information for a follow-up SAE report as information becomes available. If the FDA determines that a change to the investigators brochure, IND or protocol is needed, the Data Coordinating Center will send a copy of the IND Safety Report to all clinical centers, with instructions to forward the report to their IRB.

The DCC will maintain a list of all SAEs for reporting and review at Steering Committee meetings and DSMB meetings. The DSMB will review each SAE report and provide comments to the NIDDK program official. If requested by any member of the DSMB, a teleconference will be scheduled to discuss the SAE and recommend any actions to the NIDDK sponsor. The clinical center must submit to the NIDDK and to the Data Coordinating Center a follow-up memo within one month of the SAE (and periodic updates if needed) to report the details of the disposition of the SAE.

6.11 Procedures for unmasking treatment assignment

Treatment assignments are double masked throughout the study until all data collection for the CyNCh trial has been completed (i.e., after completion of the 24 week post trial follow-up for all participants). Every effort will be made to maintain the masking throughout the study except in emergencies. The code of specific pharmacological treatment will not be broken without the knowledge of the clinical center's principal investigator and the study leadership.

Unmasking of study medication may occur under the following conditions:

- **Severe allergic reaction (Stevens-Johnson Syndrome)**: Study medication is stopped indefinitely. The patient, primary care provider (PCP), and the investigator may be unmasked.
- **Pregnancy during the study:** Study medication will be stopped indefinitely, and the coded medication may be unmasked. The patient, PCP, and investigator will be notified of the associated risks of teratogenicity.
- **Development of hepatotoxicity:** Hepatotoxicity will be defined as the development of jaundice with a serum direct bilirubin of > 1.0 mg/dL and an increase in the baseline ALT and AST value that is two-fold or > 400 U/L during treatment. Study medication will be discontinued; however, there will be no unmasking until study conclusion.

The Data and Safety Monitoring Board will review all instances of unmasking that occur.

7. Statistical design and analysis

7.1 Primary hypothesis:

Cysteamine bitartrate DR at 9-12 mg/kg orally daily for 52 weeks is better than placebo in improving liver histologic parameters as measured by the NAFLD activity score in children with NAFLD.

Secondary aims:

- To assess safety and tolerability of cysteamine DR during the 52 week treatment period
- To assess the effect of cysteamine DR on:
  - Serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST) and serum γ-glutamyl transferase (GGT).
  - Liver fat as measured by MRI.
  - Markers of oxidation and anti-oxidant status: malondialdehyde, F2 alpha-isoprostane, total antioxidant capacity
  - Insulin resistance
  - Anthropometrics
  - Quality of Life
  - Liver related symptoms

7.2 Outcome measures

Primary outcome measure:

The primary outcome measure, improvement in NAFLD from the start of treatment to the end of treatment at 52 weeks, is defined for each patient as:

- Improvement in NAS score by 2 or more points, and
- No worsening of the fibrosis score

Secondary outcome measures:

- *Conversion from a diagnosis of NASH to not-NASH.* For the subset of patients with NASH at enrollment, determine the proportion whose 52-week liver biopsy results in a centrally determined histological diagnosis of not-NASH
- *Change in NAS*. Change in NAS, is defined for each patient, as the difference in 52 week vs. baseline NAS scores
- *Specific histologic features.* Changes in specific histological scores, defined for each patient, as the difference in 52 weeks vs. baseline, in the following histological features from centrally scores liver biopsies:
  - Fibrosis
  - Steatosis
  - Lobular inflammation
  - Portal chronic inflammation
  - Ballooning

  Change from stage 1b to 1a fibrosis. For the subset of patients with stage 1b fibrosis at baseline, determine the proportion whose 52-week liver biopsy results in a centrally determined histological diagnosis of fibrosis 1a.
- *Serum aminotransferase*. Change in serum aminotransferase, is defined for each patient, as the difference in 52 week vs. baseline serum aminotransferase

- *Gamma-glutamyl transpeptidase*. Change in serum gamma-glutamyl transpeptidase, is defined for each patient, as the difference in 52 week vs. baseline serum gamma-glutamyl transpeptidase
- *MRI-determined hepatic fat fraction.* Change in MRI-determined hepatic fat fraction is defined for each patient, as the difference in 52 week vs. baseline MRI-determined hepatic fat fraction
- *Markers of oxidation and anti-oxidant status*. Change in markers of oxidation and anti-oxidant status, is defined for each patient, as the difference in 52 week vs. baseline values of the following markers:
    - Malondialdehyde,
    - F2alpha-isoprostane,
    - Total antioxidant capacity, and
    - Oxidized LDL
- *Fasting insulin.* Change in fasting insulin, is defined for each patient, as the difference in 52 week vs. baseline fasting insulin
- *Fasting glucose*. Change in fasting glucose, is defined for each patient, as the difference in 52 week vs. baseline fasting glucose
- *Obesity measures.* Change in obesity measures, is defined for each patient, as the difference in 52 week vs. baseline values of the following obesity measures:
    - Weight,
    - Height,
    - BMI,
    - Waist circumference
- *Pediatric Quality of Life*. Change in PedsQL score, is defined for each patient, as the difference in 52 week vs. baseline PedsQL score

7.3 Statistical analysis

Primary outcome measure:

Statistical analyses for the primary hypothesis will follow the intention-to-treat paradigm, which means that all randomized patients with baseline and 52 week liver biopsies will be included in the treatment group to which they were assigned. Any randomized patient who does not have the requisite biopsies will be accounted for and compared by assigned treatment group.

Since the primary outcome measure, defined in Section 7.2, is a binary indicator of improvement in histologic activity score after 52 weeks of treatment compared to baseline and since the randomization is stratified by clinic, P-values will be derived from the Mantel-Haenszel $\chi 2$ test for stratified 2x2 tables. The proportion of patients improved in the group assigned to cysteamine DR will be compared to the group assigned to placebo and a two-tailed p-value of ≤ 0.05 will be considered statistically significant.

Secondary outcome measures:

Statistical analyses for the secondary measures that are continuous measures or scores will be carried out using separate bootstrapped mixed-effects-models corresponding to each individual change measure (defined in Section 7.2) as the response variable, with random effects for variation by clinic, and an indicator variable for comparing cysteamine DR (compared to placebo). The regression coefficient estimate for the indicator variable is the treatment effect for each secondary measure and is interpreted as the mean of the per-patient differences in each secondary outcome measure in the cysteamine DR group minus the corresponding mean differences in the placebo group. Statistical significance for each secondary treatment effect will be achieved if zero is not included in the nominal 95% bootstrapped confidence limits for each secondary measure. Bootstrapping with 2,000 replications will be performed to achieve 95% confidence limits that account for, in advance,

potential non-normality in the continuous secondary outcomes measures or scores. The bootstrapping estimation will be carried out using Stata software.

Statistical analyses for each of the secondary measures that are events (binary responses) will be carried out using the same method as for the analysis of the primary outcome measure. Events rates (proportions) in the cysteamine DR group and in the placebo group will be determined and a stratified (by clinic) Mantel-Haenszel analysis for stratified 2x2 tables will the used to obtain a weighted odds ratio as the treatment effect (OR > 1 favors cysteamine DR) and P-value for the significance of the odds ratio estimate; a nominal two-tailed p-value of ≤ 0.05 will be considered statistically significant.

We plan to use nominal P-values when reporting the statistical significance of each secondary outcome, since we have declared such outcomes in advance in hypothesis-driven discussions among the investigators. We will also determine Bonferroni-corrected 95% confidence intervals and P-values for comparison with nominal P-values.

7.4 Missing data

The occurrence of missing data in this trial is expected to be low and equally distributed across the treatment groups. We estimate that careful selection of patients during the screening phase and the consent process should result in no more than 10% missing data from patients who drop out before completing the 52 week treatment period. In primary, intention-to-treat analyses, subjects with missing data will be considered unimproved on the primary outcome measure.

The proportions with missing data will be compared across treatment groups using $\chi 2$ tests. If the amount of missing data exceeds 10%, then a variety of sensitivity analyses will be carried out to compare to the primary analysis using all available non-missing data: (1) compare pessimistic and optimistic imputations of the missing values, (2) correct for missing data using multiple imputation with 10 replicated samples, and (3) use mixed random effects logistic or linear regression models, depending on the type of outcome measure. Sensitivity analyses (2) and (3) assume that that missing data are missing at random (MAR). It is possible that the missing data are not MAR and, therefore, non-ignorable. There are a few statistical methods available when there are non-ignorable missing data and these would be employed; however; all such methods involve strong assumptions that cannot be verified from the available data.

7.5 Justification of sample size

A total of 160 participants in two groups of equal size (80 per group) will be included to compare the efficacy of cysteamine DR vs. placebo with the primary outcome measure of histological improvement in NAS (as defined in section 7.2). A sample size of 160 patients with 1:1 randomization would provide 90% power to the study hypothesis to detect a significant difference between the two groups with a two-sided type I error rate of 0.05. This sample size takes into account that 10% of participants will not have 52 week biopsies and will be considered not improved based upon intention to treat analysis. The sample size estimation is based upon the assumption that the minimum clinically important difference, defined as a 33% lower relative rate of no histological improvement in the cysteamine DR group vs. the placebo group, will be expected with the assumed response rates in the placebo group of 75% (based on TONIC data and 2 to 1 split for patients not taking vs. taking vitamin E at the start of the trial) vs. expected percent with no histological improvement in NAS in the cysteamine DR group to be 50%. These sample size estimations are conservative and would have a minimal likelihood of a type 2 error.

7.6 Interim monitoring

An independent Data and Safety Monitoring Board (DSMB), appointed by the NIDDK, will

review the protocol for the CyNCh trial and monitor the safety data as the trial progresses to ensure patient safety and to review efficacy. The DSMB is a multidisciplinary group with a written charge provided by the NIDDK. The DSMB reports to the NIDDK, which will communicate DSMB recommendations to the investigators, as appropriate. The DSMB will hold a meeting to approve the protocol. After the trial commences, the DSMB will meet twice a year to review data and other issues. The DSMB may request more frequent meetings if necessary to fulfill its charge. It may also request additional safety reports on a more frequent basis. For example, all serious adverse events are reported to the DSMB for their consideration and recommendations as they occur.

Interim data on safety measures requested by the DSMB are reviewed at each of the scheduled semi-annual full meetings. Two additional written safety reports will be reviewed by the DSMB between scheduled full meetings. Serious adverse events will be reviewed by the DSMB as they occur with the option of a teleconference discussion if any DSMB member so requests.

The DSMB will review quarterly reports by masked treatment groups of incident hepatotoxicities, as well as counts of patients who required more frequent liver function testing due to rises in ALT levels of more than 2 times baseline ALT or beyond 400 U/L. The DSMB will also examine the trends in ALT or AST levels for each patient who experiences a rise in ALT.

The DSMB also reviews the overall progress of the trial in terms of recruitment and data quality and makes a formal recommendation to the NIDDK at the end of each scheduled meeting as to whether the trial should continue unmodified, continue with protocol modifications, or be stopped.

8. Human subject issues

8.1 Overview

The study protocol, questionnaires, and consent forms will be submitted to each participating center's IRB. Sites which recruit patients will submit their recruitment materials to their IRB prior to use. A site may not initiate any patient contact about the CyNCh trial until the site has IRB approval for the trial. All study personnel must complete training in the Protection of Human Subjects per NIH guidelines. The proposed study anticipates recruiting a significant proportion of racial/ethnic minorities (African-Americans, Asian-Americans and Hispanics) as well as non-Hispanic white subjects.

8.2 Institutional Review Board (IRB) approvals

A site may not initiate patient screening and randomization activities in the CyNCh trial until the site has IRB approval for the trial and the IND is in effect. Consent forms must have IRB approval. Sites must provide the DCC with a copy of the initial IRB approval notice and subsequent renewals as well as copies of the IRB approved consent statements.

8.3 Informed consent

Template parent consent and pediatric assent documents will be prepared for the trial for screening to determine eligibility with an affirmation of consent for randomization in the trial. Individual sites may add material but may not delete material thought to be necessary for informed consent. Clinics may reformat and reword information to conform to their local requirements. The patient's guardian must sign the consent and the patient must sign the assent to be eligible for the trial. The consent documents will describe the purpose of the trial, the procedures to be followed, and the risks and benefits of participation. Copies of the signed consent/assent forms will be given to the patient and patient's guardian, and this fact will be documented in the patient's record.

8.4 Subject confidentiality

All laboratory specimens, study forms, reports, and other records that are part of the study data collection materials will be identified by coded number to maintain patient confidentiality. All records will be kept in locked file cabinets. All electronic records of study data will be identified by coded number. Clinical information will not be released without written permission of the patient, except as necessary for monitoring by the IRB. Consent procedures and forms, and the communication, transmission and storage of patient data will comply with individual site IRB and NIH requirements for compliance with The Health Insurance Portability and Accountability Act (HIPAA).

8.5 Administration of study drug

An investigator may not administer an investigational new drug to human subjects until the IND goes into effect (30 days after IND receipt by FDA) or sooner if notified. And investigational drug under IND may only be used by an investigator in compliance with 21 CFR Part 50 and 21 CFR Part 56.

9. References

1. Schwimmer JB, Deutsch R, Kahen T, Lavine JE, Stanley C, Behling C. Histopathology of pediatric nonalcoholic fatty liver disease. *Hepatology* 2005;42:641-649.

2. Szczepaniak LS, Nurenberg P, Leonard D, et al. Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. *Am J Physiol Endocrinol Metab* 2005;288:E462-468.

3. Brunt EM. Pathology of nonalcoholic fatty liver disease. *Nat Rev Gastroenterol Hepatol* 2010;7:195-203.

4. Brunt EM. What's in a Name? *Hepatology*. 2009;50:663-667.

5. Schwimmer JB, Deutsch R, Kahen T, Lavine JE, Stanley C, Behling C. Prevalence of fatty liver in children and adolescents. *Pediatrics.*2006;118:1388-1393.

6. Nobili V, Marcellini M, Devito R, et al. NAFLD in children: a prospective clinical-pathological study and effect of lifestyle advice. *Hepatology* 2006;44:458-465.

7. Schwimmer JB, Deutsch R, Rauch JB, Behling C, Newbury R, Lavine JE. Obesity, insulin resistance, and other clinicopathological correlates of pediatric nonalcoholic fatty liver disease. *J Pediatr.* 2003;143:500-505.

8. Kistler KD, Molleston J, Unalp A, Abrams SH, Behling C, Schwimmer JB. Symptoms and quality of life in obese children and adolescents with nonalcoholic fatty liver disease. *Aliment Pharmacol Ther.* 2009;31:396-406.

9. Xanthakos S, Miles L, Bucuvalas J, Daniels S, Garcia V, Inge T. Histologic spectrum of nonalcoholic fatty liver disease in morbidly obese adolescents. *Clin Gastroenterol Hepatol.* 2006;4:226-232.

10. Rashid M, Roberts EA. Nonalcoholic steatohepatitis in children. *J Pediatr Gastroenterol Nutr.* 2000;30:48-53.

11. Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 1999;116:1413-1419.

12. Bugianesi E. Non-alcoholic steatohepatitis and cancer. *Clin Liver Dis.* Feb 2007;11(1):191-207, x-xi.Review

13. Cuadrado A, Orive A, Garcia-Suarez C, et al. Non-alcoholic steatohepatitis (NASH) and hepatocellular carcinoma. *Obes Surg.* 2005;15:442-446.

14. Hashizume H, Sato K, Takagi H, et al. Primary liver cancers with nonalcoholic steatohepatitis. *Eur J Gastroenterol Hepatol.* 2007;19:827-834.

15. Rubinstein E, Lavine JE, Schwimmer JB. Hepatic, cardiovascular, and endocrine outcomes of the histological subphenotypes of nonalcoholic fatty liver disease. *Semin Liver Dis.* 2008;28:380-385.

16. Sanyal AJ, Banas C, Sargeant C, et al. Similarities and differences in outcomes of cirrhosis due to nonalcoholic steatohepatitis and hepatitis C. *Hepatology* 2006;43:682-689.

17. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation* 2002;106:3143-3421.

18. Schwimmer JB, Pardee PE, Lavine JE, Blumkin AK, Cook S. Cardiovascular risk factors and the metabolic syndrome in pediatric nonalcoholic fatty liver disease. *Circulation.* 2008;118:277-283.

19. Ekstedt M, Franzen LE, Mathiesen UL, et al. Long-term follow-up of patients with NAFLD and elevated liver enzymes. *Hepatology* 2006;44:865-873.

20. Aygun C, Kocaman O, Sahin T, et al. Evaluation of metabolic syndrome frequency and carotid artery intima-media thickness as risk factors for atherosclerosis in patients with nonalcoholic fatty liver disease. *Dig Dis Sci* 2008;53:1352-1357.

21. Nadeau KJ, Klingensmith G, Zeitler P. Type 2 diabetes in children is frequently associated with elevated alanine aminotransferase. *J Pediatr Gastroenterol Nutr* 2005;41:94-98.

22. Adams LA, Lymp JF, St Sauver J, et al. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. *Gastroenterology* 2005;129:113-121.

23. Fan JG, Li F, Cai XB, Peng YD, Ao QH, Gao Y. Effects of nonalcoholic fatty liver disease on the development of metabolic disorders. *J Gastroenterol Hepatol* 2007;22:1086-1091.

24. Brunt EM, Kleiner DE, Wilson LA, et al. Portal chronic inflammation in nonalcoholic fatty liver disease (NAFLD): a histologic marker of advanced NAFLD-Clinicopathologic correlations from the nonalcoholic steatohepatitis clinical research network. *Hepatology* 2009;49:809-820.

25. Pardee PE, Lavine JE, Schwimmer JB. Diagnosis and treatment of pediatric nonalcoholic steatohepatitis and the implications for bariatric surgery. *Semin Pediatr Surg.* 2009;18:144-151.

26. Kahn CR, Flier JS, Bar RS, et al. The syndromes of insulin resistance and acanthosis nigricans. Insulin-receptor disorders in man. *N Engl J Med.* 1976;294:739-745.

27. Moller DE, Flier JS. Insulin resistance--mechanisms, syndromes, and implications. *N Engl J Med.* 1991;325:938-948.

28. Poretsky L, Piper B. Insulin resistance, hypersecretion of LH, and a dual-defect hypothesis for the pathogenesis of polycystic ovary syndrome. *Obstet Gynecol* 1994;84:613-621.

29. Jeanrenaud B. Hyperinsulinemia in obesity syndromes: its metabolic consequences and possible etiology. *Metabolism* 1978;27:1881-1892.

30. Malhi H, Gores GJ. Molecular mechanisms of lipotoxicity in nonalcoholic fatty liver disease. *Semin Liver Dis.* 2008;28:360-369.

31. Pfaffenbach KT, Gentile CL, Nivala AM, et al. Linking endoplasmic reticulum stress to cell death in hepatocytes: roles of C/EBP homologous protein and chemical chaperones in palmitate mediated cell death. *Am J Physiol Endocrinol Metab.* 2010;298:E1027-1035.

32. Anderson N, Borlak J. Molecular mechanisms and therapeutic targets in steatosis and steatohepatitis. *Pharmacol Rev.* 2008;60:311-357.

33. Syn WK, Choi SS, Diehl AM. Apoptosis and cytokines in non-alcoholic steatohepatitis. *Clin Liver Dis.* 2009;13:565-580.

34. Syn WK, Jung Y, Omenetti A, et al. Hedgehog-mediated epithelial-to-mesenchymal transition and fibrogenic repair in nonalcoholic fatty liver disease. *Gastroenterology.* 2009;137:1478-1488.e1478.

35. Jung Y, Diehl AM. Non-alcoholic steatohepatitis pathogenesis: role of repair in regulating the disease progression. *Dig Dis Sc.* 2010;28:225-228.

36. Neuschwander-Tetri BA. Lifestyle modification as the primary treatment of NASH. *Clin Liver Dis.* 2009;13:649-665.

37. Schwimmer JB, Middleton MS, Deutsch R, Lavine JE. A phase 2 clinical trial of metformin as a treatment for non-diabetic paediatric non-alcoholic steatohepatitis. *Aliment Pharmacol Ther.* 2005;21:871-879.

38. Nobili V, Manco M, Ciampalini P, et al. Metformin use in children with nonalcoholic fatty liver disease: an open-label, 24-month, observational pilot study. *Clin Ther* 2008;30:1168-1176.

39. Lavine JE, Schwimmer JB, Molleston JP, et al. Treatment of nonalcoholic fatty liver disease in children: TONIC trial design. *Contemp Clin Trials* 2010 Jan;31(1):62-70.

40. Lavine JE, Schwimmer JB, Van Natta ML, Molleston JP, Murray KF, Rosenthal P, et al: Effect of vitamin E or metformin for treatment of nonalcoholic fatty liver disease in children and adolescents. The TONIC Randomized Controlled Trial. *JAMA* 2011; 305: 1659-1668.

41. Gastaldelli A, Harrison SA, Belfort-Aguilar R, et al. Importance of changes in adipose tissue insulin resistance to histological response during thiazolidinedione treatment of patients with nonalcoholic steatohepatitis. *Hepatology.* 2009;50:1087-1093.

42. Kintscher U, Law RE. PPARg-mediated insulin sensitization: the importance of fat versus muscle. *Am J Physiol Endocrinol Metab.* 2005;288:E287-291.

43. Ratziu V, Giral P, Jacqueminet S, et al. Rosiglitazone for nonalcoholic steatohepatitis: one-year results of the randomized placebo-controlled Fatty Liver Improvement with Rosiglitazone Therapy (FLIRT) Trial. *Gastroenterology.* 2008;135:100-110.

44. Ratziu V, Charlotte F, Bernhardt C, et al. Long-term efficacy of rosiglitazone in nonalcoholic steatohepatitis: results of the fatty liver improvement by rosiglitazone therapy (FLIRT 2) extension trial. *Hepatology.* 2010; 51:445-453.

45. Promrat K, Lutchman G, Uwaifo GI, et al. A pilot study of pioglitazone treatment for nonalcoholic steatohepatitis. *Hepatology.* 2004;39:188-196.

46. Belfort R, Harrison SA, Brown K, et al. A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. *N Engl J Med.* 2006;355(22):2297-307.

47. Aithal GP, Thomas J, Kaye P, et al. Randomized, placebo-controlled trial of pioglitazone in nondiabetic subjects with nonalcoholic steatohepatitis. *Gastroenterology.* 2008;135:1176-1184.

48. Sanyal AJ, Chalasani N, Kowdley K V, et al. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *New EnglJ Med* 2010;362:1675-1685.

49. Chang CY, Argo CK, Al-Osaimi AM, Caldwell SH. Therapy of NAFLD: antioxidants and cytoprotective agents. *Journal of Clinical Gastroenterology.* Mar 2006;40 Suppl 1:S51-60.

50. Nobili V, Manco M, Devito R, Ciampalini P, Piemonte F, Marcellini M. Effect of vitamin E on aminotransferase levels and insulin resistance in children with non-alcoholic fatty liver disease. *Aliment Pharmacol Ther.* 2006;24:1553-1561.

51. Sanyal AJ, Campbell-Sargent C, Mirshahi F, et al. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* 2001;120:1183-1192.

52. Sanyal AJ. Mechanisms of Disease: pathogenesis of nonalcoholic fatty liver disease. *Nat Clin Pract Gastroenterol Hepatol* 2005;2:46-53.

53. Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. *J Nutr* 2004;134:489-492.

54. Prescott LF, Critchley JA. The treatment of acetaminophen poisoning. *Annu Rev Pharmacol Toxicol.* 1983;23:87-101.

55. Pamuk GE, Sonsuz A. N-acetylcysteine in the treatment of non-alcoholic steatohepatitis. *J. Gastroenterol Hepatol.* 2003;18:1220-1221.

56. Lyons J, Rauh-Pfeiffer A, Yu YM, et al. Blood glutathione synthesis rates in healthy adults receiving a sulfur amino acid-free diet. *Proc Natl Acad Sci USA.* 2000;97:5071-5076.

57. Lu SC. Regulation of glutathione synthesis. *Curr Top Cell Regul* 2002;36:95-116.

58. Gahl WA, Thoene JG, JA. S. Cystinosis. *N Engl J Med.* 2002; 347:111-121.

59. Dohil R, Fidler M, Barshop BA, et al. Understanding intestinal cysteamine bitartrate absorption. *J Pediatr.* 2006;148:764-769.

60. Dohil R, Schmeltzer S, Cabrera BL, Wang T, Durelle J, Duke KB, Schwimmer JB, Lavine JE. Enteric-coated cysteamine for the treatment of paediatric non-alcoholic fatty liver disease. *Aliment Pharmacol Ther.* 2011 33:1036-1044

61. Lindor KD, Kowdley KV, Heathcote EJ, et al. Ursodeoxycholic acid for treatment of nonalcoholic steatohepatitis: results of a randomized trial. *Hepatology* 2004;39:770-778.

62. Abdelmalek MF, Sanderson SO, Angulo P, et al. Betaine for nonalcoholic fatty liver disease: results of a randomized placebo-controlled trial. *Hepatology.*2009;50:1818-1826.

63. Loomba R, Sirlin CB, Schwimmer JB, Lavine JE. Advances in pediatric nonalcoholic fatty liver disease. *Hepatology.* 2009;50:1282-1293.

64. Peterson KF, Dufour S, Befroy D, et al. Reversal of nonalcoholic hepatic steatosis, hepatic insulin resistance and hyperglycemia by moderwate weight reduction in patients with type 2 diabetes. *Diabetes* 2005;54:603-608.

65. Nobili V, Manco M, Raponi M, Marcellini M. Case management in children affected by non-alcoholic fatty liver disease. *J Paediatr Chid Health.* 2007;43:414.

66. Barlow SE. Expert committee recommendations regarding the prevention, assessment, and treatment of child and adolescent overweight and obesity: summary report.*Pediatrics* 2007;120 Suppl 4:S164-192.

67. Healthy Weight - it's not a diet isal. Tips for Parents – Ideas to Help Children Maintain a Healthy Weight. *http://www.cdc.gov/healthyweight/children/* 2009, accessed September 17, 2010.

68. We Can - Ways to Enhance Children's Activity and Nutrition. *http://www.nhlbi.nih.gov/health/public/heart/obesity/wecan/index.htm*. Accessed September 17, 2010.

69. How Many Fruits and Vegetables Do You Need? Eat a Variety of Fruits & Vegetables Every Day. *Centters for Disease Control and Prevention*. Accessed September 17, 2010.

70. For Kids. *MyPyramid.gov* 2010. Accessed September 17, 2010.

71. Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005;41:1313-1321.

72. Thomsen C, Becker U, Winkler K, et al. Quantification of liver fat using magnetic resonance spectroscopy. *Magn Reson Imaging.* 1994;12:487-495.

73. Szczepaniak LS, Babcock EE, Schick F, et al. Measurement of intracellular triglyceride stores by H spectroscopy: validation in vivo. *Am J Physiol* 1999;276:E977-E989.

74. Fischbach F, Bruhn H. Assessment of in vivo 1H magnetic resonance spectroscopy in the liver: a review. *Liver Int.* 2008;28:297-307.

75. Reeder S, Hines C, Yu H, et al. On The Definition of Fat-Fraction for In Vivo Fat Quantification with Magnetic Resonance Imaging. *Proceedings 17th Scientific Meeting, International Society for Magnetic Resonance in Medicine. Honolulu.* 2009;211.

76. Irwan R, Edens MA, Sijens PE. Assessment of the variations in fat content in normal liver using a fast MR imaging method in comparison with results obtained by spectroscopic imaging. *Eur Radiol.* 2008;18:806-813.

77. Brix G HS, Bellemann ME, et al. MR imaging of fat-containing tissues: valuation of two quantitative imaging techniques in comparison with localized proton spectroscopy. *Magn Reson Imaging.* 1993;11:977-991.

78. US Department of Health and Human Services, Office for Human Research Protections, Code of Federal Regulations (CFR). 45 CFR part 46, http://www.hhs.gov/ohrp/humansubjects/guidance/45cfr46.html

79. US Department of Health and Human Services, Code of Federal Regulations (CFR), Title 21: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRsearch.cfm?CFRPart=312

80. US National Institutes of Health, National Cancer Institute. Common Terminology Criteria for Adverse Events, v3.0 August 2006. Accessed at http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcaev3.pdf

10. Appendices

10.1 Participating Centers

Clinical Centers

- Children's Memorial Hospital, Chicago, IL (Peter Whitington, MD)
- Cincinnati Children's Hospital Medical Center, Cincinnati, OH (Stavra A. Xanthakos, MD)
- Columbia University, NY, NY (Joel Lavine, MD, PhD)
- Emory University, Atlanta, GA (Saul Karpen, MD, PhD)
- Indiana University, Indianapolis, IN (Jean Molleston, MD)
- Saint Louis University, St. Louis, MO (Ajay Jain, MD)
- Texas Children's Hospital, Houston, TX (Sarah Barlow, MD)
- University of California, San Diego, CA (Jeffrey Schwimmer, MD)
- University of California, San Francisco, CA (Philip Rosenthal, MD)
- University of Washington, Seattle, WA (Karen Murray, MD)

Radiology Reading Center

- University of California, San Diego, CA (Claude Sirlin, MD)

Data Coordinating Center:

- Johns Hopkins University, Baltimore, MD (James Tonascia, PhD)

National Institutes of Health:

- National Institute of Diabetes and Digestive and Kidney Diseases (Ed Doo, MD, Averell Sherker, MD)

NIDDK Central Repositories:

- Biosample repository: Fisher Bioservices Corporation
- Genetics repository: Rutgers, The State University of New Jersey
- Data repository: Information Management Services (IMS)

10.2 Data Collection schedule

Assessment/Procedure	Screening visits	RZ	Follow-up visits Weeks from randomization f04	f12	f24	f36	f52	f76
Consent and HIPAA authorization	X	.	.	.	.	.	.	.
Baseline medical history	X	.	.	.	.	.	.	.
Follow-up medical history	.	.	X	X	X	X	X	X
Review for adverse effects	.	.	X	X	X	X	X	X
Review for concomitant medications	X	X	X	X	X	X	X	X
Alcohol questionnaire AUDIT (A) if interim (I)	A	.	I	I	I	I	I	I
Detailed (D) or focused (F) physical exam	D	F	F	F	D	F	D	D
Liver biopsy*	X*	.	.	.	.	.	X	.
MRI for hepatic fat (optional)	X	.	.	.	.	.	X	.
Nutritional assessment	X	.	.	.	.	.	X	.
Pediatric quality of life	X	.	.	.	.	.	X	X
Liver symptoms questionnaire	X	.	.	X	X	X	X	X
Standard of care materials provided	.	X	.	.	.	.	.	.
Eligibility confirmation	.	X	.	.	.	.	.	.
Study drug dispensing	.	X	X	X	X	X	.	.
Review of study drug adherence	.	.	X	X	X	X	X	.
Labs:								
Complete blood count	X	.	X.	X	X	.X	X	X
Comprehensive metabolic panel with uric acid	X	.	.	.	X	.	X	X
Hepatic panel with GGT, PT, INR	X	.	X	X	X	X	X	X
Fasting lipid profile	X	.	.	.	X	.	X	X
Fasting serum glucose, HbA1c, and insulin	X	.	.	.	X	.	X	X
Etiologic tests	X	.	.	.	.	.	.	.
Pregnancy test	X	X	X	X	X	X	X	.
Banking:								
Fasting serum and plasma	X	.	.	X	X	X	X	X
DNA	X	.	.	.	.	.	.	.
Liver tissue	X	.	.	.	.	.	X	.
Closeout form	.	.	.	.	.	.	.	X

Complete blood count: Hemoglobin, hematocrit, mean corpuscular volume (MCV), white blood cell count (WBC), white blood cell differential, platelet count

Comprehensive metabolic panel: calcium, carbon dioxide, chloride, glucose, potassium, sodium, creatinine, blood urea nitrogen (BUN), uric acid

Hepatic panel: Total bilirubin, direct bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, gamma glutamyltransferase (GGT), albumin, total protein, prothrombin time (PT), international normalized ratio (INR)

Lipid profile: triglycerides, total cholesterol, LDL and HDL

Etiologic tests: Hepatitis B surface antigen, hepatitis C antibody, alpha-1-antitrypsin level, ceruloplasmin. Autoantibodies: (ANA, AMA ASMA), serum iron, ferritin and total iron binding capacity (TIBC)

*The liver biopsy during screening is for the patient's clinical evaluation of NAFLD

10.3 Blood collection schedule (amounts in mL)

Procedure	Screening	Study visit (wk) f04	f12	f24	f36	f52	f76	Total
Fasting glucose, HbA1c and insulin	5	.	.	5	.	5	5	20
Fasting lipid profile	5	.	.	5	.	5	5	20
Complete blood count	5	5	5	5	5	5	5	35
Comprehensive metabolic panel	5	.	.	5	.	5	5	20
Hepatic panel with GGT, PT, INR	5	5	5	5	5	5	5	35
Etiologic tests	20	.	.	.	.	.	.	20
Plasma	10	.	10	10	10	10	10	60
Serum	20	.	20	20	20	20	20	120
DNA	20	.	.	.	.	.	.	20
Total	95	10	40	55	40	55	55	**350**

Complete blood count: Hemoglobin, hematocrit, mean corpuscular volume (MCV), white blood cell count (WBC), white blood cell differential, platelet count

Comprehensive metabolic panel: calcium, carbon dioxide, chloride, glucose, potassium, sodium, creatinine, blood urea nitrogen (BUN), uric acid

Hepatic panel*:* Total bilirubin, direct bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, gamma glutamyltransferase (GGT), albumin, total protein, prothrombin time (PT), international normalized ratio (INR)

Lipid profile: triglycerides, total cholesterol, LDL and HDL

Etiologic tests: Hepatitis B surface antigen (HBsAg), hepatitis C antibody (anti-HCV), alpha-1-antitrypsin level, ceruloplasmin. Autoantibodies: (ANA, AMA, ASMA), serum iron, ferritin, and total iron binding capacity (TIBC)

10.4 Glossary

A1AT	alpha-1-antitrypsin
ALT	alanine aminotransferase
AMA	antimitochondrial antibody
ANA	anti-nuclear antibody
anti-HCV	hepatitis C antibody
ASMA	anti-smooth muscle antibody
AST	aspartate aminotransferase
AUDIT	Alcohol Use Disorders Identification Test
BMI	body mass index (kg/m^2)
BUN	blood urea nitrogen
CC	Clinical Center
CRN	Clinical Research Network
DCC	Data Coordinating Center
DDC	Drug Distribution Center
DSMB	Data and Safety Monitoring Board
EMT	epithelial-mesenchymal transition
GCRC	General Clinical Research Center
GGT	gamma glutamyltransferase
HbA1c	hemoglobin A1c
HBc	hepatitis B core antigen
HBsAg	hepatitis B surface antigen
HCC	Hepatocellular carcinoma
HCV	hepatitis C virus
HIPAA	Health Insurance Portability and Accountability Act
INR	international normalized ratio
IRB	institutional review board
MCV	mean corpuscular volume
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
NIH	National Institutes of Health
NAFL	nonalcoholic fatty liver
NAFLD	nonalcoholic fatty liver disease
NAS	nonalcoholic fatty liver disease activity score
NASH	nonalcoholic steatohepatitis
NSAIDs	nonsteroidal anti- inflammatory drugs
PPARγ	peroxisome proliferator-activated receptor-gamma
PT	prothrombin time
ROS	reactive oxidative species
SAE	serious adverse event
SOC	standard of care
TIBC	total iron binding capacity
WBC	white blood cell count

10.5 Document History

Cysteamine Bitartrate Delayed-Release for the Treatment of Nonalcoholic Fatty Liver Disease (NAFLD) in Children (CyNCh) Trial Protocol (08 March 2012)

Cysteamine Bitartrate Delayed-Release for the Treatment of Nonalcoholic Fatty Liver Disease (NAFLD) in Children (CyNCh) Trial Protocol (13 April 2012)

Numerous editorial and wording changes were made to the following sections

- IND #114,924 was added to the cover page and section 6.9
- Corrected spelling of maldondialdehyde to malondialdehyde throughout

§ Design Synopsis
- Removed "serum" from pregnancy test in inclusion and exclusion criteria
- Added seizure disorder as an exclusion criteria
- Added gastrointestinal ulcers/bleeding as an exclusion criteria
- Added Clinically significant depression (patients hospitalized for suicidal ideations or suicide attempts within the past 12 months) as an exclusion criteria

§ 4.3 Exclusion criteria
- Added seizure disorder
- Added gastrointestinal ulcers/bleeding
- Added Clinically significant depression (patients hospitalized for suicidal ideations or suicide attempts within the past 12 months)
- Removed "serum" from pregnancy test

§ 5.4 Follow-up visits
- Added complete blood count to follow-up visits at Week 4, Week 12, and Week 36
- Removed phosphate from metabolic panel

§ 10.2 Data Collection schedule
- Added complete blood count to follow-up visits at Week 4, Week 12, and Week 36
- Removed phosphate from metabolic panel

§ 10.3 Blood collection schedule
- Added complete blood count to follow-up visits at Week 4, Week 12, and Week 36
- Adjustments made to estimated blood draw amounts
- Removed phosphate from metabolic panel

§ 10.5 Document History
- Added document history

Cysteamine Bitartrate Delayed-Release for the Treatment of Nonalcoholic Fatty Liver Disease (NAFLD) in Children (CyNCh) Trial Protocol (06 September 2013)

§ Design Synopsis
- Changed Recruitment phase from 9 to 19 months
- Changed Follow-up phase: from 27 to 37 months

§ 3.5 Primary Endpoint changed to Primary outcome measure

- Clarified primary outcome measure to: "Centrally scored and masked assessment of histologic improvement in NAFLD between the baseline liver biopsy and follow-up biopsy after 52 weeks of treatment, where improvement is defined as: (1) decrease in NAS of 2 or more and (2) no worsening of fibrosis."

§ 3.6 Secondary Endpoints changed to Secondary outcome measures

§ 4.1 Recruitment

- Changed recruitment period to 19 months

§ 5.2 Screening and baseline data collection: Screening visit

- Removed albumin, total protein from metabolic panel and added to hepatic panel
- Added "Three separate 24 hour food recalls will be obtained during screening."

§ 5.4 Follow-up visits

- Removed albumin, total protein from metabolic panel and added to hepatic panel at Week 24, Week 52 and Week 72 visits.
- Added albumin, total protein to hepatic panel at Week 4, Week 12, and Week 36 visits
- Clarified that three separate NDSR 24 hour food recalls will be obtained at Week 52 visit

§ 5.5 Questionnaires

- Clarified that three separate NDSR 24 hour food recalls will be obtained during screening and at Week 52 visit

§ 6.2 Safety issues in children with NAFLD taking delayed release cysteamine bitartrate (RP103) capsules

- Added: RP103 was approved by the FDA in April 2013 for the treatment of nephropathic cystinosis (Raptor Therapeutics Inc., PROCYSBI (cysteamine bitartrate delayed release capsules, Investigator's Brochure, April 2013).

§ 7.2 Outcome measures

- Primary outcome measure revised to: The primary outcome measure, improvement in NAFLD from the start of treatment to the end of treatment at 52 weeks, is defined for each patient as:
  - Improvement in NAS score by 2 or more points, and
  - No worsening of the fibrosis score
- All secondary outcome measures were expanded to express changes from baseline to end of treatment in the cysteamine group compared to the placebo group

§ 7.3 Statistical analysis

- Primary hypothesis changed to Primary outcome measure
- Secondary outcomes statistical analyses were added to detail the planned statistical approach for each secondary outcome measure to determine changes from baseline to end of treatment in the cysteamine group compared to the placebo group

§ 10.1 Participating centers

- Changed investigator at Texas Children's Hospital, Houston, TX
- Changed NIDDK Data Repository contractor

§ 10.2 Data Collection Schedule

- Removed total protein from metabolic panel and added to hepatic panel
- Corrected spelling of "transferease" to transferase

§ 10.3 Blood Collection Schedule

- Removed total protein from metabolic panel and added to hepatic panel
- Corrected spelling of "transferease" to transferase