NASH CRN

Nonalcoholic Steatohepatitis
Clinical Research Network

Treatment of Nonalcoholic Fatty Liver Disease in Children (TONIC)

Protocol

CONFIDENTIAL

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TONIC Trial Protocol

Contents

TONIC Trial Protocol

Design synopsis

Title
- Treatment of Nonalcoholic Fatty Liver Disease in Children (TONIC)

Sponsor
- NIDDK and NICHD

Objective
- To determine whether 96 weeks of treatment with either metformin or vitamin E leads to sustained reduction in serum ALT in nondiabetic children with NAFLD compared to treatment with placebo

Type of study
- Phase III randomized clinical trial
- Multicenter, masked, placebo-controlled

Treatment groups
- Group 1: Metformin, 500 mg PO b.i.d. and vitamin E-placebo b.i.d.
- Group 2: Vitamin E, 400 IU natural form PO b.i.d. and metformin-placebo b.i.d.
- Group 3: Metformin-placebo b.i.d. and vitamin E-placebo b.i.d.

Population
- Children with NAFLD aged 8-17 years at first screening visit

Study duration – per patient
- Up to 16 weeks (112 days) of screening prior to randomization, including at least 3 months of drug washout for those using antiNAFLD or antidiabetic medications prior to randomization
- 96-week treatment
- 24-week post-treatment follow-up

Study duration – per calendar time
- Recruitment phase: August 2005 - September 30, 2007
- Follow-up phase: August 2005 - October 2009

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Design synopsis

Sample size justification

- Total of 180 patients in 3 groups of equal size (60 per group)
- Primary comparisons
  - Metformin vs placebo
  - Vitamin E vs placebo
- Error protection
  - Type I = 0.025 (0.05, Bonferroni protected)
  - Type II = 0.10 (90% power)
- Effect size for sustained reduction in ALT after 96 weeks of treatment
  - Expected percent with sustained reduction in the placebo group: 20%
  - Expected percent with sustained reduction in the metformin or vitamin E groups: 50%
- Statistical test and sample size software
  - Chi-squared test for two proportions
  - Dupont and Plummer PS software
- Source of data for response rates
  - Open-label pilot studies of metformin and vitamin E at the UCSD NASH CRN clinic
  - Consensus of NASH CRN clinicians regarding expected response in the placebo group

Number of clinical centers

- 8

Inclusion criteria

- Age 8 through 17 at first screening visit
- Histologic evidence of NAFLD – biopsy cannot be older than 6 months (183 days) as of randomization
- ALT level > 60 U/L on two separate occasions at least 30 days apart but no more than 12 months (365 days) apart. One of these measures must be obtained at the TONIC clinical center during screening for TONIC. The other measure may be historic (i.e., obtained prior to initiation of screening in TONIC)
- Consent
- Randomized within 16 weeks (112 days) of starting screening

Exclusion criteria

- History of significant alcohol intake (AUDIT questionnaire) or inability to quantify alcohol consumption
- Diabetes mellitus
  - Fasting serum glucose of 126 mg/dL or greater

  OR
  - 2-hour serum glucose of 200 mg/dL or greater (from oral glucose tolerance test, OGTT)

OR
- History of diabetes mellitus

- ALT > 400 U/L on measurement closest in time to randomization
- Clinical or histologic evidence of cirrhosis
- Evidence of other chronic liver disease
  - Alpha-1 antitrypsin deficiency
  - Bile duct anomalies
  - Hemochromatosis
  - Hepatitis - autoimmune or viral
  - Wilson disease
- Serum creatinine of 1.5 mg/dL or greater for males and 1.4 mg/dL or greater for females
- Use of drugs historically associated with NAFLD (systemic glucocorticoids, tetracyclines, anabolic steroids, valproic acid, salicylates, tamoxifen, other known hepatotoxins) for more than 2 weeks in the 2 years prior to screening
- Use of antidiabetic drugs (insulin, biguanides, glucosidase inhibitors, sulfonylureas, meglitinides, metformin, thiazolidinediones) in the 3 months prior to randomization
- Use of antiNAFLD drugs (metformin, vitamin E, thiazolidinediones, UDCA, SAM-e, betaine, milk thistle, probiotics) in the 3 months prior to randomization
- Use of any over-the-counter or herbal remedy for hyperlipidemia in the 3 months prior to randomization
- History of metabolic acidosis
- History of renal dysfunction
- History of coagulopathy
- History of bariatric or hepatobiliary surgery
- History of total parenteral nutrition during the past 3 years prior to screening
- Inability to swallow study medication
- Vitamin E supplementation of greater than 100 I/U per day
- Disease considered by study physician to be significant
- Females of childbearing potential: positive pregnancy test during screening or at randomization or unwillingness to use an effective form of birth control during the trial
- Females of childbearing potential: breast feeding
- Any other condition, which in the opinion of the investigator would impede compliance or hinder completion of study

Outcome measures

- Primary: Sustained reduction in ALT to either 50% of baseline value or ≤ 40 U/L
- Secondary:
  - Sustained reduction in serum AST
  - Sustained reduction in serum GGT

- Change in histologic feature scores determined by standardized scoring of liver biopsies at baseline and after 96 weeks of treatment
- Change in liver fibrosis, inflammation, or steatosis
- Change in body mass index
- Change in insulin resistance indices
- Change in serum vitamin E levels
- Change in serum cytokine and fibrosis marker levels
- Change in serum lipid profile
- Change in QOL scores

Randomization

- Centrally administered randomization stratified by clinical center and blocked by calendar time

Visit schedule

- Baseline/screening: at least 1 visit separated by at least 1 calendar day from randomization; screening period can last no more than 16 weeks (112 days)
- Randomization (final pre-treatment interview, dispensing of study drugs)
- Follow-up visits
  - 4 weeks after randomization
  - 12 weeks after randomization and every 12 weeks thereafter up to 96 weeks
  - 120 weeks after randomization (24 weeks after treatment ends)
- Liver biopsy at 96 week visit

Statistical analysis

- All analyses will be on an "intention-to-treat" basis

Safety monitoring

- NIDDK appointed Data and Safety Monitoring Board will monitor the data for safety and efficacy for outcomes such as metabolic acidosis, hepatotoxicity, hypoglycemia, pregnancy, new onset diabetes, and any other outcomes or events identified as safety-related

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1. Objectives

Insulin resistance and oxidative stress (resulting in lipid peroxidation) are considered to be the two most important mechanisms in the pathogenesis of NAFLD. We hypothesize that metformin and vitamin E will lead to sustained reduction in serum ALT with biopsy proven NAFLD through changes in insulin resistance or oxidative stress.

The principal objective of this randomized, multicenter, double-masked, placebo-controlled trial is to evaluate whether 96 weeks of treatment with either metformin or vitamin E leads to sustained reduction in serum ALT in nondiabetic children with NAFLD compared to treatment with placebo.

Other objectives include:

- To evaluate changes in serum AST and GGT
- To measure change in histologic feature scores of NAFLD
- To evaluate change in liver fibrosis, inflammation, or steatosis
- To evaluate change in insulin resistance indices
- To evaluate change in serum vitamin E levels
- To evaluate change in serum cytokine and fibrosis marker levels
- To evaluate change in serum lipid profile
- To evaluate changes in anthropometric measurements (weight, BMI, waist to hip ratio, waist circumference, triceps skin fold thickness, total body fat, and distribution of fat)
- To measure change in quality of life scores
- To assess the role of diet and activity in the development and treatment of NAFLD
- To elucidate the natural history regarding clinical course and histology in untreated pediatric NAFLD (placebo plus diet and exercise advice)
- To obtain serum, liver, and DNA specimens from biopsy-confirmed pediatric patients with NAFLD for subsequent studies on etiopathogenesis

2. Background and significance

2.1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a common and increasingly recognized disorder characterized by macrovesicular accumulation of fat in hepatocytes, usually found in association with obesity, insulin resistance, and hyperlipidemia. This condition occurs in both adults and children, and arguably represents the most common cause of liver disease in pre-adolescence and adolescence. Reports from North America, Europe, Australia, and Asia highlight the global magnitude of this problem, which appears to be increasing in proportion to prevalence of pediatric obesity. Liver histology in NAFLD may demonstrate steatosis alone, to more advanced and concerning findings of fat in concert with inflammation and fibrosis (NASH). Cirrhosis in the context of pediatric NASH is reported. Although noninvasive imaging is being developed to accurately quantitate hepatic fat content, no other noninvasive measures are identified to estimate degree of liver inflammation or fibrosis. Due to the fact that distinction between simple steatosis from NASH or NASH-related cirrhosis requires liver biopsy, relatively little is known in children about prevalence or natural history. With a paucity of longitudinal data, we do not know whether simple steatosis progresses to NASH, and which factors influence the rate of NASH progression to cirrhosis. Given that the location and extent of inflammation and fibrosis often differ between adult and pediatric NASH, extension of adult data to children is unwarranted.

Preliminary studies are published on promising therapies for NASH. With rare exception, they are open-label, uncontrolled, non-randomized pilot trials. Most studies lack baseline or end-of-treatment biopsies, and it is unknown whether long term treatment affects outcome. To attain adequate statistical power, multicenter studies are necessary.

Realizing the need for further investigation into the natural history, pathogenesis, and treatment of adult and pediatric NASH, the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) issued a request for applications (RFA) in February, 2001 for a multi-center network (NASH CRN). Eight Clinical Centers and a Data Coordinating Center were funded by the NIDDK, with additional support from the National Institute of Child Health and Development (NICHD) allowing expansion of the pediatric component. The NASH CRN began in the summer of 2002, and many of the protocols are being finalized and undergoing institutional review. A major effort of the network is to establish a longitudinal study of suspected or proven pediatric NAFLD cases to classify, evaluate, and follow in a standard fashion. Many of the pediatric objectives of the NASH CRN are contained within this protocol.

2.2. Definition of NAFLD and NASH

NAFLD demonstrates a range of severity from the most benign (simple steatosis) to NASH resulting in cirrhosis. Although the condition was initially recognized histologically as a complication of weight loss surgery involving jejunal bypass, Ludwig later recognized the condition in obese, nonalcoholic, middle-aged adults, and coined the term “nonalcoholic steatohepatitis”[1].

Moran et al. first described the condition in children[2]. The three reported children, two boys and one girl, were obese and without any other identifiable cause of chronic liver disease. The biopsies from these children were similar to adults with NASH, and the children demonstrated biochemical improvement of their serum aminotransferases with weight loss[2]. Subsequent reports of children with biopsy-proven NASH have appeared from Japan[3], United States[4,5], Canada[6], Australia[7], and Italy[8]. Reports now document the presence of or progression to cirrhosis in children with NASH[6,9].

Steatohepatitis, the histologic entity of fatty liver with inflammation and potential fibrosis, can result from a variety of metabolic, infectious, nutritional, or toxic insults. Many of these etiologies are listed in the differential diagnosis section. When steatohepatitis fits certain histologic criteria, in the context of insulin resistance or metabolic syndrome, the entity is termed NASH. Criteria for staging and grading NASH in adults have been developed[10]. Recently, a large analysis of NASH histology in children was performed detailing the histologic features of pediatric NASH using criteria developed for adults[11]. Adult NASH histology differs from pediatric NASH histology, particularly with regard to extent and location of hepatic inflammation and fibrosis. We define pediatric NASH to be a biopsy-proven diagnosis of predominantly macrovesicular steatosis with evidence of either lobular or portal inflammation, evidence of cellular injury, with or without portal or pericellular fibrosis. Lipogranulomas are considered sufficient evidence of cellular injury, as the adult feature of hepatocellular ballooning or Mallory hyaline are infrequent in children.

2.3. Epidemiology

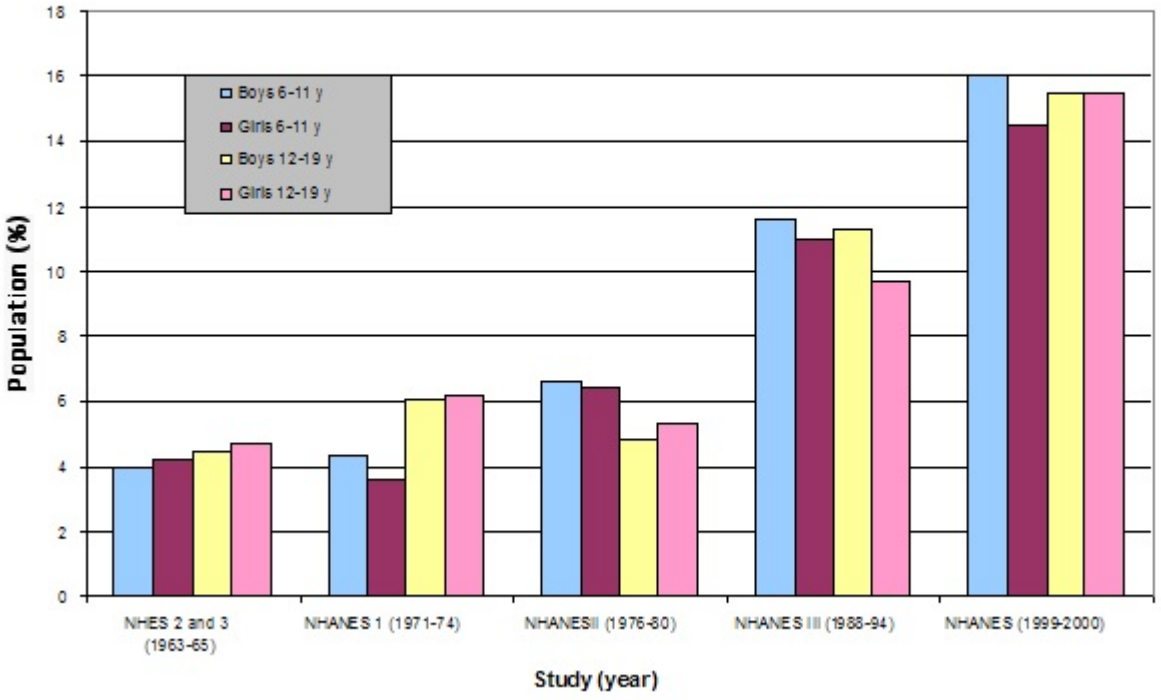
The prevalence of NASH in the pediatric population is not known. Determination of prevalence is derailed by the requirement for examination of liver histology to make a diagnosis. Estimates of prevalence can be inferred from data on the prevalence of childhood obesity, the frequency of "bright liver" on ultrasound in obese children, the frequency of abnormal ALT tests in obese children with echogenic liver, and the frequency of NASH versus simple steatosis in obese children with echogenic livers and elevated ALT who undergo biopsy.

The prevalence of child and adolescent obesity has risen dramatically over the past 20 - 30 years. Data from the National Health and Nutrition Examination Survey from 1999 - 2000 show that 14 - 16% of boys and girls between 6 and 19 years of age are obese, with obesity defined as being greater than the 95th percentile for body mass index (BMI) adjusted for age[12]. This is a dramatic increase from the approximate 5% prevalence reference population found in the National Health Examination Surveys 2 and 3 in 1963 - 1965. Evident in Figure 1, the prevalence has increased with every survey since the 1960s in the United States, with no promise of plateau. The increased prevalence of obesity is blamed on a multitude of changes in American lifestyle, such as increased sedentary activities and increased caloric intake of high-fat foods and soda with refined sugars.

Given that more than 85% of children with NAFLD are obese, the next question is how many of them have imaging studies consistent with fatty infiltration by ultrasound or MRI? Franzese et al.[13] performed ultrasonographic examinations on 72 consecutive otherwise healthy obese children with a mean age of 9.5 years. Fifty-three percent of these children exhibited a bright liver consistent with steatosis. If the prevalence of obesity in Italy were the same as in the United States, one would calculate that 8% of the pediatric population were obese with echogenic liver. In Japan, an

epidemiological ultrasonographic survey was performed on 810 school children age 4 - 12 years. No children were found with echogenic liver before age 4 years, but the overall incidence of presumed fatty liver ranged from 1.8% in girls to 3.4% in boys, 2.6% overall. Likelihood of fatty liver was best predicted by measurement of subcutaneous fat thickness[14]. Since ultrasound imaging is insensitive

Figure 1. Prevalence of Paediatric Obesity in the USA (1963-2000)
(>95th %ile BMI for age)



for demonstration of hepatic fat, these two studies hint that a minimum of 2.6 - 8% of children have NAFLD. Using the more sensitive technique of hepatic MRI for fat quantitation, Fishbein et al.[15] found that 21 of 22 obese children aged 6 - 18 years with modest hepatomegaly demonstrated elevated fat fractions. Data from this study, in conjunction with current NHANES data, suggest that as many as 16% of American children have NAFLD.

A number of investigators performed studies of fatty liver prevalence using serum ALT as a screening tool[3,8,16]. Whether ALT is a sensitive enough measure to evaluate NASH or NAFLD is not known, as recent evidence in adults provides ample evidence that "normal ALT NASH" occurs[17]. Further complicating interpretation is the realization that elevated ALT may not be due to fatty liver in some cases. Realizing that ALT as a surrogate marker in obese children likely underestimates the prevalence of NASH, it appears that 10 - 25% of obese children have abnormal ALT in these studies. Using American data for obesity prevalence, this would indicate that 1.6 - 4% of children have NAFLD.

Publications describing pediatric NASH series over the past 20 years demonstrate remarkable concordance for gender and age (Table 1). In all series, boys are reported twice as often as girls. The mean age at diagnosis in all series ranges between 11.6 and 13.5 years. Nothing is known as to why boys may be predisposed to NASH or why NASH appears at this age. Puberty is associated with dynamic changes in body composition and hormone levels. Children experience a stage of physiological insulin resistance beginning at the onset of puberty. While prepubertal children and postpubertal young adults are equally sensitive to insulin, adolescents are insulin resistant compared with either of these groups. An intriguing question about pathogenesis involves the potential role of pubertal development and sex hormones which may promote (in boys) or protect against (in girls) liver injury in susceptible individuals. Insulin resistance is reported to change at various stages of pubertal development, independent of changes in body composition with pubertal stage[18,19]. Recently, we are noting increasing numbers of children as young as 8 years presenting with NASH in our clinics. These children are still prepubertal Tanner stage I. This observation may indicate that earlier and more severe obesity may abrogate the need for puberty-related "promoters." Alternatively, the remarkable concordance among series in age and gender may reflect uniform selection bias.

Table 1. Demographic comparisons between studies of pediatric NASH

Study (year)	Location	# Boys/ # Girls	Age (mean)	Ethnicity
Moran et al (1983)	USA	2 / 1	12.6 y	White Non Hispanic (all)
Kinugasa et al (1984)	Japan	6 / 2	11.8 y	Asian (all)
Baldridge et al (1995)	USA	10 / 4	13.5 y	NS
Rashid et al (2000)	Canada	21 / 15	12 y	NS
Manton et al (2000)	Australia	8 / 4	11.6 y	NS
Schwimmer et al (2003)	USA	30 / 13	12.4 y	White Non Hispanic 25% White Hispanic 53% Black Non Hispanic 5% Other 17%

The series in Table 1 reflect populations of children in Asia, Australia, North America, and Europe. Races/ethnicities most often reported are Asian, White Hispanics, and White Non-Hispanic. Whether some races or ethnicities are more prone to develop NASH given a particular body mass index is unknown. Body fat distribution varies by race. In San Diego, NASH is diagnosed 3 times more commonly in Mexican-American children than in other children, despite the fact that only 24% of the children in San Diego are Hispanic. Studies have demonstrated that when adjusted for body size, Hispanic male children have significantly higher body fat and percentage fat than white or black males[20]. Obese Hispanic peripubertal children are reported to have increased risk for development of type 2 diabetes, indicative of severe insulin resistance[21]. The increased fat in Hispanic males for a

given BMI along with the increased insulin resistance in this population coincident with puberty may explain why a proportionately larger numbers of Hispanic males are observed in the San Diego NASH population.

A summary of pediatric NASH associations is presented in Table 2.

Table 2. Pediatric NASH associations

1)	Obesity
2)	Male gender
3)	Age > 9 years
4)	Insulin resistance
5)	Hyperlipidemia
6)	Race/ethnicity

2.4. Clinical presentation

Most children with NAFLD are asymptomatic and identified incidentally. Many pediatricians and family practice physicians are unfamiliar with NASH in children. How children present is subject to selection bias reporting by centers. Asymptomatic children are usually identified because of persistently elevated serum aminotransferases, or an echogenic liver detected on ultrasound of the abdomen. In the general pediatric gastroenterology clinic in San Diego, obese children greater than 6 years are screened for NASH, irrespective of the reason for referral. Clearly, most children found with NASH with this approach will differ from those identified elsewhere.

Children presenting with symptoms generally complain of either diffuse or right upper quadrant abdominal pain in 42 to 67% of reported series (Table 3). Those with right upper quadrant pain often have tenderness of the liver margin exacerbated by inspiratory effort. Occasionally, those complaining of right upper quadrant pain may be found to have gallstones, particularly frequent in obese Hispanic girls with associated hypercholesterolemia.

On physical examination, the most common findings are obesity, hepatomegaly, and acanthosis nigricans. Comparing published studies of biopsy-confirmed NASH, between 83 - 100% of pediatric patients are obese, 29 - 51% demonstrate hepatomegaly, and 36 - 49% exhibit acanthosis nigricans. Most patients are greater than 120% ideal body weight or have a BMI greater than 30 kg/m^2. Hepatomegaly may be difficult to appreciate by palpation or percussion due to overlying fat. On occasion, particularly in those complaining of right upper quadrant pain, the liver edge may be tender to palpation and exacerbated by palpation during inspiration. Acanthosis nigricans is a prominent discoloration usually presenting on the posterior neck folds, extending variable degrees anteriorly with increasing severity of insulin resistance. Hypertension may also be present, and comparison

must be made for age-appropriate norms. Rarely, normal weight patients present with pediatric NASH. These patients have insulin-resistance, often type 2 diabetes. These patients should be carefully examined for congenital or acquired lipodystrophies. Patients with NAFLD generally do not have ascites, caput medusae, or jaundice. Those rare patients with cirrhosis may demonstrate physical findings such as ascites, splenomegaly, and palmar erythema.

Table 3. Comparison of clinical findings in pediatric NASH

Study (year)	Obesity (%)	Obesity BMI or % IBW	Acanthosis Nigricans (%)	IDDM (%)	Hepatomegaly (%)	Presenting Symptoms (%)
Moran et al (1983)	100	30.1 kg/m^2	NS	0	33	Abdominal pain (67%)
Kinugasa et al (1984)	100	144% IBW	NS	13	NS	Obesity clinic (all)
Baldridge et al (1995)	100	150% IBW	NS	0	29	Abdominal pain (64%)
Rashid et al (2000)	83	147% IBW	36	11	44	Abdominal pain "most patients"
Manton et al (2000)	94	147% IBW	NS	0	47	Abdominal pain (59%)
Schwimmer et al (2003)	88	31.3 kg/m^2	49	14	51	Abdominal pain (42%)

2.5. Laboratory evaluation

In all series of biopsy-proven pediatric NAFLD, patients uniformly demonstrate elevated serum animotransferases. Generally, children with NAFLD have serum ALT anywhere from the upper limit of normal to 10 times the upper limit of normal. Children with normal ALT may also have NASH, but due to lack of referral of children with normal enzymes (detection bias), and reluctance of pediatric hepatologists to biopsy children with normal enzymes, we know little about "normal-ALT" NASH. This entity is recently described in adults[17]. The UCSD center has a biopsy-proven example of normal-ALT NASH, obtained in the context of performing a CT-guided liver biopsy for a focal unrelated lesion. Also, in many centers, it appears that the upper limit of normal for the normal range of serum aminotransferases has been creeping up over years. Certain centers periodically sample a "normal healthy population" which includes overweight or obese individuals who skew the upper end of "normal." Other centers use historical norms and report lower normal ranges. Thus,

many children with higher ALT may be erroneously reported as having normal ALT. In pediatric series of biopsy-proven NASH, mean serum ALT values range from 103 - 208 U/L, and mean AST values range from 63 - 104 U/L (Table 4). As in adults, the ALT/AST ratio is greater than one, with remarkable concordance between pediatric series reporting the ratio ranging from 1.5 - 1.7. This contrasts with a ratio generally less than one in alcoholic steatohepatitis. In series reporting serum GGT or alkaline phosphatase, the values are mildly abnormal. Other significantly elevated serum tests include fasting cholesterol and triglycerides. Interpretation of these results requires comparison to age- and gender-specific norms. Total and direct bilirubin should be normal.

Table 4. Comparison of laboratory findings in pediatric NASH

Study (year)	ALT (U/L)	AST (U/L)	ALT/AST ratio	GGT (U/L)	Alkaline Phosphatase (U/L)	Cholesterol (mg/dl)	Triglycerides (mg/dl)
Moran et al (1983)	156	94	1.7	NR	304	179	198
Kinugasa et al (1984)	107	73	1.5	NR	al l normal	204	174
Baldridge et al (1995)	129	77	1.7	NR	201	189	218
Rashid et al (2000)	179	104	1.7	NR	NR	171	195
Manton et al (2000)	[208]	[81]	--	[90]	NR	NR	221
Schwimmer et al (2003)	103	63	1.6	66	260	190	163

2.6. Pathogenesis

2.6.1. Insulin resistance and fat accumulation

There is strong evidence of an association between NAFLD and conditions known to be associated with insulin resistance in adults[22]. These conditions include type 2 diabetes, obesity and hyperlipidemia. Studies have demonstrated insulin resistance in adult patients with NASH[23]. A recent retrospective study in children (N=43) was performed to determine clinicopathological predictors of pediatric NASH. Criteria for insulin resistance were met by 95% of the subjects. Fasting insulin levels were also strongly predictive on univariate regression analysis for portal inflammation and perisinusoidal fibrosis[5]. Thus, in both adult and pediatric NASH, it appears that insulin resistance and accumulation of fat in the liver is a requisite first insult. The mechanism by which insulin resistance leads to steatosis is usually attributed to insulin's action in increasing peripheral lipolysis, delivery of free fatty acid to the liver, inhibition of free fatty acid release from the liver, and induction of hepatic gluconeogenesis[22]. Apparently, secondary mechanisms are required for provoking inflammation and fibrosis in susceptible fatty livers, since many individuals exhibit insulin resistance with simple steatosis only. In this "two-hit" hypothesis[24], the second hit results from oxidative stress and generation of increased reactive oxygen species (ROS). Hypothetically, increased ROS can result from particular genetic predispositions (such as polymorphisms in pro-inflammatory cytokine genes or cytochrome detoxification genes) or

environmental induction (such as diet, medications, and bacterial flora in the colon). Nothing is known about secondary mechanisms in children contributing to pediatric NASH.

2.6.2. Oxidative stress and injury

Lipid peroxidation has been demonstrated both in animal and human models of NASH, and the proinflammatory, profibrogenic properties of its aldehype end-products such as malondealdehyde (MDA) and 4-hydroxynonelol (4-HNE) potentially can account for all of the typical histological features observed in this disorder[25]. The correlation between the degree of lipid peroxidation and magnitude of hepatic steatosis also provides an explanation for the association between severity of steatosis and the risk of NASH[26].

2.7. Imaging studies

Imaging has a limited role in the diagnosis of NAFLD due to the variation in the sensitivity of the techniques, the inability of all modalities to discriminate simple steatosis from NASH, and the lack of general availability. The most commonly used is ultrasonography. Livers infiltrated with fat are hyperechogenic, or "bright." Detection of bright liver with milder degrees of fatty infiltration becomes relatively subjective, with modest sensitivity. The brightness of the liver echo is compared to either the kidney, spleen, intrahepatic portal veins, or fall in echo intensity with increasing depth from the transducer[27]. For the detection of fat, a more sensitive technique is computerized tomographic (CT) scanning. Estimates of degree of fatty infiltration is reported in Hounsfield units. Neither CT nor ultrasonography can distinguish between NASH and simple steatosis.

The most sensitive technique for detecting and quantitating hepatic fat is fast magnetic resonance imaging (MRI) or magnetic resonance spectroscopy. The fat fraction is derived from signal differences of in-phase and out-phase signals between fat and water[28]. Using this technique, Fishbein et al., recently demonstrated a correlation between quantity of hepatic fat and serum ALT in obese children with hepatomegaly[15].

2.8. Histology

Steatohepatitis is a morphological pattern of liver injury which results from a wide number of etiological insults. The histopathological features of steatohepatitis can result from alcoholism, drug-toxicity, type 2 diabetes, and a variety of inborn metabolic errors. NASH is a diagnosis requiring liver tissue examination as well as exclusion of other causes of steatohepatitis. Adult NASH is generally considered to include macrovesicular steatosis, mixed acute and chronic lobular inflammation with evidence of cellular injury, and zone 3 perisinusoidal fibrosis. Recently, attempts have been made to establish a grading and staging system for adult NASH. The purpose of grading and staging is to standardize diagnosis, establish criteria associated with presumed progression, and arrive at a "score" which can be useful in the design of treatment trials or natural history studies. Brunt et al. established a grade for necroinflammatory activity and a stage for extent of fibrosis with or without architectural remodeling. The necroinflammatory grade is derived from a combination of features of hepatocellular steatosis, cell ballooning, and inflammation. The staging of fibrosis reflects the pattern as well as the extent of fibrosis[10].

Pediatric NASH demonstrates striking differences and some similarities to the adult NASH findings (Table 5). By definition, pediatric NASH includes hepatocellular steatosis and inflammation with evidence of cellular injury[3,4,6,7]. These reports highlight the usually moderate to severe steatosis, mild mixed portal tract inflammation and megamitochondria, increased glycogen, occasional lipogranulomas, and mild lipofuscinosis. Presence of fibrosis in the portal and pericellular space is also found. However, none attempted to grade or stage the findings. Recently, UCSD sought to grade and stage their patients with pediatric NASH. Forty-three patients <18 years were identified with NAFLD from a computerized database at Children's Hospital, San Diego from 1999 - 2002. Two independent board-certified pathologists reviewed slides of tissue stained with H&E, trichrome, PAS, and Oil Red O. Slides were assessed for percentage of hepatocytes with fat, presence or absence of hepatocellular ballooning, mixed acute and chronic lobular inflammation, Mallory hyaline, lipid granulomas, megamitochondria, lipofuschin, and perisinusoidal fibrosis. Steatosis was moderate to severe in 96% of the cases. In contrast to adult data, signs of liver injury such as ballooning, lobular inflammation, and Mallory hyaline were found less than 5% of the time. Glycogen nuclei and lipogranulomas were found in the majority. In contrast to adults, portal inflammation was common but lobular inflammation infrequent. Also, in contrast, mild portal inflammation was common but perisinusoidal fibrosis was only found in 19%. Using the criteria of Brunt et al.[10], no biopsies were stage 3 or 4. Seventy percent of the biopsies with portal fibrosis lacked findings of pericellular or perisinusoidal fibrosis[11]. Thus, significant differences are appreciated between pediatric and adult NASH as presented in Table 5.

Table 5. Histological differences between pediatric and adult NASH

Quality	Pediatric NASH	Adult NASH
Steatosis	Marked	Less pronounced
Inflammation	Portal more common	Lobular more common
Ballooning	Rare	Frequent
Fibrosis	Portal more common	Lobular more common
Cirrhosis	Infrequent	More frequent

Albeit rare, cirrhosis occurs in children with NASH[3,5,6,9]. In our experience, cirrhosis with NASH is more common in children with precedent or other concurrent precipitants of liver injury, such as HCV. In adults, cryptogenic cirrhosis is thought to often result from "burned-out NASH"[29]. Cryptogenic cirrhosis occurs in adults generally suspectible to NASH, and is found in some individuals with precedent biopsies demonstrating NASH. Why the characteristic hallmark of steatosis disappears in those with cryptogenic cirrhosis is unknown. No cases of cryptogenic cirrhosis from pediatric NASH are described, although a case in an insulin-resistant obese girl was recently identified in San Diego.

2.9. Review of published treatment trials

2.9.1. Lifestyle modification

Rational treatment strategies require informed knowledge of pathogenesis. As proposed by Oliver and Day, NASH may require two hits. The first is fat accumulation within the liver, the second involves excessive production or concentration of free radicals with increased oxidative stress. Increased oxidative stress to the liver may be generated by environmental or genetic factors. Treatment strategies are mainly geared to diminish hepatic fat or reduce oxidative stress. Since NASH is a component of the metabolic syndrome, a rational therapy to treat NASH along with other co-morbidities of the metabolic syndrome is to encourage steady and sustainable weight loss. Weight loss may be achieved by either decreasing caloric intake relative to needs or increasing caloric expenditure. Thus, a few trials in children have examined the role of diet in conjunction with exercise to treat NAFLD (Table 6). In both open-label trials of weight loss, obese children with a "bright liver" on ultrasound were provided instruction on diet and exercise and encouraged to lose greater than 10% of their ideal body weight (IBW). Vajro et al.[30] found that in 7 of 9 patients who were able to lose this much weight that a decrease in the intensity of the liver echogenicity was found, and that serum ALT became normal. A subsequent weight loss trial in 28 children treated for 3 - 6 months demonstrated resolution (24 patients) or improvement (4 patients) in liver echogenicity with this degree of weight loss. Whether or not all of the subjects in these trials had NASH or NAFLD was not ascertained and follow-up liver biopsies were not performed. Many health care providers to adults and children alike find it difficult to motivate or maintain patients with lifestyle habits that promote sustained weight loss. While this strategy is most appealing, how to help patients succeed stymies providers of health care everywhere.

Table 6. Summary of pediatric treatment trials

Intervention	Reference	Sample size	Entry criteria	Duration (mo)	Outcome
Vitamin E	[31]	11	Obese U/S bright > ALT	4-10	Normal ALT same BMI
Metformin	[34]	10	Biopsy > ALT	6	Decreased ALT decreased hepatic fat on MRI decreased insulin resistance
UDCA	[30]	7	Obese > ALT	4	Unchanged ALT unchanged U/S
Weight loss 1	[8]	7	Obese > ALT	2-6	Normal ALT decreased U/S "bright liver"
Weight loss 2 (> 10% of IBW)	[13]	28	Obese U/S bright	3-6	Bright liver resolved (N=24) or improved (N=4) on U/S

2.9.2. Vitamin E

A second treatment strategy is to decrease oxidative stress by providing supplemental antioxidants. Obese children studied in the National Health and Nutrition Examination Survey (NHANES III) were found to have deficiency of serum alpha-tocopherol relative to normal weight controls. An open-label treatment trial of oral vitamin E in 11 obese children with elevated serum ALT and echogenic livers demonstrated normalized serum ALT in all patients[31]. In this pilot trial, treatment consisted of escalating doses of vitamin E between 400 - 1200 IU once a day. These patients did not have liver biopsies to confirm diagnosis or histologic response. How diminution of serum ALT corresponds with clinically relevant outcomes is uncertain, and future pediatric studies with vitamin E or other antioxidants should have baseline and follow-up liver biopsies after appropriate duration of therapy. A non-randomized treatment trial using vitamin E 300 mg/day for one year in Japanese adults with biopsy-proven NASH (N=12) demonstrated significant reduction in serum ALT and improvement in histological findings including steatosis, inflammation, and fibrosis[32]. A subsequent randomized masked trial of vitamin E 400 IU/day for NASH in adults was performed with biopsies at the start and end of the therapeutic trial. After six months, patients demonstrated normalization of serum ALT and improvement in the degree of hepatic steatosis[33]. Inflammatory cytokines will be analyzed in TONIC trial to investigate the oxidative stress benefits of vitamin E.

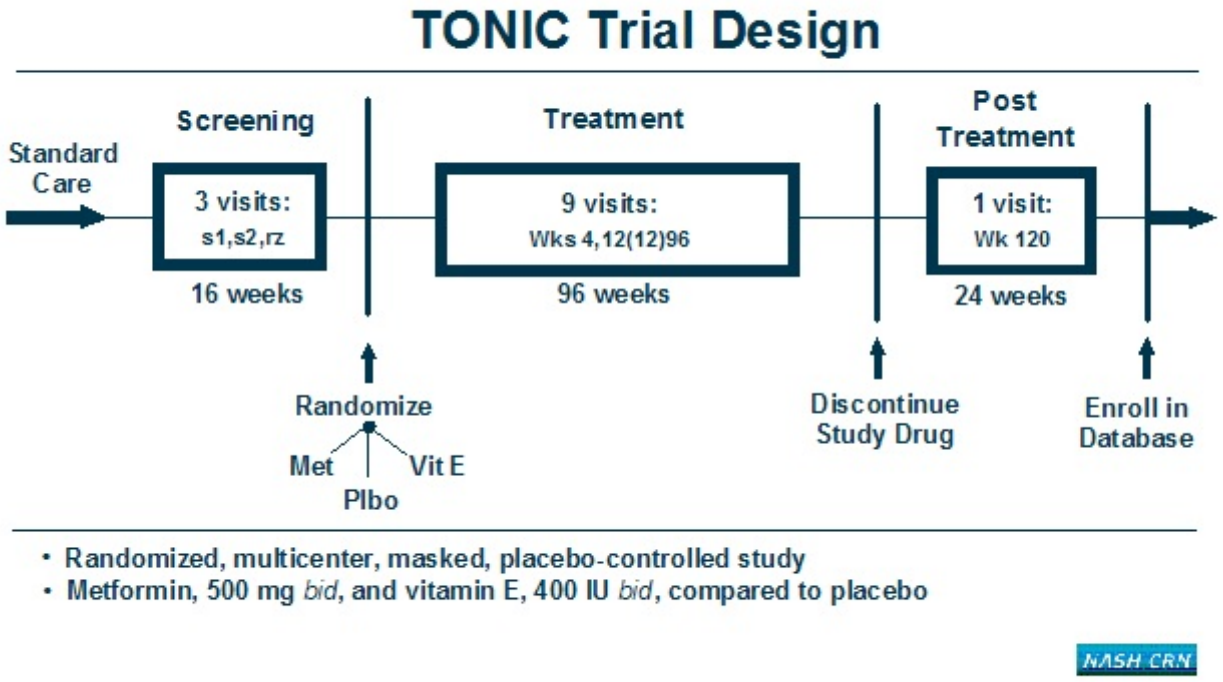
2.9.3. Metformin

Another target for treatment in NASH is reduction in insulin resistance[23]. Insulin resistance is present in over 95% of pediatric NAFLD cases, and the degree of resistance significantly predicts the presence of inflammation and fibrosis present in liver[5]. Adults with NASH demonstrated significant improvement in serum ALT after completing a 4-month trial of treatment with metformin, an insulin-sensitizing reagent[34]. Recently, an open-label pilot trial of metformin for biopsy-proven pediatric NASH was completed. Ten patients were treated for six months with metformin 500 mg orally twice a day. Significant improvement was found in serum ALT, hepatic steatosis (by MRI spectroscopy), and insulin sensitivity[35]. Median serum ALT decreased from 149 U to 51 U, median liver fat from 41% to 32%, and pediatric quality of life increased from a score of 69 to 81[35]. Thiazolidinediones, another class of insulin-sensitizing drugs, are being tested for safety and efficacy in adult NASH. However, severe cholestatic hepatitis has been reported in an adult NASH patient treated with troglitazone[36], and inadequate experience using other thiazolidinediones in children with or without preexisting liver disease warrants caution in considering its use in clinical trials in pediatric NASH.

3. Study design

3.1. Design overview

TONIC is a randomized, placebo-controlled, double-masked, multicenter trial of treatment with metformin, vitamin E, or placebo for patients with NAFLD. Screening for eligibility and collection of baseline data will span up to 16 weeks. Eligible patients will be randomized to receive either metformin (500 mg b.i.d.), or vitamin E (400 IU b.i.d.), or placebo for 96 weeks. 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. Patients will be asked to remain in the NAFLD Database with annual follow-up visits after the trial has ended. The primary comparisons will be made using an intention-to-treat analysis of the change in serum ALT at baseline and at week 96. Secondary outcome measures include change in serum AST and GGT, change in histologic feature scores, change in anthropometric measurements, change in insulin resistance, change in serum vitamin E levels, and change in pediatric quality of life scores. The study design can be schematically shown as below:



3.2. Treatment groups

Patients who have signed an informed consent statement and who meet the eligibility criteria will be randomly assigned to one of three groups for 96 weeks of treatment:

- Metformin (500 mg b.i.d.) and vitamin E-placebo (b.i.d)
- Vitamin E (400 IU, natural form, b.i.d.) and metformin-placebo (b.i.d.)
- Metformin-placebo (b.i.d.) and vitamin E-placebo (b.i.d.)

The randomization scheme will assign patients in randomly permuted blocks of assignments stratified by clinical center; block size will be determined randomly. This scheme will ensure that the three 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 data system shows that the patient is eligible, has signed the consent statement, and has had all required baseline data keyed to 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 investigator with masked study drug based on the DCC randomization schedule. Patients will be dispensed two bottles labeled ***"metformin or placebo"*** and two bottles labeled ***"vitamin E or placebo"***.

Each day in the morning and evening, one pill from the "metformin or placebo" bottle and one pill from the "vitamin E or placebo" bottle will be taken with food. One of the pills will be either 500 mg metformin or its placebo. The second pill will be 400 IU soft gel capsule of vitamin E or its placebo. The placebos for the metformin tablet and vitamin E capsule will be identical to the active study drugs. Possible treatment group combinations include the following:

	Metformin bottle		**Vitamin E bottle**		**Total** (AM + PM dose)
A.	Metformin tablet (500 mg)	+	Vitamin E placebo	=	1000 mg Metformin
B.	Metformin placebo	+	Vitamin E (400 IU)	=	800 IU Vitamin E
C.	Metformin placebo	+	Vitamin E placebo	=	0 (placebo only)

The dosage of metformin administered in this study is 500 mg twice daily. However, its administration will take place in two phases: a titration phase and a treatment phase. The use of metformin at the dose of 500 mg twice daily is associated with gastrointestinal side effects at the onset of treatment. These side effects are reduced if the medication is taken with food and the dose titrated from once daily to twice daily over two weeks.

Metformin titration phase: During the first 2 weeks following randomization, the metformin dose will be 500 mg once daily (q.d.) with the evening meal or bedtime snack. After the first 2 weeks, the metformin dose will then be raised to the full 500 mg twice daily (b.i.d.).

The dose of metformin may be reduced if intolerable gastrointestinal symptoms develop, based on the investigator's discretion, as follows:

- If the patient has intolerable symptoms while on 500 mg once daily (q.d.) dosing during the first 2 weeks after randomization, a 2 week hiatus followed by a 2 week re-attempt at 500 mg once daily (q.d.) dosing will occur. The rationale for the re-attempt is to rule out an alternative reason such as intercurrent illness.

- If the patient has intolerable symptoms while on 500 mg twice daily (b.i.d.) dosing after successful completion of 2-week titration phase, the patient dosing will be reduced to 500 mg once daily (q.d.) dosing.

Metformin treatment phase: At the end of 2-week titration phase, dose will be 500 mg twice daily, AM dose with morning meal and PM dose with the evening meal or bedtime snack.

Restarts and titration due to side effects: If participants develop side effects thought to be due to study drug and require cessation of study drug, the drug will be stopped for two weeks. If the side effects disappear, a second attempt will be made to reintroduce the study drug after two weeks. If the symptoms reappear, the study physician will discuss termination of study drug with the members of the Executive Committee.

In case of gastrointestinal side effects, investigators can initially attempt dose reduction instead of stopping therapy. Metformin dose can be cut back during the treatment phase to once daily dosage.

3.3.1. Metformin

Metformin will be administered as a single capsule of 500 mg per day orally with the evening meal or bedtime snack during the first 2 weeks after randomization. After titration, metformin will be administered 500 mg twice a day (b.i.d.) with the morning and evening meals. A similar appearing metformin-placebo capsule will be taken once daily during the 2-week titration phase by patients assigned to either the placebo group or the vitamin E group and twice a day after titration.

Marchesini et al.[34] conducted a 4 month study on 20 adults with NASH treated with metformin at 500 mg t.i.d. Metformin was found to be well tolerated in patients with normoglycemia. Three out of the 20 patients stopped treatment within a few days due to increased gastrointestinal symptoms. Drop-outs due to gastrointestinal distress were not seen once the metformin dose was increased gradually.

Schwimmer et al.[35] conducted a 1 year study on 10 children with NASH treated with metformin at 500 mg b.i.d. There was a 1-week titration period at 500 mg once daily. There were no drop-outs due to gastrointestinal distress. All children who reported symptoms did so during the first one to two weeks only and did not complain of further symptoms after this period.

3.3.2. Vitamin E

The formulation of vitamin E to be used in this study is the natural form of vitamin E (*RRR*-α-tocopherol, formerly known as d-α-tocopherol) at a dose of 400 IU administered orally via a soft gel capsule (b.i.d.) with the morning and evening meals (total dose of 800 IU/day). A similar appearing placebo soft gel capsule will be taken twice a day by patients assigned to either the placebo group or the metformin group with the morning and evening meals.

Double-masked trials and large population studies have shown that oral vitamin E at 800 IU daily dose is safe with no significant side effects[37,38,39]. The vitamin E dose chosen for this trial (400 IU b.i.d.) is within the range of vitamin E dosage that has been tested for the treatment of NASH in previous pilot studies[31,33,40,41]. For example, in a pilot study, Kugelmas et al.[41] tested the effect of step I American Heart Association diet plus exercise with or without 800 IU of vitamin E daily.

3.4. Rationale for placebo treatment design

Currently, there is no proven drug available for the treatment of NAFLD in children. In this study, we intend to test the hypothesis that therapeutic agents will lead to sustained reduction in serum ALT with biopsy proven NAFLD through changes in insulin resistance (metformin) or oxidative stress (vitamin E). The efficacy of each of these agents will be compared to placebo. As there is no proven drug for NAFLD in children, using a placebo for comparative purposes is justified. Patients will take metformin-placebo and vitamin E-placebo.

3.5. Standard treatment recommendations

In addition to the study drug, patients will receive a standardized set of recommendations about life-style modification (dietary modification, weight loss, exercise), use of prescription or non-prescription medicines or herbal remedies or dietary supplements, consumption of alcohol, and management of various co-morbid illnesses. These recommendations have been prepared by the NASH CRN Standards of Care Committee and are approved by the NASH CRN Steering Committee. This will help ensure that the patients in all groups receive standard of care treatment for NAFLD.

4. Patient selection

4.1. Recruitment

Approximately 180 patients will be recruited from the eight clinical centers of the NASH CRN (averaging 23 patients per center) over an 18-month period:

- Case Western Reserve University, Cleveland, OH
  PI: Arthur McCullough, MD
  Pediatric Co-PI: Margaret Stager, MD
- Duke University, Durham, NC/Johns Hopkins University, Baltimore, MD
  PI: Anna Mae Diehl, MD
  Pediatric Co-PI: Ann Scheimann, MD
- Indiana University, Indianapolis, IN
  PI: Naga Chalasani, MD
  Pediatric Co-PI: Jean Molleston, MD
- Saint Louis University, St Louis, MO
  PI: Brent Tetri, MD
  Pediatric Co-PI: Sarah Barlow, MD
- University of California, San Diego, CA
  PI: Joel Lavine, MD
  Pediatric Co-PI: Jeffrey Schwimmer, MD
- University of California, San Francisco, CA
  PI: Nathan Bass, MD
  Pediatric Co-PI: Philip Rosenthal, MD
- University of Washington, Seattle, WA
  PI: Kris Kowdley, MD
  Pediatric Co-PI: Karen Murray, MD
- Virginia Commonwealth University, Richmond, VA
  PI: Arun Sanyal, MD
  Pediatric Co-PI: Daphne Bryan, MD

Eligible patients will be identified and recruited at the participating clinical centers subject to the inclusion and exclusion criteria listed later in this chapter. Clinical centers will be required to recruit sufficient overall numbers of minorities and females so that results can be generalized to these populations.

Each clinical center will develop a recruitment plan. These plans will vary from clinic to clinic depending on the available pools of patients and local recruitment resources.

4.2. Inclusion criteria

- Age 8 through 17 years at first screening visit
- Histologic evidence of NAFLD based on a liver biopsy obtained in the 6 months (183 days) prior to randomization, a minimum of 5% of hepatocytes with macrovesicular fat, without other etiologies for the presence of fat being identified
- Serum alanine aminotransferase elevation (ALT > 60 U/L) on two separate occasions at least 30 days apart but no more than 12 months (365 days). One of these measures must be obtained at the TONIC clinical center during screening for TONIC. The other measure may be historic (i.e., obtained prior to initiation of screening in TONIC)
- Consent
- Randomized within 16 weeks (112 days) of starting screening

4.3. Exclusion criteria

Patients who satisfy any of the following exclusion criteria will be ineligible for enrollment in the trial:

- History of significant alcohol intake (AUDIT questionnaire) or inability to quantify alcohol consumption
- Diabetes mellitus
  - Fasting serum glucose of 126 mg/dL or greater
  OR
  - 2-hour serum glucose of 200 mg/dL or greater from oral glucose tolerance test (OGTT) with 2 g/kg (maximum 75 g) glucose load
  OR
  - History of diabetes mellitus
- ALT > 400 U/L on measurement closest in time to randomization
- Clinical or histologic evidence of cirrhosis
- Evidence of other chronic liver disease
  - Alpha-1 antitrypsin deficiency
  - Bile duct anomalies
  - Hemochromatosis
  - Hepatitis - autoimmune or viral
  - Wilson disease
- Serum creatinine of 1.5 mg/dL or greater for males and 1.4 mg/dL or greater for females
- Use of drugs historically associated with NAFLD (systemic glucocorticoids, tetracyclines, anabolic steroids, valproic acid, salicylates, tamoxifen, other known hepatotoxins) for more than 2 weeks in the 2 years prior to randomization
- Use of antidiabetic drugs (insulin, biguanides, sulfonylureas, metformin, thiazolidinediones) in the 3 months prior to randomization

- Use of antiNAFLD drugs (metformin, vitamin E, thiazolidinediones, UDCA, SAM-e, betaine, milk thistle, probiotics) in the 3 months prior to randomization
- Use of any over-the-counter or herbal remedy for hyperlipidemia in the 3 months prior to randomization
- History of metabolic acidosis
- History of renal dysfunction
- History of coagulopathy
- History of bariatric or hepatobiliary surgery
- History of total parenteral nutrition during the past 3 years prior to screening
- Inability to swallow study medication
- Vitamin E supplementation of greater than 100 I/U per day
- Disease considered by study physician to be significant
- Females of childbearing potential: positive pregnancy test during screening or at randomization or unwillingness to use an effective form of birth control during the trial
- Females of childbearing potential: breast feeding
- Any other condition, which in the opinion of the investigator would impede compliance or hinder completion of study

4.4. Run-in period

Patients must not have used any prescription or over-the-counter medication or herbal remedy taken with an intent to improve or treat NAFLD or liver disease or obesity or diabetes for the 3 months prior to randomization. AntiNAFLD agents include: metformin, vitamin E, thiazolidinediones, UDCA, SAM-e, betaine, milk thistle, and probiotics. Antidiabetic agents include but are not limited to: insulin, biguanides, sulfonylureas, metformin, and thiazolidinediones. These agents are not to be used during screening nor for the duration of the trial (except in the form of assigned study treatment or treatment for new onset diabetes) .

Any over-the-counter medication or herbal remedy that is being taken with an intent to improve hyperlipidemia will not be allowed for at least 3 months prior to randomization and will be discouraged after randomization. Patients will be allowed to continue on prescription anti-hyperlipidemic agents. Patients will be interviewed in a detailed fashion at screening, randomization, and at every clinic visit to document the absence of such use.

5. Trial protocol

5.1. Visit schedule overview

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

- Screening for eligibility for enrollment (2 visits over a maximum of 16 weeks)
- Randomization to treatment (1 visit)
- Treatment phase (9 visits over 96 weeks)
- Post-treatment follow-up phase (1 visit at 120 weeks)

The visit and data collection schedule described below in detail is summarized in Appendix 10.3.

5.2. Screening and baseline data collection

Patients who appear to be eligible after chart review and completion of standard of care tests and procedures including baseline liver biopsy for NAFLD will be invited to undergo screening. Recording of screening data on TONIC 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 plasma glucose, and routine liver tests. Prior therapy for NAFLD will be reviewed, and patients will be asked to stop any specific antiNAFLD treatment such as metformin, vitamin E, thiazolidinediones, UDCA, SAM-E, milk thistle, betaine, or probiotics. Patient charts will be reviewed for historical information and previous standard of care liver biopsy findings.

All participants who sign the consent statement will be registered in the TONIC 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. Reason for screening failure will be recorded in the TONIC database.

Screening and baseline data collection will be conducted over two clinic visits completed on separate calendar days. The goal of the first screening visit is to obtain consent and record data regarding TONIC inclusion and exclusion criteria; the goal of the second screening visit is to complete collection of baseline data on patients who appear eligible. The procedures completed during screening are:

- **Baseline standard of care liver biopsy:** Patients who have not had a liver biopsy within 6 months of randomization or whose previous liver biopsy is not available for review or whose previous liver biopsy is of inadequate quality must have a standard of care liver biopsy prior to randomization. Biopsy material must be of adequate size (1.5 cm or more) and slides of adequate quality for interpretation. The patient must have histologic evidence of NAFLD determined by the study pathologist locally (as described in section 5.7 of this protocol).

  In the case of a biopsy done previously as standard of care, the NASH CRN study physician should check if tissue blocks and or additional slides can be obtained from the original biopsy. If a standard of care liver biopsy is completed as part of screening for TONIC, the liver tissue will be prepared for light microscopy and stains including hematoxylin and eosin, Masson's trichrome and iron stain.

  Clinic staff should note that the date of the biopsy establishes a hard window for completion of screening for randomization – randomization must take place within 6 months (183 days) of the date of biopsy. Clinic staff will have to monitor completion of screening procedures and speed things up if the closing date on the 6 months biopsy window is getting close.

- **Screening visit 1**: Determination of eligibility will be based mostly on chart review of standard of care tests and procedures including baseline liver biopsy that were completed before screening visit 1. The patient will sign the consent at screening visit 1 to obtain any tests and procedures needed to finalize eligibility after chart review and will undergo a history and detailed physical examination including Tanner staging and acanthosis nigricans staging to identify other illness and contraindications for participation. Anthropomorphic assessments (body weight [kg], body height [m], waist circumference [cm], hip circumference [cm], triceps skin fold thickness [mm], mid-upper arm circumference [cm]; vital signs (systolic and diastolic blood pressure, heart rate, body temperature); and general physical findings, including hepatosplenomegaly, peripheral manifestations of liver disease, ascites, wasting or fetor, will be collected and recorded. Use of antiNAFLD and antidiabetic medications will be obtained and recorded. Laboratory test results that need to be recorded from chart review or obtained as part of screening visit 1 include: tests for hepatitis B (HBsAg, anti-HBc) and hepatitis C (anti-HCV), anti-nuclear antibody (ANA), anti-smooth muscle antibody (ASMA), anti-mitochondrial antibody (AMA), ceruloplasmin, alpha-1-antitrypsin (A1AT) concentration iron, total iron binding capacity, ferritin, fasting serum glucose, CBC, metabolic panel (sodium, potassium, chloride, bicarbonate, calcium, phosphate, BUN, creatinine, uric acid, albumin, total protein), fasting lipid profile (total cholesterol, triglyceride, LDL, HDL) and ALT. Frequency and amount of alcohol intake (AUDIT) will be obtained. Patients of childbearing potential must have a negative urine pregnancy test.

- **Screening visit 2**: Blood sample for fasting serum glucose, insulin, and C-peptide will be obtained after an overnight fast. After administration of glucose solution in a dose of 2 g per kilogram of body weight (up to a maximum of 75 g), blood sample for 2-hour serum glucose and insulin will be obtained for the oral glucose tolerance test (OGTT). Patients will provide blood for banking (measurements to be done on banked serum will include vitamin E level). Patients will complete a nutritional questionnaire (Block Brief), a health-related quality of life questionnaire (PedsQL), physical activity questionnaire, and liver symptoms questionnaire. Laboratory tests include: HbA1c, hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase), GGT, prothrombin time, vitamin B12, free fatty acid, leptin, C-reactive protein. Patients of childbearing potential must have a negative urine pregnancy test. Patients will undergo a DEXA scan to estimate total body fat. Patients will also undergo an MRI to estimate total body fat if available at the participating clinical center. All patients will be given information on a healthy life style and diet appropriate for their weight and other factors.

  DEXA - Dual energy X-ray absorptiometry: Total body composition and an estimate of fat/water ratio will be determined by DEXA using a Hologic scanner (Delphi W SIN 70517) or similar equipment, which is calibrated before scans according to manufacturer's recommendations. Patients will be scanned in light clothing lying flat on their backs and with arms by their side. Total body scan requires about 7 minutes and emits about 1.5 mrad. There is a weight limit of about 285 pounds.

  MRI - Magnetic Resonance Imaging/Spectroscopy: MR imaging is performed on a 1.5 Tesla Siemens Symphony whole body scanner or similar equipment. Axial gradient echo abdominal images are obtained at multiple values of TE, from which fat/water ratios can be calculated. Also, MR spectroscopy should be performed which provides a second independent estimate of the fat/water ratio.

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 TONIC. Thus staff can use this task to identify the items that still need to be completed, keyed, or verified after data from screening visit 1 are keyed and again after data from screening visit 2 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 drug randomly assigned to the patient. Randomization is the act of generating the random study drug 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

screening visits 1 and 2 have been keyed to the trial database. Since these processes take time, randomization cannot be done at screening visit 2, and since study medication needs to be issued to the patient, the randomization visit must be completed in person with the patient present. Therefore a visit separate from screening visit 2 is necessary. Since this will be a visit on a different calendar day and study drug 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 patients of childbearing potential, verification that the patient is feeling well, affirmation of consent, and generation of the random treatment assignment. The randomization process includes the same electronic check on eligibility that the staff may run prior to the randomization visit. The study drug assignment will not be generated unless the check finds that the patient is eligible, and the clinical center staff indicate 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 by the NASH CRN Drug Distribution Center. The research pharmacy 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 (ie, 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 and the data collected at the visit may be used in the trial.

5.4. Follow-up visits

Patients will return for follow-up visits at 4, 12, 24, 36, 48, 60, 72, 84, 96, and 120 weeks after randomization. Thus, starting 12 weeks after randomization, patients will be seen at 12 week (3 month) intervals through 96 weeks. 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 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**: Interim history including review of medications and adverse effects; height, weight, waist and hip measurements, vital signs (temperature, heart rate, blood pressure), focused physical examination; blood draw for metabolic panel (sodium, potassium, chloride, bicarbonate, calcium, phosphate, BUN, creatinine, uric acid, albumin, total protein) and hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase); urine pregnancy test (for patients of childbearing potential).

- **Week 12 visit**: Interim history including review of medications and adverse effects; height, weight, waist and hip measurements, vital signs (temperature, heart rate, blood pressure), focused physical examination; blood draw for metabolic panel (sodium, potassium, chloride, bicarbonate, calcium, phosphate, BUN, creatinine, uric acid, albumin, total protein) and hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase); urine pregnancy test (for patients of childbearing potential).

- **Week 24 visit**: Interim history including review of medications and adverse effects; height, weight, waist and hip measurements, vital signs (temperature, heart rate, blood pressure), focused physical examination; blood draw for CBC, fasting serum glucose, metabolic panel (sodium, potassium, chloride, bicarbonate, calcium, phosphate, BUN, creatinine, uric acid, albumin, total protein), hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase), vitamin B12, fasting lipid profile (total cholesterol, triglyceride, LDL, HDL); urine pregnancy test (for patients of childbearing potential); blood draw for banking at central repository.

- **Week 36 visit**: Interim history including review of medications and adverse effects; height, weight, waist and hip measurements, vital signs (temperature, heart rate, blood pressure), focused physical examination; blood draw for metabolic panel (sodium, potassium, chloride, bicarbonate, calcium, phosphate, BUN, creatinine, uric acid, albumin, total protein) and hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase); urine pregnancy test (for patients of childbearing potential).

- **Week 48 visit**: Blood sample for fasting serum glucose, insulin, and C-peptide will be obtained after an overnight fast. After administration of glucose solution in a dose of 2 g per kilogram of body weight (up to a maximum of 75 g), blood sample for 2-hour serum glucose and insulin will be obtained for the OGTT. Interim history including review of medications and adverse effects; height, weight, waist and hip measurement, triceps skin fold thickness, mid-upper arm circumference; vital signs (temperature, heart rate, blood pressure), detailed physical examination including Tanner staging and acanthosis nigricans staging; blood draw for CBC, metabolic panel (sodium, potassium, chloride, bicarbonate, calcium, phosphate, BUN, creatinine, uric acid, albumin, total protein), hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase), GGT, prothrombin time, fasting lipid profile (total cholesterol, triglyceride, LDL, HDL), HbA1c, vitamin B12, free fatty acid, leptin, C-reactive protein; urine pregnancy test (for patients of childbearing potential); alcohol, nutritional, physical activity, HR-QOL and liver symptom questionnaires; blood draw for banking at central repository.

- **Week 60 visit**: Interim history including review of medications and adverse effects; height, weight, waist and hip measurements, vital signs (temperature, heart rate, blood pressure), focused physical examination; blood draw for metabolic panel (sodium, potassium, chloride, bicarbonate, calcium, phosphate, BUN, creatinine, uric acid, albumin, total protein) and hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase); urine pregnancy test (for patients of childbearing potential).

- **Week 72 visit**: Interim history including review of medications and adverse effects; height, weight, waist and hip measurements, vital signs (temperature, heart rate, blood pressure), focused physical examination; blood draw for CBC, fasting serum glucose, metabolic panel (sodium, potassium, chloride, bicarbonate, calcium, phosphate, BUN, creatinine, uric acid, albumin, total protein), hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase), vitamin B12, fasting lipid profile (total cholesterol, triglyceride, LDL, HDL); urine pregnancy test (for patients of childbearing potential); blood draw for banking at central repository.

- **Week 84 visit**: Interim history including review of medications and adverse effects; height, weight, waist and hip measurements, vital signs (temperature, heart rate, blood pressure); focused physical examination; blood draw for metabolic panel (sodium, potassium, chloride, bicarbonate, calcium, phosphate, BUN, creatinine, uric acid, albumin, total protein) and hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase); urine pregnancy test (for patients of childbearing potential).

- **Week 96 (final treatment phase) visit**: Blood sample for fasting serum glucose, insulin, and C-peptide will be obtained after an overnight fast. After administration of glucose solution in a dose of 2 g per kilogram of body weight (up to a maximum of 75 g), blood sample for 2-hour serum glucose and insulin will be obtained for the OGTT. Interim history including review of medications and adverse effects; height, weight, waist and hip measurement, triceps skin fold thickness, mid-upper arm circumference; vital signs (temperature, heart rate, blood pressure), detailed physical examination including Tanner staging and acanthosis nigricans staging; blood draw for CBC, metabolic panel (sodium, potassium, chloride, bicarbonate, calcium, phosphate, BUN, creatinine, uric acid, albumin, total protein), hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase), GGT, prothrombin time, fasting lipid profile (total cholesterol, triglyceride, LDL, HDL), HbA1c, vitamin B12, free fatty acid, leptin, C-reactive protein; urine pregnancy test (for patients of childbearing potential); alcohol, nutritional, physical activity, HR-QOL and liver symptom questionnaires; DEXA scan for body fat composition; MRI for body fat composition (optional); liver biopsy; blood draw for banking at central repository; withdrawal of study medication.

- **Follow-up liver biopsy:** A follow-up liver biopsy will be obtained at the week 96 visit. Guidelines for obtaining the biopsy specimen are provided in the NASH CRN Liver Biopsy Procedure and NAFLD/NASH Histology Scoring System Manual; a 16 gauge needle is preferred and the specimen should be least 1.5 cm in length. The slides must be

of adequate size (1.5 cm or more) and adequate quality for interpretation. The liver tissue will be prepared for light microscopy and stains including hematoxylin and eosin, Masson's trichrome and iron stain.

- **Week 120 (24 weeks after withdrawal of study medication) visit**: Blood sample for fasting serum glucose, insulin, and C-peptide will be obtained after an overnight fast. After administration of glucose solution in a dose of 2 g per kilogram of body weight (up to a maximum of 75 g), blood sample for 2-hour serum glucose and insulin will be obtained for the OGTT. Interim medical history including review of medications and adverse effects; height, weight, waist and hip measurement, triceps skin fold thickness, mid-upper arm circumference; vital signs (temperature, heart rate, blood pressure), detailed physical examination including Tanner staging and acanthosis nigricans staging; blood draw for CBC, metabolic panel (sodium, potassium, chloride, bicarbonate, calcium, phosphate, BUN, creatinine, uric acid, albumin, total protein), hepatic panel (total and direct bilirubin, AST, ALT, alkaline phosphatase), fasting lipid profile (total cholesterol, triglyceride, LDL, HDL), HbA1c, vitamin B12, free fatty acid, leptin, C-reactive protein; urine pregnancy test (for patients of childbearing potential); alcohol, nutritional, physical activity, HR-QOL and liver symptom questionnaires.

5.5. Standardized questionnaires

Several standardized questionnaires will be administered to patients enrolled in the TONIC trial. Questionnaires will be administered at baseline (prior to randomization) and during follow-up at specified intervals (see Appendix 10.3 for the data collection schedule). The purpose of the questionnaires is to obtain important information regarding alcohol intake, nutrition, physical activity, health-related quality of life, and liver-related symptoms.

Alcohol questionnaires: Patients enrolled in the treatment protocol will complete the AUDIT questionnaire during screening. Patients will complete interim alcohol questionnaires (AUDIT-C) at follow-up visits (included in the Followup Medical History). The purpose of these questionnaires is to ascertain that there is no significant alcohol consumption at enrollment or during the study period and to evaluate the effect of alcohol consumption on the response to various interventions.

Nutrition Questionnaire: The Block Brief nutrition questionnaire will be administered to each patient during screening and after 48 and 96 weeks of treatment, and at the 120 week visit (24 weeks after withdrawal of study medication). This instrument is detailed and likely to take 30 minutes to complete. It estimates food frequency and quantity over the preceding 12-month period. The objectives of administering nutrition questionnaire are (a) to assess the baseline nutritional comparability among randomized patients and (b) to assess the possible effect of nutrient intake on the response to various treatments.

Measure of functional activity: The Modifiable Activity Questionnaire (MAQ) for Adolescents will be administered during screening and after 48 and 96 weeks of treatment, and at the 120 week visit (24 weeks after withdrawal of study medication).

Health-related quality of life: The Pediatric Quality of Life (PedsQL) questionnaire will be administered during screening and after 48, and 96 weeks of treatment, and at the 120 week visit (24 weeks after withdrawal of study medication).

Liver-related symptoms: A questionnaire on liver symptoms for children with NAFLD has been developed by the NASH CRN Measures and Assessments Committee and will be administered during screening and after 48 and 96 weeks of treatment, and at the 120 week visit (24 weeks after withdrawal of study medication).

The NASH CRN Steering Committee noted and acknowledged during the questionnaire selection process that some questionnaires were not validated for all eligible participants in TONIC trial.

5.6. Specimen repository

Specimens will be collected and stored in a central repository for use as approved by the Steering Committee of the NASH CRN (see Appendix 10.4 for whole blood draw schedule). Specimens include serum, plasma, and DNA. The blood collected into a serum separation tube at screening visit 2, and at 24, 48, 72, and 96 week visits will be divided into 0.5 mL aliquots. Aliquots will be kept in a storage facility at -70°C. Blood will be collected at the screening visit 2 for extraction of DNA which will be stored at -20°C.

5.7. Overview of scoring of liver biopsies

Liver biopsies will be obtained from each patient in the 6 months prior to randomization and after 96 weeks of treatment. Standard of care baseline liver biopsies will be reviewed and scored by the local NASH CRN pathologist to determine eligibility. Standard of care slides accessed with patient's permission and shipped to the Histology Review Center will be scored by central review of the slides by the Pathology Committee. TONIC trial follow-up liver biopsy slides will be reviewed and scored by the local NASH CRN pathologist first for patient care (data not collected) and then shipped to the Histology Review Center for scoring by central review of the slides by the Pathology Committee.

The tissue will be examined by light microscopy and scored based upon the NASH CRN NAFLD/NASH scoring system. The scoring system is detailed in the Liver Biopsy Procedure and NAFLD/NASH Histologic Scoring System Manual prepared by the NASH CRN Pathology Committee and approved by the NASH CRN Steering Committee. Pediatric and adult biopsies will be scored according to the same criteria. However, since change in histology is not the primary outcome for TONIC trial and since children may show a different pattern of progression than adults, criteria for improved histology in children will not be specified at this time. Briefly, biopsies will be assessed for the following:

(1) Adequacy of the biopsy sample for reading;

(2) Steatosis (including grade of 0-3, location, and whether microvesicular);

(3) Fibrosis based upon Masson's trichrome stain as 0-4 with:
0 = none
1a = mild zone 3 perisinusoidal
1b = moderate zone 3 perisinusoidal
1c = portal/periportal only
2 = zone 3 and periportal
3 = bridging
4 = cirrhosis

(4) Lobular inflammation as 0, 1 (<2 areas seen), 2 (2-4 areas seen), or 3 (>4 areas seen under 20-fold magnification) and as presence or absence of microgranulomas and lipogranulomas

(5) Portal inflammation as 0 (none), 1 (mild), or 2 (greater than mild)

(6) Liver cell injury: Ballooning as 0 (none), 1 (few) and 2 (many), with notation of presence or absence of acidophilic bodies (0 = rare or 1 = many), pigmented macrophages (0 = rare/absent, 1 = many), and megamitochrondria (0 = rare/absent, 1 = many)

(7) Mallory bodies as 0 (rare/absent) or 1 (many)

(8) Glycogen nuclei as 0 (rare/absent) or 1 (many)

(9) Hepatocellular iron grade based on iron stain as 0 (absent or barely discernible, 40x), 1 (barely discernable granules, 20x), 2 (discrete granules resolved, 10x), 3 (discrete granules resolves, 4x), and 4 (masses visible by naked eye)

(10) Steatohepatitis as 0 (no), 1a (suspicious/borderline/indeterminate; Zone 3 pattern), 1b (suspicious/borderline/indeterminate; Zone 1, periportal pattern), and 2 (yes, definite)

The steatosis grade 1 or greater will be used to determine eligibility and all component scores will constitute secondary histologic outcome measures defined elsewhere in this protocol. Baseline liver biopsies will be scored by the local NASH CRN pathologist for eligibility. Baseline and follow-up liver biopsies will be scored by central review of the slides by the Pathology Committee. The scores obtained centrally will be used for data analysis.

5.8. 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 pills in the patient's returned pill containers
- Conducting a brief, structured interview, the study coordinator will assist the patients to identify problems in taking the study drug and to estimate adherence to the assigned study drug 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.

Certificates of appreciation may be given at enrollment and at conclusion as an incentive.

5.9. Management of concomitant conditions

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

General anesthesia during major surgical procedures may pose a risk of metabolic acidosis. Study drug will be suspended prior to such anticipated surgical procedures, with the last dose administered on the day prior to surgery. Serum creatinine should be less than 1.5 mg/dL for males and less than 1.4 mg/dL for females to restart the study drug after the surgical procedure.

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 at once daily dosage and then progressing to twice daily dosage after another two weeks. If local skin reactions recur with restarting the study drug, study drug should be discontinued. In cases where the study drug has been discontinued, the study drug will be unmasked and the patient, investigator, and primary care provider will be notified in order to prevent future exposures.

6. Safety monitoring

Safety issues can be divided into adverse effects relating to the therapeutic interventions, safety issues related to liver biopsy, concerns related to patient privacy, and issues related to the central specimen repository.

6.1. Safety issues related to metformin

The following paragraphs discuss the important potential adverse effects of metformin and the proposed safeguards to minimize the risks involved.

- **Gastrointestinal side effects**: As many as 30% of patients receiving metformin report diarrhea, nausea, metallic taste, abdominal bloating, flatulence, or anorexia. We intend to minimize these side effects by recommending that metformin be taken with food and by gradually titrating the dose over two weeks.

- **Anemia**: About 6-9% of patients receiving metformin may develop reduced vitamin B12 levels. However, megaloblastic anemia is rare and metformin use has not been reported to cause peripheral neuropathy. Vitamin B12 levels will be measured at baseline and every 24 weeks until the end of TONIC trial. Patients with low B12 levels at baseline or those with diminishing B12 levels while on the protocol will be treated according to standard clinical practice.

- **Lactic acidosis** is a rare, but serious, metabolic complication that can occur due to metformin accumulation during treatment with metformin. The reported incidence of lactic acidosis in patients receiving metformin is very low (approximately 0.03 cases/l,000 patient years). However, when it occurs, the case fatality rate is 50%. We intend to minimize the lactic acidosis risk (a) by not enrolling patients with renal dysfunction in TONIC trial, (b) by not including patients with cirrhosis of the liver, and (c) by thoroughly educating the patients and investigators about avoiding intravenous contrast during the study period. If intravenous contrast is required either electively or emergently, patients will discontinue study drug on the day of dye administration for 3 days. The study drug will be restarted if the serum creatinine measured 48 hours after the contrast administration is less than 1.5 mg/dL for males and less than 1.4 mg/dL for females.

  Patients in whom the study drug has been discontinued due to significant adverse effects will be followed according to the protocol and will be offered a liver biopsy at the conclusion of the study.

6.2. Safety issues related to vitamin E

From numerous publications on the "prophylactic" and "therapeutic" use of vitamin E, it may be concluded that the toxicity of vitamin E is very low. It has been demonstrated in animal experiments that vitamin E has neither mutagenic, teratogenic nor carcinogenic properties. Based on studies in humans, a daily dosage of 100-300 mg vitamin E can be considered harmless from a toxicological point of view. Using double-blind studies involving a large number of subjects, it has been demonstrated that oral doses up to 3,200 units/day led to no consistent adverse effects[37,38,39]. From a large body of published data, dosage ranges have been deduced which can be characterized as safe for human subjects even where their use extends over a long period of time. Oral intake of high levels of vitamin E can exacerbate the blood coagulation defect of vitamin K deficiency caused by malabsorption or anticoagulant therapy. High levels of vitamin E intake in these subjects are therefore contraindicated.

In the Diabetes Prevention Program (DPP), the incidence of diabetes in the adult aged placebo group was 11.0 cases per 100 person years[42]. Based on this, it is anticipated that approximately 13 subjects in the placebo group will develop new-onset diabetes mellitus during their participation in the TONIC trial. Although use of antioxidants may improve insulin resistance and thereby may reduce the incidence of new-onset diabetes, it is safe to assume that the magnitude of risk of new-onset diabetes mellitus in patients randomized to vitamin E is same as placebo (ie, 13 subjects during the 24 months treatment). Measures to identify patients who have developed new-onset diabetes mellitus and their management strategy are described in section 6.3.

6.3. Safety issues related to placebo

As presented in the section above, it is anticipated that about 13 subjects in the placebo group will develop new-onset diabetes mellitus during their participation in the TONIC trial. Participants will be seen frequently during the study period for assessment of symptoms consistent with uncontrolled hyperglycemia and will have fasting glucose levels measured at 24, 48, 72, 96, and 120 weeks after randomization (at 48, 96, and 120 weeks this measurement is part of OGTT). Patients with fasting glucose $\geq$126 mg/dL will have the same test repeated within 6 weeks to confirm the diagnosis of new-onset diabetes. Patients with a diagnosis of new onset diabetes will be managed according to the following stepped care approach and the primary care physician will be notified.

(a) Lifestyle counseling to reduce excess body weight and increase exercise
(b) Education on diabetic diet
(c) If fasting glucose remains greater than 140 mg/dL after three months or if patient develops symptomatic diabetes, treatment with sulfonylurea will be initiated.
(d) If patient still is unable to maintain glycemic control, primary care physician may request unmasking of the study medication and should institute necessary measures for diabetes management. Study medication will not be unmasked to the investigator. These patients will be followed at scheduled intervals to collect other outcome data; however, they will

not have the OGTT scheduled for the visits at 48, 96, and 120 weeks but will have only fasting glucose levels measured.

During the trial, if a participant develops side effects thought to be due to the study drug and requires cessation of study drug, 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 be followed in the study according to the protocol, in keeping with the "intention-to-treat" paradigm.

6.4. Safety issues related to percutaneous liver biopsy

Patients will have one follow-up liver biopsy for research purposes during their participation in the TONIC trial. 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. Other rare complications of liver biopsy include infection, perforation of another organ, and severe bleeding such that a blood transfusion or even radiological/surgical interventions are required to stop the bleeding (less than 1 in 1,000). Very rarely (less than 1 in 10,000 reported 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 patients with cirrhosis or coagulopathy, (c) by adhering to good clinical practice in performing the liver biopsy, (d) by assuring that an attending hepatologist or radiologist directly supervises if a physician trainee is performing the procedure, and (e) by considering a transjugular liver biopsy in morbidly obese patients in whom a percutaneous, mid-axillary approach may not be feasible.

6.5. Concerns related to patient privacy

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

6.6. Issues related to specimen repository

Serum, plasma, and DNA from the participants will be stored for future studies related to NAFLD and possibly other liver/metabolic diseases. These samples will be stored in a central repository. The NASH CRN Steering Committee will develop specific guidelines addressing the

issues such as (a) obtaining a separate informed consent, (b) storage, (c) transportation of the material, (d) who will have access to the material, and (e) what investigations are to be conducted.

6.7. Food and Drug Administration

The TONIC trial will be conducted under an Investigational New Drug (IND) # 71,217 held by the NIDDK Project Officer. The investigators will complete a Statement of Investigator (FDA Form 1572) per the Code of Federal Regulations before the initiation of the TONIC trial. 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.

6.8. Adverse event reporting

The TONIC trial 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", shared by 17 Departments and Agencies and (2) 21 CFR 312, 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 requires 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 TONIC trial definitions and procedures for adverse events are designed to satisfy both sets of requirements.

6.8.1. Definitions

Adverse event. An adverse event is any untoward medical occurrence that may present itself during treatment or administration with a pharmaceutical product or clinical procedure and which may or may not have a causal relationship with the treatment. Adverse events include any unanticipated problems involving risks to participants, or breaches of protocol which might entail risk to participants. The term "unanticipated problem" includes both new risks and increased rates of anticipated problems.

Serious adverse event (SAE). A serious adverse event is an adverse event occurring at any time during the study that results in death, life-threatening adverse drug experience, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant disability/incapacity, or a congenital anomaly/birth defect. Other events may also be considered an SAE if, based on medical judgement, the event jeopardized the patient to the point of requiring medical or surgical intervention to prevent the occurrence of any of the conditions for an SAE listed above.

Unexpected adverse event. An unexpected adverse event is any adverse event with specificity or severity that is not consistent with the risk information in the study protocol, current investigator brochure, or current package insert.

Associated with the use of the drug means that there is a reasonable possibility that the adverse experience may have been caused by the drug.

6.8.2. 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 one of the study drugs. 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 at its semi-annual 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 the Common Terminology Criteria for Adverse Events[43].

After each meeting, the DSMB will issue a written summary of its 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.

6.8.3. Reporting serious adverse events

Serious adverse events (SAE) must be reported upon discovery at the clinical center. This will involve completing an SAE data form describing the severity and details of the event. The SAE form, together with a memo summarizing the circumstances of event and the current status of the patient, must be faxed to the Data Coordinating Center and to the NIDDK project officer within one working day of the discovery of the SAE. Also within one day, the clinical center must notify the NIDDK and Data Coordinating Center of the SAE by telephone or confirmed e-mail. The NIDDK project officer will work with the Data Coordinating Center to transmit the SAE form and memo to all study centers and to the DSMB.

If the SAE is unexpected AND associated with a study drug, then the NIDDK project officer will notify the FDA no later than 15 days from the discovery of the SAE (no later than 7 days if the SAE is fatal or life threatening). The pharmaceutical manufacturer will also be notified. The clinical center investigator may also be responsible for completing an FDA MedWatch 3500 form.

The DSMB will review each SAE report and provide comments to the NIDDK project officer within one week of receipt of the report. 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 project officer 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. The NIDDK project officer will work with the Data Coordinating Center to distribute the follow-up memo to the clinical center and to the DSMB.

6.9. Procedures for unmasking treatment assignment

Treatment assignments are double masked throughout the study until after all data collection for the TONIC trial has been completed (ie, after completion of the 24 week post trial follow-up for all patients). Every effort will be made to maintain the masking throughout the study except in emergency situations. The code of specific study drug will not be broken without the knowledge of the clinical center's principal investigator.

Unmasking of study drug will occur under the following conditions:

- **Severe allergic reaction (Stevens-Johnson Syndrome)**: Study drug is stopped indefinitely. The patient, primary care provider (PCP), and the investigator will be unmasked.

- **New onset diabetes and fasting blood glucose at least 140 mg/dL on two separate occasions:** Study drug will be stopped indefinitely. The patient will be referred to their PCP with standard American Diabetes Association recommendations. The patient and PCP will be unmasked to metformin assignment.

- **Pregnancy during the study**: Study drug will be stopped indefinitely, and the coded study drug will be unmasked. The patient and PCP will be notified of the assigned treatment and 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 or a doubling of the serum baseline ALT **and** ALT value > 400 U/L during treatment. There will be no unmasking until study conclusion and subjects will be advised to avoid metformin and vitamin E until the unmasking is done. One exception is if any of these subjects with hepatotoxicity develop diabetes during the study duration. In this situation, the study medication will be unmasked and subject and PCP will be notified.

In unforeseen situations where the clinical center principal investigator considers unmasking is in the best interest of the participant's health and well being, unmasking could be done after notifying and obtaining approval of the NASH CRN Executive Committee.

The Data and Safety Monitoring Board will review all instances of unmasking that occur. The Data and Safety Monitoring Board may develop its own set of unmasking guidelines for monitoring purposes.

7. Statistical design and analysis

7.1. Hypotheses

Primary hypotheses:

- In pediatric nondiabetic patients with NAFLD, improvement in insulin resistance over 96 weeks of treatment with metformin will result in sustained reduction in serum alanine aminotransferase (ALT) compared to treatment with placebo

- In pediatric nondiabetic patients with NAFLD, improvement in oxidative stress status over 96 weeks of treatment with vitamin E will result in sustained reduction in serum ALT compared to treatment with placebo

Secondary hypotheses:

- Treatment with metformin or vitamin E will result in sustained reduction in serum AST and GGT compared to treatment with placebo

- Metformin and vitamin E are equally effective in achieving histologic improvement in nondiabetic children with NAFLD

- Levels of proinflammatory cytokines and serum markers for fibrosis will decrease with treatment with metformin and with vitamin E compared to treatment with placebo

- Pediatric nondiabetic patients with NAFLD have an impaired quality of life, and these scores will improve as their serum ALT improves upon treatment with metformin or vitamin E compared to treatment with placebo

Other questions to be addressed:

- We predict that insulin resistance is common in pediatric nondiabetic patients with NAFLD and will be significantly related to waist-to-hip ratio, waist circumference, serum ALT, and hepatic histology

- We predict that degree of obesity will be significantly related to hepatic histology in pediatric nondiabetic patients with NAFLD and more severe obesity (BMI or BMI z score) will be related to greater elevations in ALT and more advanced hepatic histology

- We predict that serum markers for steatosis, inflammation, and fibrosis will be significantly related to hepatic histology in pediatric nondiabetic patients with NAFLD

- We predict that worsening hepatic histology will be significantly related to a worsening in health-related quality of life in pediatric nondiabetic patients with NAFLD and more advanced histology will have worse health-related quality of life as measured by scores on the PedsQL

- We predict that improvement in the measures of insulin resistance will be significantly related to improvements in liver histology, serum ALT, and serum markers for steatosis, inflammation, and fibrosis in pediatric nondiabetic patients with NAFLD

- We predict that therapy with vitamin E will improve insulin resistance in pediatric nondiabetic patients with NAFLD

- We predict that improvement in liver histology upon treatment with insulin sensitizers will be significantly related to improvements in waist-to-hip ratio and waist circumference in pediatric nondiabetic patients with NAFLD

- We predict that therapy with same dose metformin across different age, height, and weight groups will have similar weight loss effects in pediatric nondiabetic patients with NAFLD

- We predict that maturation and growth (Tanner staging, anthropometric measures) will be similar across treatment groups in pediatric nondiabetic patients with NAFLD

7.2. Outcome measures

The primary outcome measure is the reduction in serum ALT after 96 weeks of treatment to either 50% of the baseline value OR to 40 U/L or lower which is sustained for at least one year. Other outcome measures are as follows:

- Reduction in serum aspartate transferase (AST) after 96 weeks of treatment to either 50% of the baseline value OR 40 U/L or lower which is sustained for at least one year.

- Reduction in serum gamma glutamyl transferase (GGT) after 96 weeks of treatment to either 50% of the baseline value OR 40 U/L or lower which is sustained for at least one year.

- Change in anthropometric measurements (weight, BMI, BMI z score, waist-to-hip ratio, waist circumference, triceps skin fold thickness and total body fat) after 96 weeks of treatment compared to baseline.

- Change in Tanner staging after 96 weeks of treatment compared to baseline.

- Change in insulin resistance (assessed by HOMA), serum vitamin E levels, cytokines, fibrosis markers, and lipid profile after 96 weeks of treatment compared to baseline.

- Change in health-related quality of life (PedsQL) after 96 weeks of treatment compared to baseline.

The histologic outcome measures require improvement in NAFLD activity after 96 weeks of treatment as determined by liver biopsies pre- and post-treatment. The histologic measures are derived from changes from baseline to the end of treatment in the NASH CRN NAFLD/NASH activity score (NAS). 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 score (0-3) +
Lobular inflammation score (0-3) +
Hepatocyte ballooning score (0-2)

Outcome measures derived from the NAS will include, but not be limited to:

- Change in NAS after 96 weeks of treatment compared to baseline NAS

- Changes in fibrosis, steatosis, lobular inflammation, hepatocyte ballooning and other specific feature scores from the histologic scoring system after 96 weeks of treatment compared to baseline

- Improvement in NAS and in specific features defined as any improvement in the corresponding scores

7.3. Statistical analysis

Primary hypotheses:

Statistical analyses for the two primary hypotheses will follow the intention-to-treat paradigm, which means that all randomized patients with baseline and 96 week serum ALT values will be analyzed in the treatment group to which they were assigned. Any randomized patient who does not have the requisite ALT values will be accounted for and compared by assigned treatment group. Patients not able to be included in the intention-to-treat analyses will be compared to those who are included with respect to demographic and other characteristics.

Since the primary outcome measure, defined in Section 7.2, is a binary indicator of sustained reduction in ALT after 96 weeks of treatment compared to baseline and since the randomization is stratified by clinic, P-values for the primary hypotheses will be derived from the Mantel-Haenszel χ_2 test for stratified 2x2 tables[44]. Two P-values will be derived: one comparing proportions improved in the group assigned to metformin compared to the group assigned to placebo and another comparing the group assigned to vitamin E to the group assigned to placebo. Since two primary comparisons are planned, a P-value of 0.025 will be considered significant, applying a Bonferroni correction for multiple comparisons.

Given the randomized design and adequate size planned for the TONIC trial, it is unlikely that confounding of the treatment groups by covariates related to the change in serum ALT will occur. However, if confounding should occur, logistic regression analyses with sustained reduction in ALT as the binary response and treatment group indicators and any suspected confounders as covariates will be carried out to determine the sensitivity of the primary P-values to confounding.

Secondary hypotheses:

Statistical analyses for the first secondary hypothesis, that treatment with metformin or vitamin E will result in sustained reduction in serum AST and GGT compared to treatment with placebo, will be the same as described for primary hypotheses.

The second secondary hypothesis, that metformin and vitamin E are equally effective in achieving histologic improvement in nondiabetic children with NAFLD, involves the histologic outcome measure. Equivalence will be assessed by means of 95% confidence intervals on the difference in proportions improved.

Analyses for outcomes related to other secondary hypotheses will be conducted in two ways. Improvement will be analyzed both as a binary outcome (improved vs. not improved) and also in terms of the numerical change in the outcome. Binary outcomes will be compared using the Mantel-Haenszel χ^2 test for stratified 2x2 tables. Numerical changes will be analyzed by descriptively comparing the between-treatment group differences in mean and median changes; P-values will be derived from Wilcoxon rank sum tests for comparison of the distribution of changes in each group. If concerns about confounding arise, logistic regression models for improvement outcomes and linear regression models for numerical change outcomes will be used to correct for the confounding. Analyses for secondary hypotheses will generally involve three separate analyses, one for each treatment group comparison: metformin vs. placebo, vitamin E vs. placebo, and metformin vs. vitamin E. No adjustments for multiple comparisons will be applied to the secondary hypotheses; however, any significant findings must be interpreted taking into account the strength of the finding and its biologic plausibility.

Other questions to be addressed:

A series of other questions of interest were listed in Section 7.1. Analyses related to these questions will involve a mix of appropriate exploratory and confirmatory analyses, similar, but not limited to those described for the primary and secondary hypotheses.

7.4. Missing data

The occurrence of missing data in TONIC trial is expected to be low and, when present, is expected to be equally distributed across the 3 treatment groups. We estimate that careful selection of patients during the 16 weeks screening phase and the consent process should result in no more than 10% missing data from patients who drop out before completing the 96 week treatment period. In primary, intention-to-treat analyses, patients with missing data will be considered unimproved on the primary outcome measure.

The proportions with missing data will be compared across treatment groups using χ^2 tests. If the amount of missing data exceeds 10% and the frequency of missing data differs by treatment group, then a variety of sensitivity analyses will be carried out to compare with 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.

7.5. Justification of sample size

The planned sample size for the TONIC trial is 180 patients with equal allocation to each of the three treatment groups (60 per group).

We based the sample size estimates on a two-group, binomial comparison of the proportions of patients satisfying the primary outcome, reduction in serum ALT to either 50% of the baseline value OR to 40 U/L or lower which is sustained for at least one year (defined in Section 7.2) over the 96 week course of treatment. Since TONIC is a three group trial with two primary hypotheses, we assume that the two primary comparisons, metformin vs. placebo and vitamin E vs. placebo, require the same sample size and reduce the type I error from 0.05 to 0.025 (Bonferroni correction). Expected proportions improved were approximated using pilot data from a 48-week metformin study (Jeff Schwimmer, personal communication) and from a consensus among our investigators as to the response in the placebo group. Currently, there are no available data to estimate the response in vitamin E, which is assumed, for purposes of planning the trial, to be the same as for metformin.

The sample size calculations were performed using the PS[45] software using the formula:

$$n = \frac{\left[z_{1-\alpha/2}\sqrt{2\bar{\pi}(1-\bar{\pi})} + z_{1-\beta}\sqrt{\pi_1(1-\pi_1)+\pi_2(1-\pi_2)}\right]^2}{(\pi_1-\pi_2)^2}$$

where,

n = sample size per group

π_1 = expected proportion improved in placebo group (assumed=0.20)

π_2 = expected proportion improved in metformin or vitamin E group (0.50)

$\bar{\pi}$ = average of π_1 and π_2

α = Two-sided type I error (0.025, Bonferroni corrected for two comparisons)

β = Type II error (0.10; ie, 90% power)

The number per group, using the above formula is 60 patients; or a total of 180 for the trial. As indicated in Section 7.4, patients with missing primary outcome data will be considered unimproved and will be included in the 180 patients.

7.6. Interim analysis

An independent Data and Safety Monitoring Board (DSMB), appointed by the NIDDK, is responsible for approving the protocol for the TONIC trial and for monitoring the accumulating interim 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, in turn, communicate DSMB recommendations to the investigators, as appropriate. The DSMB meets in person to approve the protocol. After the trial commences, the DSMB meets twice a year to review data or other issues – once in person and once by telephone conference. 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 (as close to real time as possible) 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 300 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 reviews one planned interim analysis of the primary outcome measure. O'Brien-Fleming statistical stopping guidelines for efficacy apply. This interim efficacy analysis will occur when approximately 50% of the data are complete or when approximately 90 of the 180 patients have completed the 96 week course of treatment.

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 TONIC 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 approval

A site may not initiate patient activities in the TONIC trial until the site has Institutional Review Board (IRB) approval for the trial. 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

A prototype consent 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 must sign the consent to be eligible for the trial. The consent form will describe the purpose of the trial, the procedures to be followed, and the risks and benefits of participation. Copies of the signed consent forms will be given to the patient, and this fact will be documented in the patient's record.

8.4. Patient 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).

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10. Appendices

10.1. Participating centers

Clinical Centers

- Case Western Reserve University
- Duke University/Johns Hopkins University
- Indiana University
- St. Louis University
- University of California, San Diego
- University of California, San Francisco
- University of Washington
- Virginia Commonwealth University

Data Coordinating Center:

- Johns Hopkins University

National Institutes of Health:

- National Institute of Diabetes and Digestive and Kidney Diseases
- National Cancer Institute
- National Institute of Child Health and Human Development

10.2. Committees

- Steering Committee
- Executive Committee
- Database Committee
- Measures and Assessments Committee
- Pathology Committee
- Standards of Care Committee
- Pediatrics Committee
- Ancillary Studies Committee
- Presentations and Publications Committee
- TONIC Eligibility Committee
- Treatment Trial Protocol Committee
- Pilot and Feasibility Studies Committee

10.3. Data collection schedule

Assessment/Procedure	Screening visits S1	Screening visits S2	RZ	Follow-up visits Weeks from randomization 4	12	24	36	48	60	72	84	96	120
Consent	X	.	X	.	.	.	.	.	.	.	.	.	.
Baseline (B) or interim (I) medical history	B	.	.	I	I	I	I	I	I	I	I	I	I
Review for adverse effects	X	.	.	X	X	X	X	X	X	X	X	X	X
Review of concomitant medications	X	.	.	X	X	X	X	X	X	X	X	X	X
AUDIT (A) or interim (I) alcohol quest	A	.	.	I	I	I	I	I	I	I	I	I	I
Study drug dispensing	.	.	X	.	X	X	X	X	X	X	X	.	.
Review of study drug adherence	.	.	.	X	X	X	X	X	X	X	X	X	.
Detailed (D) or focused (F) physical exam	D	.	.	F	F	F	F	D	F	F	F	D	D
DEXA scan for body fat	.	X	.	.	.	.	.	.	.	.	.	X	.
MRI for body fat (optional)	.	X	.	.	.	.	.	.	.	.	.	X	.
Liver biopsy (H for ≤6 months)	H	.	.	.	.	.	.	.	.	.	.	X	.
Block Brief nutrition questionnaire	.	X	.	.	.	.	.	X	.	.	.	X	X
Modifiable activity questionnaire	.	X	.	.	.	.	.	X	.	.	.	X	X
Pediatric quality of life	.	X	.	.	.	.	.	X	.	.	.	X	X
Liver symptom questionnaire	.	X	.	.	.	.	.	X	.	.	.	X	X
Labs													
Metabolic panel	X	.	.	X	X	X	X	X	X	X	X	X	X
Hematology	X	.	.	.	.	X	.	X	.	X	.	X	X
Fasting lipid profile	X	.	.	.	.	X	.	X	.	X	.	X	X
Fasting serum glucose	X	.	.	.	.	X	.	.	.	X	.	.	.
Hepatic panel	.	X	.	X	X	X	X	X	X	X	X	X	X
Vitamin B12	.	X	.	.	.	X	.	X	.	X	.	X	X
GGT, prothrombin time, HbA1c	.	X	.	.	.	.	.	X	.	.	.	X	X
OGTT with insulin and C-peptide	.	X	.	.	.	.	.	X	.	.	.	X	X
Free fatty acid, leptin, C-reactive protein	.	X	.	.	.	.	.	X	.	.	.	X	X
Urine pregnancy test (females)	X	X	X	X	X	X	X	X	X	X	X	X	X
Banking													
Fasting serum, plasma banking	.	X	.	.	.	X	.	X	.	X	.	X	.
Serum vitamin E (on banked specimen)	.	X	.	.	.	.	.	X	.	.	.	X	.
DNA for banking	.	X	.	.	.	.	.	.	.	.	.	.	.
Closeout form (CO)	.	.	.	.	.	.	.	.	.	.	.	.	X

Note: **Detailed (D) physical** includes measurement of height, weight, waist, hips; vital signs (temperature, heart rate, blood pressure); triceps skin fold thickness; mid-upper arm circumference; examination for scleral icterus and pedal edema and auscultation of the heart and lungs; general physical findings (hepatosplenomegaly, peripheral manifestations of liver disease, ascites, wasting, fetor); Tanner staging. **Focused (F) physical** includes measurement of height, weight; vital signs (temperature, heart rate, blood pressure); examination for scleral icterus and pedal edema and auscultation of heart and lungs.

OGTT: Blood samples will obtained only at baseline and at 2 hours after administration of glucose solution.

Lipid profile: total cholesterol, triglyceride, LDL, HDL.

Hematology: Hemoglobin, hematocrit, WBC, platelet count.

Metabolic panel: sodium, potassium, chloride, bicarbonate, calcium, phosphate, BUN, creatinine, uric acid, albumin, total protein.

Hepatic panel: total bilirubin, direct bilirubin, AST, ALT, alkaline phosphatase.

Fasting visits: All visits except RZ.

10.4. Whole blood draw schedule

	Study visit (wk)												
Procedure	**s1**	**s2**	**4**	**12**	**24**	**36**	**48**	**60**	**72**	**84**	**96**	**120**	**Total**
OGTT w/insulin	.	10	.	.	.	.	10	.	.	.	10	10	40
Fasting lipid	5	.	.	.	5	.	5	.	5	.	5	5	30
Hematology	5	.	.	.	5	.	5	.	5	.	5	5	30
Metabolic panel	5	.	5	5	5	5	5	5	5	5	5	5	55
Hepatic panel	.	5	5	5	5	5	5	5	5	5	5	5	55
HbA1c and others	.	5	.	.	.	.	5	.	.	.	5	5	20
Plasma: banking	.	5	.	.	.	.	5	.	.	.	5	.	15
Serum: fibrosis	.	10	.	.	.	.	10	.	.	.	10	.	30
Serum: banking	.	25	.	.	20	.	25	.	20	.	25	.	115
Serum: Vit E	.	5	.	.	.	.	5	.	.	.	5	.	15
DNA	.	20	.	.	.	.	.	.	.	.	.	.	20
Total	15	85	10	10	40	10	80	10	40	10	80	35	**425**

OGTT w/insulin: also includes c-peptide and fasting glucose (baseline and 2-hour samples only are collected)
Hematology: hemoglobin, hematocrit, WBC, platelet count
Metabolic panel: sodium, potassium, chloride, bicarbonate, calcium, phosphate, BUN, creatinine, uric acid, albumin, total protein
Hepatic panel: total bilirubin, AST, ALT, alkaline phosphatase
HbA1c and others: HbA1c, GGT, prothrombin time (INR)

Note: Fasting serum glucose (visits s1, f024, f072), **Vitamin B12** (visits s2, f024, f048, f072, f096, f120), and **free fatty acid, leptin, and C-reactive protein** (visits s2, f048, f096, f120) are also collected, but these measures can be obtained from the blood drawn at these visits for one of the other tests specified in the table (ie, these measures do not add to the volume of blood needed to be drawn at the visit)

Fasting visits: s1, s2, f024, f048, f072, f096, and f120.

Fasting TONIC study visits need to be scheduled for early morning. Fasting is defined as nothing by mouth except water in the 12 hours prior to blood draw

10.5. Glossary

3-NT	-	3-nitrotyrosine
ANA	-	anti-nuclear antibody
AST	-	aspartate aminotransferase
ALT	-	alanine aminotransferase
anti-HBc	-	hepatitis B core antibody
anti-HBs	-	hepatitis B surface antibody
anti-HCV	-	hepatitis C antibody
BMI	-	body mass index (kg/m^2; calculated as the weight in kilograms divided by the square of the height in meters)
BUN	-	blood urea nitrogen
DCC	-	data coordinating center
DEXA	-	dual energy x-ray absorptiometry
DPP	-	Diabetes Prevention Program
FDA	-	Food and Drug Administration
FFA	-	unesterified free fatty acid
GGT	-	gamma glutamyl transferase
HBsAg	-	hepatitis B surface antigen
HCV	-	hepatitis C virus
HOMA-IR	-	homeostasis model assessment method for insulin resistance (calculated as [fasting insulin (μU/ml) * fasting glucose (mmol/L)]/22.5)
HRQOL	-	health-related quality of life
IBW	-	Ideal body weight
NAFL	-	nonalcoholic fatty liver
NAFLD	-	nonalcoholic fatty liver disease
NAS	-	NASH activity score
NASH	-	nonalcoholic steatohepatitis
NIDDK	-	National Institute of Diabetes and Digestive and Kidney Diseases
PCOS	-	polycystic ovary syndrome
PCP	-	primary care provider
PPARγ	-	peroxisome proliferator-activated receptor-gamma
SAM-e	-	S-adenosyl methionine
UDCA	-	ursodeoxycholic acid (aka, URSO)
ULN	-	upper limit of normal
URSO	-	ursodeoxycholic acid (aka, UDCA)

10.6. Document history

TONIC trial protocol (22 October 2004)

TONIC trial protocol (27 January 2006)

Numerous editorial and wording changes were made to the following sections:

§ Design synopsis:
- Added "study duration – per calendar time"
- Changed Population – "aged 8-15" to "aged 8-17"
- Changed Inclusion criteria – "age 8 through 15" to "age 8 through 17"

§3.1 Design overview
- Changed "washout" to "post-treatment follow-up" in the first paragraph

§4.2 Inclusion criteria
- The 3rd inclusion criterion "Serum alanine aminotransferase elevation (ALT > 60 U/L) at time of screening and on one previous occasion made at least one month, but no greater than 6 months prior to the screening ALT (a total of 2 determinations separated in time by 1 - 6 months)" was changed to "Serum alanine aminotransferase elevation (ALT > 60 U/L) on two separate occasions at least 30 days apart but no more than 6 months (183 days). One of these measures must be obtained at the TONIC clinical center during screening for TONIC. The other measure may be historic (i.e., obtained prior to initiation of screening in TONIC)"
- Added a 5th inclusion criterion "Randomized within 16 weeks (112 days) of starting screening"
- Changed upper age limit of inclusion criteria from "15 years" to "17 years"

§4.3 Exclusion criteria
- The 2nd exclusion criterion
  "Diabetes mellitus by oral glucose tolerance test (OGTT) with 2 g/kg (maximum 75 g) glucose load
  – Fasting plasma glucose of 126 mg/dL or greater OR
  – 2-hour plasma glucose of 200 mg/dL or greater" was changed to

  "Diabetes mellitus
  - Fasting serum glucose of 126 mg/dL or greater
  OR
  - 2-hour serum glucose of 200 mg/dL or greater from oral glucose tolerance test (OGTT) with 2 g/kg (maximum 75 g) glucose load

OR
- History of diabetes mellitus"

- Added "on measurement closest in time to randomization" to the 3rd exclusion criterion
- The 10th exclusion criterion "Metabolic acidosis" was changed to "History of metabolic acidosis"
- Moved the 11th exclusion criterion "History of diabetes mellitus" to the 2nd exclusion criterion
- The 15th exclusion criterion "Current, untreated disease and/or considered by study physician to be significant (cardiac, renal, pulmonary, psychiatric, neoplastic, chronic inflammatory disease besides liver, coagulopathy)" was changed to "Disease considered by study physician to be significant"
- Added "Use of any over-the-counter or herbal remedy for hyperlipidemia in the 3 months prior to randomization" as an exclusion
- Added "History of renal dysfunction" as an exclusion
- Added "History of coagulopathy" as an exclusion
- Added "Vitamin E supplementation of greater than 100 I/U per day" as an exclusion

§4.4 Run-in period
- Changed "Any over-the-counter medication or herbal remedy that is being taken with an intent to improve hyperlipidemia will not be allowed for at least 3 months prior to randomization nor after randomization" to "Any over-the-counter medication or herbal remedy that is being taken with an intent to improve hyperlipidemia will not be allowed for at least 3 months prior to randomization and will be discouraged after randomization" in the 2nd paragraph

§5.1 Visit schedule overview
- Changed "post-treatment washout phase" to "post-treatment follow-up phase"

§5.2 Screening and baseline data collection, Baseline standard of care liver biopsy
- 2nd paragraph "In the case of a biopsy done previously as standard of care, the NASH CRN study physician should check if tissue blocks and or additional slides can be obtained from the original biopsy. If a standard of care percutaneous liver biopsy is completed as part of screening for this trial, patient will present fasted with no liquids for at least 12 hours. Intravenous sedation will be done on prepped and draped patient. Under sterile field, a needle biopsy device will be positioned by ultrasound guidance via the right axillary approach. One core will be biopsied from the right lobe of the liver to be placed in formalin for later preparation of slides for light microscopy and stains including hematoxylin and eosin, Masson's trichrome and iron stain. Following the procedure, subjects will be placed on their right side and monitored for 4 hours to include vital check every 30 minutes, and hematocrit values. All abnormalities will be followed to resolution and per good clinical practices guidelines." was changed to

"In the case of a biopsy done previously as standard of care, the NASH CRN study physician should check if tissue blocks and or additional slides can be obtained from the original biopsy. If a standard of care liver biopsy is completed as part of screening for TONIC, the liver tissue will be prepared for light microscopy and stains including hematoxylin and eosin, Masson's trichrome and iron stain."

§5.2 Screening and baseline data collection, Screening visit 1

- Deleted body mass index [BMI], BMI z score, and waist-to-hip ratio from the list of anthropometric measures since they will be derived from other measures
- Added mid-upper arm circumference to the list of anthropometric measures
- Added iron, total iron binding capacity, ferritin, and ALT to laboratory test results to be obtained
- Deleted anti-liver-kidney microsomal antigen (anti-LKM), anti-soluble liver antigen (anti-SLA), and protease inhibitor (Pi) phenotype from the list of laboratory tests
- Changed plasma glucose to serum glucose in this section and throughout
- Deleted carbon dioxide, lactate, and globulin from the metabolic panel

§5.2 Screening and baseline data collection, Screening visit 2

- 2nd sentence "After administration of glucose solution in a dose of 1.75 g per kilogram of body weight (up to a maximum of 75 g), blood sample for 2-hour plasma glucose, insulin, and C-peptide will be obtained for the oral glucose tolerance test (OGTT)." was changed to "After administration of glucose solution in a dose of 2 g per kilogram of body weight (up to a maximum of 75 g), blood sample for 2-hour serum glucose and insulin will be obtained for the oral glucose tolerance test (OGTT)."

§5.3 Randomization visit

- Deleted height, weight, vital signs (temperature, heart rate, blood pressure); brief interim medical history including menstrual history from the procedures list in 2nd paragraph
- Changed "The study drug assignment will not be generated unless the check finds that the patient is eligible." to "The study drug assignment will not be generated unless the check finds that the patient is eligible, and the clinical center staff indicate that they want to randomize the patient."
- Changed "medication kit" to "medication bottles"

§ 5.4 Follow-up visits, Week 4 visit

- Added waist and hip measurements
- Deleted carbon dioxide, lactate, and globulin from the metabolic panel

§ 5.4 Follow-up visits, Week 12 visit

- Added waist and hip measurements
- Deleted carbon dioxide, lactate, and globulin from the metabolic panel

§ 5.4 Follow-up visits, Week 24 visit

- Added waist and hip measurements
- Added vitamin B12 measurement
- Deleted carbon dioxide, lactate, and globulin from the metabolic panel

§ 5.4 Follow-up visits, Week 36 visit
- Added waist and hip measurements
- Deleted carbon dioxide, lactate, and globulin from the metabolic panel

§ 5.4 Follow-up visits, Week 48 visit
- Changed the dose of glucose solution to 2 g per kilogram
- Deleted 2-hour OGTT c-peptide measurement
- Added triceps skin fold thickness, mid-upper arm circumference
- Deleted carbon dioxide, lactate, and globulin from the metabolic panel

§ 5.4 Follow-up visits, Week 60 visit
- Added waist and hip measurements
- Deleted carbon dioxide, lactate, and globulin from the metabolic panel

§ 5.4 Follow-up visits, Week 72 visit
- Added waist and hip measurements
- Added vitamin B12 measurement
- Deleted carbon dioxide, lactate, and globulin from the metabolic panel

§ 5.4 Follow-up visits, Week 84 visit
- Added waist and hip measurements
- Deleted carbon dioxide, lactate, and globulin from the metabolic panel

§ 5.4 Follow-up visits, Week 96 visit
- Changed the dose of glucose solution to 2 g per kilogram
- Deleted 2-hour OGTT c-peptide measurement
- Added triceps skin fold thickness, mid-upper arm circumference
- Deleted carbon dioxide, lactate, and globulin from the metabolic panel

§ 5.4 Follow-up visits, Follow-up liver biopsy
- Changed the paragraph "A follow-up liver biopsy will be obtained at the week 96 visit. Guidelines for obtaining the biopsy specimen are provided in the NASH CRN Liver Biopsy Procedure and NAFLD/NASH Histology Scoring System Manual; a 16 gauge needle is preferred and the specimen should be least 1.5 cm in length. Patient will present fasted with no liquids for at least 12 hours for follow-up percutaneous liver biopsy. Intravenous sedation will be done on prepped and draped patient. Under sterile field, a needle biopsy device will be positioned by ultrasound guidance via the right axillary approach. The liver tissue must be of adequate size (1.5 cm or more) and slides of adequate quality for interpretation. One core will be biopsied from the right lobe of the

liver to be placed in formalin for later preparation of slides for light microscopy and stains including hematoxylin and eosin, Masson's trichrome and iron stain. Following the procedure, subjects will be placed on their right side and monitored for 4 hours to include vital check every 30 minutes, and hematocrit values. All abnormalities will be followed to resolution and per good clinical practices guidelines." to

"A follow-up liver biopsy will be obtained at the week 96 visit. Guidelines for obtaining the biopsy specimen are provided in the NASH CRN Liver Biopsy Procedure and NAFLD/NASH Histology Scoring System Manual; a 16 gauge needle is preferred and the specimen should be least 1.5 cm in length. The slides must be of adequate size (1.5 cm or more) and adequate quality for interpretation. The liver tissue will be prepared for light microscopy and stains including hematoxylin and eosin, Masson's trichrome and iron stain."

§ 5.4 Follow-up visits, Week 120 visit

- Changed the dose of glucose solution to 2 g per kilogram
- Deleted 2-hour OGTT c-peptide measurement
- Added triceps skin fold thickness, mid-upper arm circumference
- Deleted carbon dioxide, lactate, and globulin from the metabolic panel

§ 5.5 Standardized questionnaires - Alcohol questionnaires

- Clarified that the interim alcohol questionnaire is included in the Follow-up Medical History form, and is not a separate form

§ 5.7 Overview of scoring of liver biopsies

- Under (3) Fibrosis based upon Masson's trichrome stain as 0-4, added "perisinusoidal" to 1a and 1b
- Under (9), changed "Iron as 0 to 4" to "Hepatocellular iron grade based on iron stain as 0 (absent or barely discernible, 40x), 1 (barely discernable granules, 20x), 2 (discrete granules resolved, 10x), 3 (discrete granules resolves, 4x), and 4 (masses visible by naked eye)"
- Under (10), changed "Steatohepatitis finding (No; Suspicious/borderline/indeterminate; or Yes, definite)" to "Steatohepatitis as 0 (no), 1a (suspicious/borderline/indeterminate; Zone 3 pattern), 1b (suspicious/borderline/indeterminate; Zone 1, periportal pattern), and 2 (yes, definite)"

§ 6.4 Safety issues related to percutaneous liver biopsy

- 4th sentence "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 (less than 1 in 1,000)." was changed to "Other rare complications of liver biopsy include infection, perforation of another organ, and severe bleeding such that a blood transfusion or even radiological/surgical interventions are required to stop the bleeding (less than 1 in 1,000)."

§ 6.7 Food and Drug Administration
- Added IND # 71,217

§ 7.4 Missing data
- Changed "4 months" to "16 weeks" in the first paragraph
- Changed 2nd paragraph "The proportions with missing data will be compared across treatment groups using χ^2 tests. Non-significant P-values indicate that the data are missing at random (MAR). If this is the case, analyses of the non-missing data are not threatened. If the amount of missing data exceeds 10% and the data are MAR, 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. Although unlikely in a large trial, it is possible that the missing data are not MAR and the missing data are non-ignorable. A few statistical methods are 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." to

  "The proportions with missing data will be compared across treatment groups using χ^2 tests. If the amount of missing data exceeds 10% and the frequency of missing data differs by treatment group, then a variety of sensitivity analyses will be carried out to compare with 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."

§ 7.5 Justification of sample size
- Changed last sentence "The number per group, using the above formula is 54. Inflating this number by the 10% expected missing data rate yields approximately 60 patients per group, or a total of 180 for the trial." to "The number per group, using the above formula is 60 patients; or a total of 180 for the trial. As indicated in Section 7.4, patients with missing primary outcome data will be considered unimproved and will be included in the 180 patients."

§ 10.2 Committees
- Added TONIC Eligibility Committee

§ 10.3 Data collection schedule was revised

§ 10.4 Whole blood draw schedule was revised

§ 10.5 Glossary
- Deleted definition of MAR

§ 10.6 Document history was added

TONIC trial protocol (22 August 2006)

§ Design synopsis
- The 3rd inclusion criterion "ALT level > 60 U/L on two separate occasions at least 30 days apart but no more than 6 months (183 days) apart. One of these measures must be obtained at the TONIC clinical center during screening for TONIC. The other measure may be historic (i.e., obtained prior to initiation of screening in TONIC)" was changed to: "ALT level > 60 U/L on two separate occasions at least 30 days apart but no more than 12 months (365 days) apart. One of these measures must be obtained at the TONIC clinical center during screening for TONIC. The other measure may be historic (i.e., obtained prior to initiation of screening in TONIC)"

§ 4.1 Recruitment
- Added Jeffrey Schwimmer, MD as Pediatric Co-PI for the University of California, San Diego, CA
- Replaced Martin Graham, MD with Daphne Bryan, MD as the Pediatric Co-PI for Virginia Commonwealth University, Richmond, VA

§ 4.2 Inclusion criteria
- The 3rd inclusion criterion "Serum alanine aminotransferase elevation (ALT > 60 U/L) on two separate occasions at least 30 days apart but no more than 6 months (183 days). One of these measures must be obtained at the TONIC clinical center during screening for TONIC. The other measure may be historic (i.e., obtained prior to initiation of screening in TONIC)" was changed to "Serum alanine aminotransferase elevation (ALT > 60 U/L) on two separate occasions at least 30 days apart but no more than 12 months (365 days). One of these measures must be obtained at the TONIC clinical center during screening for TONIC. The other measure may be historic (i.e., obtained prior to initiation of screening in TONIC)"

TONIC trial protocol (25 May 2007)

§ Design Synopsis
- Study duration - per calendar time revised
- Recruitment phase extended thru September 30, 2007
- Follow-up phase extended thru October 2009

§ Data collection schedule revised to add Closeout form (CO) at f120 visit

10.6. Document history

TONIC trial protocol (15 Nov 2010)

§ Design Synopsis

- Primary outcome measure was corrected to match Section 7. Statistical design and analysis" (ALT < 40 U/L was changed to ALT ≤ 40 U/L)