CLINICAL RESEARCH PROTOCOL

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

PROTOCOL NUMBER: 13DK0002

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TITLE: **TREATMENT OF NON-ALCOHOLIC FATTY LIVER WITH DIFFERENT DOSES OF VITAMIN E**

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Short Title: Vitamin E for NAFLD

Identifying Words: Non-alcoholic fatty liver disease, Non-alcoholic steatohepatitis, steatosis, vitamin E, lifestyle intervention, diet

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Estimated Duration of Study: 7 years

Start Date: 8/1/2012 **End Date:** 8/1/2019

Number and Type of Patients: Up to 90 patients with non-alcoholic fatty liver disease, aged 18 years or above, both males and females. No healthy volunteers.

Project Uses Ionizing Radiation: Research indicated, RSC approval 2346-B, expiring June 2021

Project does not use durable power of attorney, is not a multi-site study and does not use an off-site location

Précis

Non-alcoholic fatty liver disease (NAFLD) is the most common cause for liver test abnormalities in the western world, and an increasingly rising cause for liver-related morbidity and mortality. Vitamin E, a fat-soluble anti-oxidant was recently found to be an effective treatment for NAFLD; however, its mechanism of action is unclear. In a controlled clinical trial vitamin E treatment was shown to significantly reduce the hepatic fat burden, suggesting mechanisms other than reducing oxidative stress are involved. Furthermore, the optimal dose of vitamin E to treat NAFLD is unknown.

We propose a phase IIa study to determine the optimal dose of vitamin E and its mechanism and site of action. In this study we aim to enroll up to 90 patients with NAFLD. Initially, all patients will undergo 12 weeks of intensive lifestyle modification. Following that, all patients will be randomized to treatment with 3 different doses of natural vitamin E (rrr-α-tocopherol at 200, 400 or 800 IU/d) for 24 weeks. The primary end points for efficacy are normalization of liver enzymes and reduction in liver fat contents by magnetic resonance spectroscopy. Patients will undergo liver and adipose tissue biopsies before vitamin E treatment and after 4 weeks of therapy, and the biopsy samples will be used to measure changes in gene expression and markers of oxidative stress. This will be coupled with extensive phenotyping before and after treatment using serological, radiological and dynamic endocrine testing and is aimed at finding the dose-response characteristics of vitamin E in NAFLD, and allowing us to understand the mechanism of its action.

After 24 weeks of randomized treatment, all patients will be switched to a dose of 800 IU/ml and will continue treatment for up to 30 months, at the end of which another liver biopsy will be performed. From this phase we will assess the effects of dose increase of vitamin E on liver enzymes and fat content, and will determine the effect of long-term treatment on histological outcome.

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Introduction

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is the most common cause for liver enzyme abnormalities in the western world. This disorder, consisting of abnormal accumulation of triglycerides in the liver, is estimated to affect approximately 30% of the US population[1]. NAFLD commonly exists with features of the metabolic syndrome (obesity, hypertension, diabetes mellitus and hypertriglyceridemia) and its rising prevalence seems to accompany the increasing rates of obesity.

A subset of patients with NAFLD develop non-alcoholic steatohepatitis (NASH), where the fat accumulation is accompanied by hepatocellular injury, inflammation and fibrosis. NASH can be a progressive disease, leading to cirrhosis, liver failure and hepatocellular carcinoma. NASH and NAFLD are also associated with increased cardiovascular mortality, even in patients without other "classical" cardiovascular risk factors.

The pathogenesis underlying NAFLD and NASH is not clearly understood. Insulin resistance is commonly, but not universally, seen in these patients but it is unclear whether it is causing the fat accumulation or is being caused by it. Triglyceride storage in the liver may have a physiological role as a protective mechanism in the face of increased free fatty acid (FFA) flow from adipose tissue. When the ability of the hepatocytes to store these fatty acids as non-toxic triglycerides is overwhelmed, hepatic lipotoxicity ensues, while peripheral lipotoxicity worsens muscle insulin resistance and pancreatic beta-cell function[2]. The accumulation of free fatty acids in hepatocytes causes endoplasmic reticulum (ER) stress, an increase in reactive oxygen species (ROS) and apoptosis. This is accompanied by inflammatory cell infiltration, an increase in inflammatory cytokines[3], activation of macrophages and stellate cells and fibrosis.

Innate immune cells such as natural killer T (NKT) cells and natural killer (NK) cells are thought to contribute to the disease pathogenesis. NKT cells, for example, get activated by lipids and, in a TIM-1-mediated fashion, by apoptotic hepatocytes[4], produce cytokines that promote fibrosis and then undergo apoptosis themselves. CD1d-deficient mice that lack NKT cells are protected from fibrosis[5]. NK cells are activated by cytokines and by altered surface marker expression by hepatocytes, and are able to exert rapid effector functions such as cytokine production and cytotoxicity. Both NKT and NK cells constitute the major lymphocyte population in the liver.

There is a marked heterogeneity in the presentation and phenotype of patients with NAFLD. Racial and ethnic variability is significant; for example, Asian patients tend to have NAFLD at lower BMIs than their Caucasian counterparts, African-Americans seem to be relatively protected and Hispanics are at increased risk. Genetic factors clearly play a role, with most data pointing towards variants in the PNPLA3 (adiopnutrin) gene as strongly associated with disease occurrence and severity[6]. However, it is still unclear why some patients develop NASH and progressive disease, while others remain in the relatively benign steatotic stage. To date, no test apart from a liver biopsy accurately differentiates NASH from steatosis.

Currently, there is no standard approved therapy for NASH. Life style modification is routinely advocated, although only recently it was actually shown to benefit patients[7]. Diet, weight loss and physical exercise improve insulin sensitivity not only at the hepatic level, and thus have an important role in reducing the release of FFA from adipose tissue[8]. For patients who are not able to gain benefit from lifestyle changes, a logical choice is pharmacological intervention.

Vitamin E

Vitamin E is a fat-soluble antioxidant that is believed to be important in maintaining the integrity of lipid membranes and preventing oxidative injury to lipids, cholesterol and cell signaling molecules. Clinically apparent vitamin E deficiency is rare and is usually associated with severe malabsorption syndromes, advanced liver disease or inherited deficiencies in vitamin E transport proteins. In adults, vitamin E deficiency is associated with neuropathy and ataxia. In animals, vitamin E deficiency is associated with reproductive failure and with poor fetal neurological development.

The term "Vitamin E" is a collective name for several fat-soluble compounds; naturally occurring vitamin E exists in multiple chemical forms (α, β, γ, and δ-tocopherol and –tocotrienol), which have variable levels of antioxidant activity and biologic effects in different cell systems. In humans, however, only α-tocopherol appears to have significant biologic effects[9]. There are 3 chiral centers in the α-tocopherol molecule, yielding 8 possible racemic isomers; of these, RRR-α-tocopherol is the most active form and the one that is present in nature, while isomers with S chirality in the first chiral center are inactive and are not accumulated in tissues.

Vitamin E is present naturally in many foods and is absorbed in the intestine only in the presence of fats. The recommended daily intake for adults is 15 mg (22.4 IU) of d-alpha-tocopherol. Even this low amount is actually more than the average American consumes daily; in fact, it is estimated that about 90% of Americans are marginally deficient in vitamin E intake from dietary sources[10]. Multiple vitamin E supplements are available and an estimated 11% of the adult US population takes vitamin E supplement daily (which usually have 400 IU). Vitamin E is also present in many multivitamins, but usually in lower amounts (40-50 IU). Vitamin E supplements are manufactured from different sources and by different techniques. Synthetic supplements are the least expensive and contain and all-racemic mixture of all isomers, and thus have a potency of ~50% that of natural vitamin E, which contains mostly or purely RRR-α-tocopherol. Both formulations are available over the counter.

The absorption of vitamin E is dependent upon the presence of dietary fat. Thus, even if vitamin E supplements are taken, if not taken with a meal that has at least some fat, absorption will be minimal. Vitamin E from the intestines is taken up into chylomicrons, transported to the circulation via the thoracic duct and then rapidly and almost completely extracted from the post-prandial blood by the liver. In the liver, rrr-α-tocopherol is bound by the tocopherol transfer protein (TTP), which can deliver it into and across lipid membranes. The other forms of vitamin E (l-α-tocopherol and γ-tocopherol) for instance) are not bound to TTP and are metabolized by the liver and excreted in bile. Thus, the specificity of TTP for rrr-α-tocopherol is what determines that this form of vitamin E is the

biologically active form in humans. The hepatic TTP also allows for vitamin E to be secreted into the blood where it is taken up by HDL and LDL lipoprotein particles. These lipoprotein particles deliver vitamin E to other tissues. Inside of cells, vitamin E remains bound to lipids and can act to protect intracellular membranes from free radical damage probably by forming transient oxidative intermediates and delivering the free electrons to other anti-oxidants such as vitamin C[11]. The overall effects of vitamin E are multiple, perhaps because it protects membrane bound enzymatic and cell-signaling molecules non-specifically which are involved in a multitude of activities. Vitamin E also appears to have its major effect in the presence of environmental oxidative stress, so that cells are more able to resist injury when vitamin E replete.

The antioxidant activities of vitamin E and epidemiologic evidence linking vitamin E intake to several diseases led to extensive, large scale studies of vitamin E for prevention of cancer as well as cardiovascular and cerebrovascular disease. While controversial, overall these studies showed little or no effect of vitamin E supplementation on subsequent cancer or cardiovascular events. Indeed, perhaps the most reproducible effect seen was a slight increase in hemorrhagic stroke[12] that has been attributed to the anti-platelet effects of higher doses of vitamin E. For these reasons, vitamin E supplementation has become more controversial.

Vitamin E for NAFLD

The anti-oxidant effect of vitamin E can potentially ameliorate the lipotoxicity and ROS-induced damage in NASH and affect disease progression. Several treatment trials were conducted with inconclusive results seen in the smaller studies[13]. However, two recent studies have suggested that vitamin E may be beneficial in nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) and that a surrogate marker for its effect might be serum alanine aminotransferase (ALT) levels.

The PIVENS trial[14] was a double-blind, double-dummy placebo-controlled trial conducted at 8 U.S. medical centers in 286 adults with liver biopsy proven NASH who took either pioglitazone (30 mg), vitamin E (800 IU of rrr-α-tocopherol) or placebo daily for 2 years (96 weeks) with regular monitoring of serum enzymes and liver biopsy done before and at the end of treatment. Follow up also occurred 24 weeks after stopping the interventions. The primary endpoint was improvement in liver histology which was statistically significant comparing placebo to vitamin E with decreases in steatosis, inflammation and cell injury scores, but little change in fibrosis (Figure 1, Panel A). In addition, there were marked differences in ALT levels during treatment and this effect was seen within 6 months of starting therapy (Figure 2, Panel A). Overall, histological and biochemical response were achieved in 43% of patients treated with vitamin E in this study.

The TONIC trial[15] was a similar double-blind, double-dummy placebo-controlled trial conducted at the same 8 U.S. medical centers in 116 children or adolescents with biopsy-proven NAFLD who took either metformin (1000 mg), vitamin E (800 IU of rrr-α-tocopherol) or placebo daily for 2 years (96 weeks) with regular monitoring of serum enzymes and liver biopsy done before and at the end of treatment. The primary endpoint was improvement in ALT, even though liver histology was also obtained, largely because liver histologic changes had never been documented in children before this study. There

were statistically significant differences in ALT levels between vitamin E and placebo treated children at 6 months and one year, but the differences were no longer significant at 2 years largely because of improvements in the placebo group (Figure 2, panel B). Importantly, there were improvements in liver histology in steatosis, inflammation and cell injury, although they were significant only for cell injury (Figure 1, panel B).

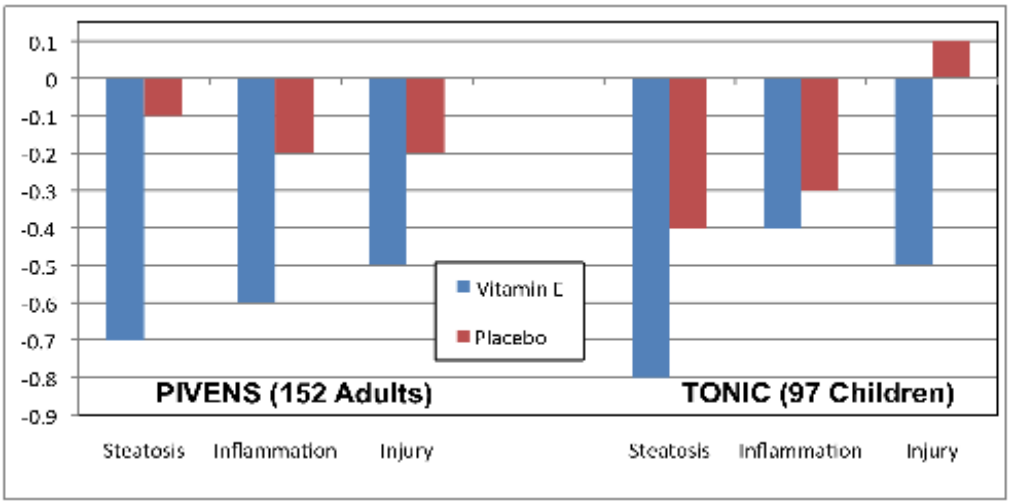

Figure 1 – Histological changes in vitamin E studies

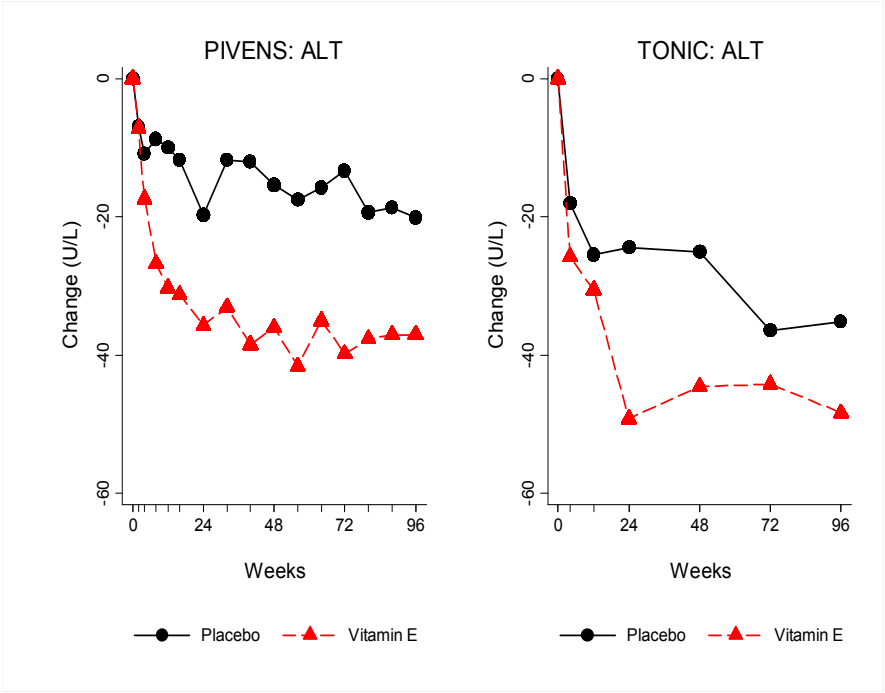

Figure 2 – Changes in serum ALT in vitamin E studies

While these two studies showed convincing evidence that vitamin E has an effect on NAFLD and NASH, they also raised many issues regarding optimal treatment and

mechanism of action. Vitamin E was anticipated to affect inflammation and injury through its anti-oxidant effect. However, there was also marked and significant decrease in liver fat, which was not associated with improvement in insulin sensitivity, through an unclear mechanism. Theoretically, vitamin E could affect liver fat by modulating the expression of lipogenic or lipolytic enzymes, by altering the delivery of free fatty acids from adipose tissue to the liver or by affecting the release of triglycerides from the liver. Vitamin E was shown to activate the nuclear receptor pregnane X receptor (PXR)[16], to induce the expression of an endogenous PPARγ agonist in adipocytes[17] and it was also suggested that vitamin E changes the expression of lipogenic enzymes in hepatocytes (Sanyal, A, unpublished results).

In addition, the clinical studies of vitamin E in NAFLD did not address the important problems of vitamin E dosage. The preparation used was "natural" vitamin E (rrr-α-tocopherol) and was >40 times the recommended daily allowance for vitamin E. This dose was higher than those used in almost all previous studies of vitamin E such as for cancer and heart disease prevention that typically used 100-400 IU of racemic, dl-α-tocopherol, equivalent to 10% to 25% of the dose used in PIVENS. The dose of vitamin E is particularly an issue in patients who might be at risk of hemorrhagic stroke such as those on anti-platelet medications or with hypertension and diabetes, features that are commonly found in patients with NASH and NAFLD.

Finally, the studies did not identify markers of anti-oxidant status that might be correlated with effects of vitamin E on fatty liver disease. In preliminary studies of vitamin E levels and of serum markers of anti-oxidant status (including proteomics studies), no marker was identified that was different between vitamin E and placebo treated subjects.

We propose to conduct a phase IIa study of natural vitamin E in patients with NAFLD, to determine the optimal dose of vitamin E, its mechanism of action, and to help understand the pathophysiology of the disease.

Aims

Primary Aims:

1. Primary clinical aim: Defining the optimal dose of vitamin E to treat NASH
2. Primary scientific aim: Determining the mechanism leading to fat loss with vitamin E treatment.

Secondary aims:

1. Will patients who do not respond to low-dose vitamin E benefit from a higher dose?
2. Is the vitamin E-induced fat loss due to reduction in hepatic de-novo lipogenesis, decrease in free fatty acid supply to the liver, increase in β-oxidation or increase in VLDL secretion?
3. Is the vitamin E action mediated through its anti-oxidant effect or through direct effect on gene induction?
4. Is there a dose response curve for the anti-platelet effect of vitamin E?

5. Liver fat has been suggested as a cause of peripheral and hepatic insulin resistance. Since vitamin E is not thought to affect insulin sensitivity directly, does the reduction in fat caused by vitamin E drive a change in liver and peripheral insulin sensitivity?
6. What is the role of liver-resident innate immune cells, especially NKT and NK cells, in NASH, and how are these affected by treatment with vitamin E?
7. What effect does genetic variability have on hepatic and adipose tissue physiology in NAFLD, and on the response to treatment?

Study design and methods

Overall design (Figure 3)

A prospective randomized trial. Eligible patients will be screened and enrolled. After enrollment, patients will undergo an initial period of 12 weeks of lifestyle modification. At the conclusion of that phase, the patients will be admitted to the clinical center for an extensive in-patient evaluation and a liver and adipose tissue baseline biopsy. Patients will then be randomized to receive vitamin E 200 IU/d, 400 IU/d or 800 IU/d for 24 weeks. 4 weeks after starting vitamin E, the patients will be admitted again, to undergo a second liver and adipose tissue biopsy. At the end of the 24 week period, all patients will undergo an extensive in-patient evaluation identical to the baseline admission (but without the biopsies) and will then be offered the option to switch to treatment with open-label vitamin E 800 IU/d for up to 120 weeks more.

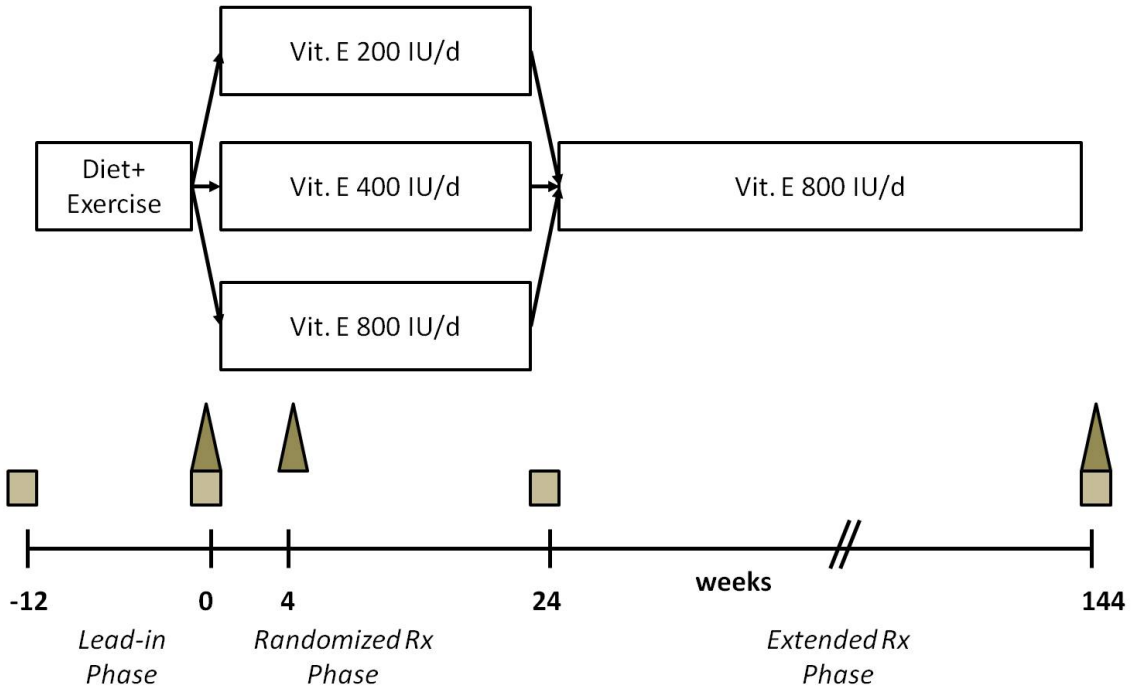

Figure 3 – Overall study design. Extended studies highlighted in squares, liver and adipose tissue biopsies in triangles.

Initial screening and enrollment

Patients will be screened during one or more visits in the Outpatient Clinic with history, physical examination, review of outside medical records, routine blood tests and bleeding time. Alcohol history will be assessed in the medical interview and will be quantified using the AUDIT questionnaire, which has been validated as a screening tool for excessive alcohol consumption[18]. We will observe the total score (reflecting the risk for alcohol-related problems) as well as quantify alcohol intake (using the first 3 items on the questionnaire). The initial screening visit to assess eligibility for the protocol will be done under protocol 91-DK-0214, "Evaluation of Patients with Liver Disease"; only eligible patients will then be enrolled into this protocol. All patients will be asked to stop any medication being used for their liver disease, including herbal medications and vitamins. Patients meeting the eligibility criteria will be enrolled and consented. Enrolled patients will be asked to complete baseline dietary and exercise questionnaires and will be given food and exercise logs as well as a pedometer. Following enrollment, patients will undergo a magnetic resonance spectroscopy to quantitatively measure liver fat content (see below), to be performed at or before the day of their first appointment with the nutritionist.

Run-in phase and lifestyle intervention

One to four weeks after enrollment, patients will meet with a Clinical Center nutritionist for an individual counseling session. All patients will be instructed on a balanced diet, including decreased intake of saturated and trans fats and increased intake of fiber. In overweight patients (BMI >= 25 kg/m2), the dietary intervention will focus on a calorie-restricted diet, aiming for a weight reduction of 5-8% from their baseline weight, preferably attained over a 6-month period. The calorie-restricted diets will be planned to create a deficit of 500-700 kcal/day and will be individualized for each study subject. Patients will be encouraged to achieve a regimen of moderate-intensity physical activity, with gradual increases in intensity or duration, aiming at eventually exercising for at least 150 minutes per week. Patients will be instructed to stop taking any vitamins or supplements and a standard dose multi-vitamin will be prescribed for the duration of the study.

After enrollment, the patients will follow every two weeks with the nutritionist. On the first follow-up visit (2 weeks after enrollment), there will be an individual session with the nutritionist during which progress, including keeping of food logs, will be reviewed and diet plans will be adjusted and reinforced as needed. On this visit and on subsequent visits throughout the run-in phase, patients will attend 30-45 minute educational/support group sessions. Sample topics to be covered during these group sessions will include heart healthy eating guidelines, food-label reading, appropriate portion sizes and healthy strategies for eating out.

Baseline dietary intake will be assessed using the NCI Diet History Questionnaire II (DHQ II) utilizing the past month, with portion size version. Patients will be advised to complete the online version of the questionnaire at home, the day before coming to clinic, or on a computer in the clinic waiting room. Patients who do not have access to the internet, or who are not comfortable with using the internet version will fill out the paper version of the questionnaire in the clinic. Before enrollment, and again at the end of the 12 week run-in phase, patients will be directed to complete a 3-day food record which will serve as

the primary measure of change in food intake. Food records will be reviewed for completeness and accuracy and will be analyzed using Nutrition Data System for Research (NDS-R) software. Patients will also be instructed to track their intake in a daily food log, mainly for self-monitoring purposes.

On enrollment, patients will be provided with pedometers and will be instructed to record their daily step-count in a diary. Physical activity levels will also be monitored using the International Physical Activity Questionnaire (IPAQ), which will be completed on enrollment and again at the end of the 12 week lifestyle modification program. Patients will be encouraged to achieve a regimen of moderate-intensity physical activity, with gradual increases in intensity or duration, aiming at eventually exercising at least 150 minutes per week.

During the randomized vitamin E treatment phase, patients will be continue to be seen by the nutritionist every 8 weeks for individual follow-up visits and to reinforce components covered during the initial 12 week lifestyle intervention. During the extended treatment phase, the patients will be seen by the nutritionist every 12 weeks. Patients will complete the 3-day food intake diary and IPAQ every 24 weeks during the randomized treatment and extended treatment phases. The data obtained from these diaries and questionnaires will be used to ensure that differences in outcomes observed between the different vitamin E groups are not due to differences in adherence to lifestyle changes. Although part of the effect of treatment may be due to lifestyle intervention, it should affect all treatment groups in a similar manner and the effect of vitamin E will be deduced from the dose response.

Detailed in-patient assessment

At the beginning of the randomized-dose treatment phase, patients will be admitted to the Clinical center for a detailed histological, physiological and biochemical analysis, including:

1. History and physical examination: including vital signs, weight and anthropomorphic measurements.
2. Fasting blood tests: chem-20, GGT, CBC, PT, PTT, serum insulin, c-peptide, lipid panel, apolipoproteins, total free fatty acids, hemoglobin A1c, alpha-fetoprotein, ferritin, iron, transferrin saturation, immunoglobulins, TSH, C-reactive protein, PSA and vitamin E levels
3. Research blood: 10 ml of serum will be separated from collected blood and stored in 2 separate aliquots in -80°C for determining serum levels of hormones, cytokines and adipokines, injury markers (such as CK-18 fragments), and for archival storage. 10 ml of plasma will be separated from collected blood and stored at -80ºC for analysis of oxidative stress measures.
4. NKT and NK cell analysis: 40 ml of blood will be drawn for analysis of NKT and NK cell phenotype and effector function. Additional 8 ml of serum tube (8 ml) will be used to provide autologous serum for in vitro functional assays. The frequency of performing these assays is detailed in table 1.
5. Urine tests: urinalysis, pregnancy test (for women of child-bearing potential) and 24-hour urine collection for creatinine clearance and protein and uric acid

excretion. A 5 ml urine sample will be stored at -80ºC for oxidative stress measurements.

6. Imaging:
    a. Magnetic resonance imaging (MRI):
        i. MR spectroscopy to quantitatively measure liver fat content.
        ii. Liver volume estimation
        iii. Adipose tissue distribution: measurement of total abdominal adipose tissue, visceral adipose tissue and subcutaneous adipose tissue volumes.
    b. Total body mass, total body fat mass and bone mineral density will be determined using dual energy x-ray absorptiometry (DEXA).
7. Insulin sensitivity:
    a. Insulin-modified frequently sampled intravenous glucose tolerance test (FSIVGTT)[19] – after a 12-14 hour fast, intravenous access will be achieved in both arms. After 15 minutes of comfortable rest, blood samples will be drawn at, 10 and 1 minute before glucose injection. At time 0, 300mg/kg of glucose, in the form of dextrose 50% in water, will be injected over 1-2 minutes. Blood samples will be drawn at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16 and 20 minutes. At 20 minutes, IV insulin will be given at a dose of 4 mU/min*kg for 5 minutes. Blood sampling will continue at 22, 23, 24, 25, 26, 27, 28, 30, 40, 50, 60, 70, 80, 100, 120, 150 and 180 minutes. All samples will be analyzed for plasma glucose, insulin and free fatty acid concentrations[20]. Glucose effectiveness and insulin sensitivity will be calculated according to the minimal model using the MINMOD software.
    b. Hepatic insulin sensitivity will be estimated using the HOMA-IR2 model.
    c. Adipose tissue insulin sensitivity will be estimated at the basal state by the HOMA-FFA model.
8. Liver biopsy: a percutaneous liver biopsy will be performed under local anesthesia, with the patient in a fasting state. Tissue will be used for histological evaluation, RNA analysis, immunological studies, measurement of oxidative stress and archival storage.
9. Subcutaneous adipose tissue biopsy: subcutaneous adipose tissue will be removed from the abdominal region by needle aspiration under local anesthesia. The sample will be submitted for histological evaluation, RNA analysis and an additional fraction will be used to for ex vivo studies (see below).
10. Resting energy expenditure (REE) measurement will be performed at the patient bedside.
11. Studies under other protocols. Participation in the following protocols will be offered to all patients in the study if eligible. Refusal to enroll in these additional studies will not preclude patients from participating in this protocol.

a. Genetic analysis: peripheral blood mononuclear cells will be extracted from 20 ml of blood and stored for DNA extraction and genotyping (one time only). This will be done under a separate protocol and consent (91-DK-0214).

b. Cardiovascular imaging: imaging by CT scan and MRI of the heart. This will be done under a separate protocol and consent (11-DK-0168).

c. Fatty acid oxidation will be assessed by measurement of ^{13}C-palmitate oxidation with a breath test device to detect expired $^{13}CO_2$. This will be performed under a separate future protocol and consent.

12. Fetal hemoglobin (HbF) levels may be measured in some of the patients at baseline and during treatment, to assess the effects of vitamin E on HbF induction.

Vitamin E Treatment

Patients will be evaluated again in the outpatient clinic 1-2 weeks after the admission and will be given the biopsy results. Patients whose liver biopsy did not show evidence of NAFLD will be excluded at this visit and will end the study. All other patients will be randomized to receive 200 IU/d, 400 IU/d or 800 IU/d of vitamin E and will receive the medication for a planned duration of 24 weeks.

The vitamin E formulation will be "natural" RRR-α-tocopherol, provided by PharmaVite Inc. (Northridge, CA) under a clinical trial agreement. The medication will be supplied in 200 IU and 400 IU softgel capsules on an annual basis and will be stored and dispensed by the NIH Clinical Center Pharmaceutical Development Section. As described earlier, RRR-α-tocopherol is thought to be the predominantly biologically active metabolite in synthetic vitamin E formulations (that contain a mixture of racemic enantiomeres), due to the specificity of affinity to hepatic TTP. There is no data suggesting difference in toxicity of different vitamin E formulations that is separate from differences in efficacy. Depending on their group allocation, patients will take one 200 IU capsule, one 400 IU capsule or two 400 IU capsules, once daily.

Randomization will be done by the Clinical Center pharmacy Protocol Support Service and will be stratified by the result of the liver biopsy (classified as definite steatohepatitis, steatosis without steatohepatitis, or borderline[21, 22]) and by the presence or absence of diabetes mellitus type 2 at enrollment. Patients will be monitored on weeks 1, 2, 3, 4, 8, 12, 16, and 20 of treatment. On each visit, fasting blood tests for Chem-20, insulin, C-peptide, free fatty acids and vitamin E levels will be drawn, vital signs including weight will be measured and a symptom questionnaire will be administered. CBC, lipid panel, apolipoproteins and urinalysis will be drawn every 4 weeks. A nurse will determine weight, vital signs and symptoms on every visit. A physician will evaluate the patients every 4 weeks. A serum sample for research purposes will be drawn on each visit and stored in -80ºC.

On week 4 of treatment, patients will be admitted for a second liver biopsy and adipose tissue biopsy, and will have a repeat of the MR spectroscopy and FSIVGTT.

On week 24 of treatment, all patients will be admitted for an exit work-up, identical to the detailed in-patient assessment described above, but without the biopsies.

During the treatment, patients will be recording medication intake on a diary that will be collected every visit and will be used to assess compliance.

Extended Treatment Phase

On discharge, all patients will be offered to continue treatment with open-label vitamin E 800 IU/d (two 400 IU capsules taken once daily) for up to 120 more weeks, after which, an exit liver biopsy will be performed. Enrollment into the extended treatment phase is optional and refusal will not exclude the patient from the study. The medication intake diary will be used in this phase as well.

After 24 weeks in the extended phase (week 48 of the study), patients will undergo an additional MR spectroscopy. Based on the results of the MRS and liver enzymes, the response will be assessed prior to deciding on treatment continuation. For this purpose, response is defined as a decline of >=25% in ALT or normal ALT or normal AST or an absolute decline of more than 2% in liver fat content by MRS. The response criteria are selected to be sensitive, but not necessarily specific, aiming to predominantly identify patients who are not responding to treatment and in which continuation is futile. Based on the response assessment there are three possible outcomes (Figure 4):

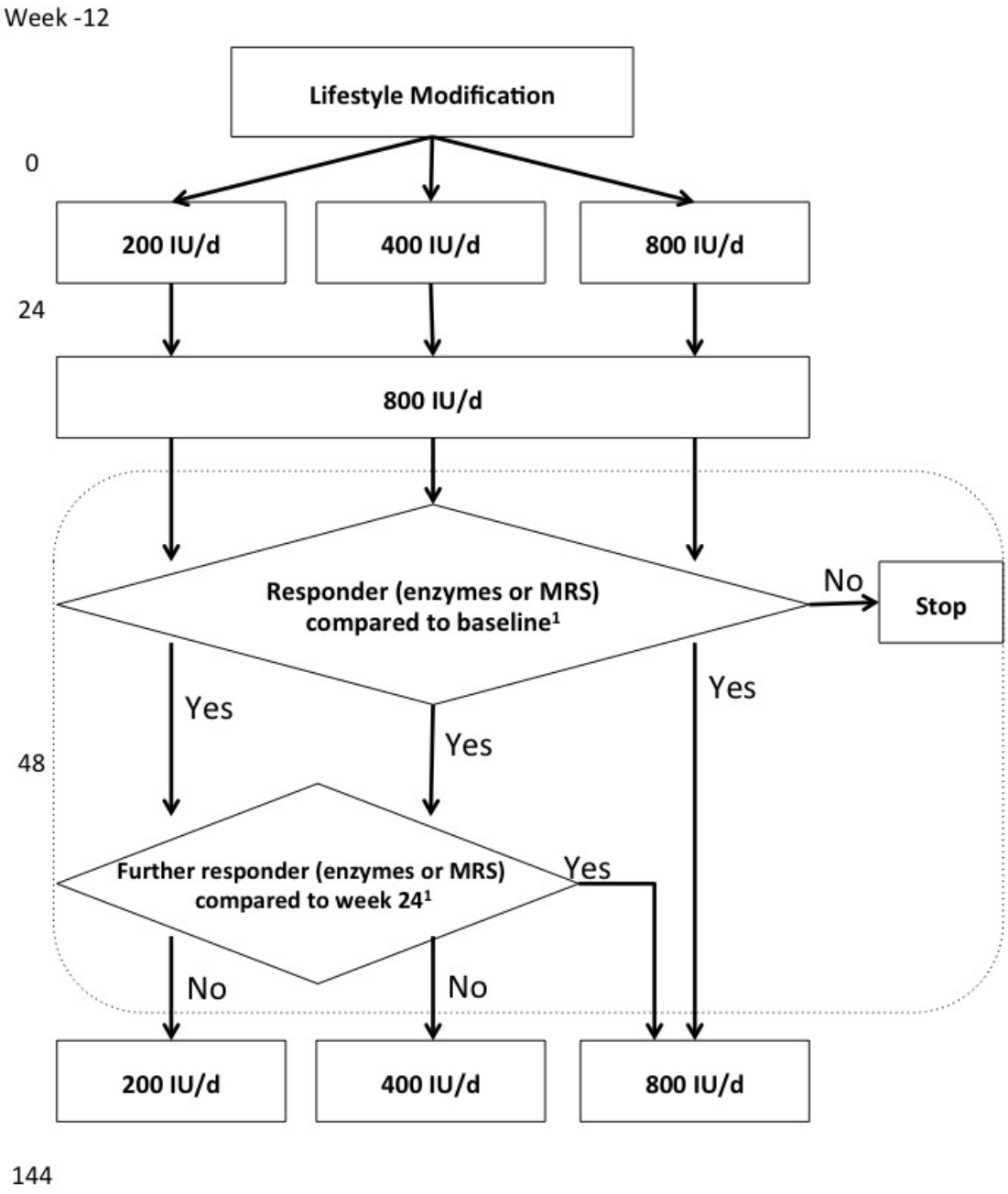

Figure 4 – Stopping rule based on week 48 results. [1]Response defined as ≥ 25% decrease in ALT, or normal ALT or AST, or absolute decrease of >2% in liver fat by MRS.

1. Patients who did not meet any of the response criteria compared to baseline will discontinue treatment for futility.
2. Patients treated with 200 or 400 IU/d during the first 24 weeks, will be reverted after 48 weeks to their original dose if they had a response at week 48 compared to baseline, but no incremental response when switching to 800 IU/d at week 24 (i.e., did not meet any of the response criteria comparing week 48 to week 24).

3. Patients who were treated initially by 800 IU/d and had a response at week 48 or patients who were treated with a lower dose initially and had an improvement when switching at week 24 to the higher dose, will be offered to continue treatment with the 800 IU/d dose.

Details of procedures

Adipose Tissue Biopsy

Subcutaneous adipose tissue (1-10 g) will be removed from the abdominal region by aspiration with a 2.5 or 3 mm blunt Spirotri cannula under local anesthesia (1% lidocaine). The tissue obtained will be used to obtain isolated adipocytes and organ culture using standard procedures[23], as well as isolation of preadipocyte cell lines that will be expanded and stored in liquid nitrogen. The remaining tissue will be separated into aliquots of approximately 500 mg for RNA isolation and protein extraction, flash frozen in liquid nitrogen, and stored at -80 ºC. The relatively large sample of fat from each subject will ensure extraction of sufficient RNA analysis.

Liver Biopsy

A percutaneous liver biopsy will be performed under conscious sedation and local anesthesia using standard technique with the Klatzkin biopsy needle or a spring-loaded cutting needle ("biopsy gun"). A bedside ultrasound examination will be used to confirm an optimal biopsy site before the procedure. Following the biopsy the patients will be monitored overnight for recovery from sedation and for the development of complications.

Accurate interpretation of liver histology is significantly impacted by the size of the histological specimen[24, 25]. Because the initial study biopsy will be used for staging as well, it is critical that adequate tissue be submitted for histological evaluation. A specimen of at least 2 cm length will be reserved for histological evaluation; the remainder (0.5-1 cm) will be split to 2 or 3 aliquots, one to be stored in RNALater (Qiagen, Valencia, CA) or a similar preservative and used in later analyses, and one flash-frozen in liquid nitrogen at bedside for archiving and for measurement of oxidative stress. If a sufficient specimen was obtained allowing for a third aliquot, 3-5 mm of tissue will be transported in RPMI medium for extraction of liver-resident lymphocytes and immunological studies.

If inadequate tissue is obtained for clinical purposes, a second or third pass may be attempted, as is in keeping with clinical practice. The risk of complication from liver biopsy increases only after 3 biopsy passes are made. If after a single pass inadequate tissue is available for the research specimen (0.5 cm), one additional pass will be performed. If two passes have already been made in order to obtain adequate clinical material, a third pass will not be permitted to obtain a research sample. No more than three passes will be made for clinical purposes, in keeping with current practice guidelines. The potential need for one additional pass is discussed in the consent form.

Histological evaluation will be performed by an expert liver pathologist in a qualitative manner and the presence or absence of NASH will be determined. Biopsies will be classified as showing definite steatohepatitis, borderline or not steatohepatitis but with steatosis[21, 22]. The biopsy sample will be scored semi-quantitatively using the scoring

system utilized by the NASH Clinical Research Network trials[26]. The NAFLD activity score (NAS) will be calculated as the arithmetic sum of the scores for steatosis, ballooning injury and lobular inflammation.

Magnetic Resonance Spectroscopy

Experiments will be performed on an MRI scanner using TIM phased-array coils. B_0 shim parameters are optimized with a breath hold B_0 mapping method[27]. Navigator gated T_2 weighted (T2w) and breath-hold T_1 weighted (T1w) images in coronal and axial orientations will be used to prescribe a 20x20x20 mm^3 spectroscopy volume in an area with high signal to noise ratio, free of obvious fatty structures or blood vessels. After additional manual shimming, and optimization of transmit power and WS RF level 32 signals will be averaged with a minimum repetition time (TR) of 3s and 35 ms echo time (TE) with WET water suppression (WS) followed by 4 signals averaged with WS RF power set to zero. Spectral data will be fitted with AMARES[28] using a model of four resonances for the lipid signals on the WS spectra. Additionally, the apparent T_2's of the summed lipid CH_2 and CH_3 resonances lipid and the T_2 of water will be measured with five quick expiration breath hold non-WS spectra averaging two signals each at TR 3s and TE 24, 36, 48, 96 and 144 ms. These T_2 values will be used to correct the water and total lipid signals for signal decay in the 35 ms echo delay. Fat fraction will be then calculated as from the T2 corrected signals S_{water} and S_{lipid} as: fat fraction = $S_{lipid}/(S_{water}+S_{lipid})$.

Justification for study design

Run-in phase

To date, most trials in NAFLD enrolled patients based on the results of a pre-enrollment liver biopsy, which was not necessarily performed immediately prior to enrollment. However, in clinical practice a decrease in liver enzymes is often seen in the first few months after a patient is given a diagnosis of NAFLD, probably reflecting a serious attempt at lifestyle modification. Similar effects were noted in placebo-treated patients[7, 29]. This is especially important in studies with a histological end-point, in which the true baseline status may thus be different than the one suggested by a "distant" enrollment biopsy. Furthermore, it may not be justified to suggest pharmacological interventions to patients who are controlling their disease adequately with dietary modification and physical activity.

In this study, patients can be enrolled based on non-invasive studies that are suggestive of the presence of NAFLD but not definitive. All patients will undergo a standardized lifestyle intervention period with a biopsy immediately preceding pharmacological therapy. This will ensure that the biopsy will serve as a true baseline. Furthermore, this design allows us to evaluate physiological changes induced by lifestyle modification alone.

Liver biopsies

In this study, patients will undergo 2-3 liver biopsies. The first biopsy is done for clinical and research purposes. Clinically, the biopsy is indicated as the gold-standard for diagnosing fatty liver disease and to date, the only reliable method to distinguish NASH

from simple steatosis. Thus, the first biopsy will serve clinically to confirm the diagnosis of NAFLD and to assess severity of disease. This biopsy is also crucial for research purposes, as it will provide a baseline for the comparison to the on-treatment and exit biopsies.

A few patients may have had a liver biopsy done before enrollment into the protocol, either by a referring physician or at the LDB (for example in a previous protocol). However, a previous biopsy can not serve as a true baseline clinically (because of possible changes in disease behavior or management over time), nor will it provide the tissue for research this protocol requires. For that reason, even patients with a previous biopsy will have to agree to undergo an entry biopsy in order to enroll. If a patient had a previous liver biopsy, the baseline biopsy may not provide a direct clinical benefit. In such a case, the fact that the procedure is purely for research purposes will be clearly explained to the patient during enrollment and consent and will be documented in the patient's chart.

Patients will be informed of the findings in the entry biopsy before being randomized. If the biopsy does not show any evidence of NAFLD, the patients will be withdrawn from the study. We plan to treat all patients with NAFLD in the study, whether they have NASH or steatosis only. However, the severity (or lack of) of liver disease in the biopsy will be conveyed to the patients in laymen terms and patients will be given the option to withdraw themselves from the treatment phase if they wish so.

The second biopsy will be performed 4 weeks after starting vitamin E. The goal of this procedure is to obtain tissue to observe the effect of vitamin E on hepatic and adipose tissue gene expression. The histological response to pharmacological agents is slow and we do not anticipate a marked decrease in liver fat during these 4 weeks. This biopsy will allow us to detect an effect of vitamin E on gene expression and fatty acid metabolism without being confounded by the effects on the transcriptome of the decrease in liver fat. This biopsy is a purely research procedure and is not anticipated to provide clinical benefit; this will be clarified to the patients on enrollment, and again before the procedure.

A third liver biopsy will be performed in patients who agree to continue with extended vitamin E therapy, after 3 years of treatment. This biopsy will allow determination of histological response, especially whether the beneficial effects of vitamin E extend to halting progression of fibrosis. Although repeating liver biopsies in patients with NAFLD is not standard clinical practice, this biopsy is anticipated to provide important clinical information regarding the individual patient response and restaging, as well as research data.

At the liver diseases branch, we have acquired considerable experience in the performance of liver biopsies for clinical and research indications. Several LDB past and present studies, as well as studies in other institutions in viral hepatitis and in fatty liver diseases employed more than one biopsy, with good patient acceptance, safety track and important data acquired.

Absence of a control arm

A placebo-controlled arm could be considered in this study. However, by including a fourth arm we would markedly reduce our sample size in each arm and the statistical power to detect differences. Furthermore, we do not think it would be justified to perform a second

liver biopsy on patients treated with placebo. The lead-in phase should eliminate most of the placebo effect, and the use of paired biopsies for the analysis will allow us to control for inter-individual changes.

Duration of treatment

There is no clear accepted duration of treatment for NASH and similar to other metabolic conditions like diabetes or hypertension, treatment is probably required for life. The kinetics of biochemical response in the previous vitamin E trials (figure 2) show near-plateauing at week 24, and suggest little incremental benefit will be achieved by extending treatment beyond that.

Inclusion and exclusion criteria

Inclusion

1. Clinical suspicion of NAFLD, defined by the presence of at least two of the following criteria:
    a. Suggestion of liver fat by an imaging study (ultrasound, CT scan, MRI or MR spectroscopy) performed in the 6 months prior to enrollment.
    b. Elevated aminotransferase levels (ALT > 31 U/L for men or > 19 U/L for women, or AST > 30 U/L) on at least two occasions in the 6 months preceding enrollment.
    c. Presence of the metabolic syndrome, defined according to the modified AHA/NCEP criteria[30] as the presence of at least three of:
        i. Abdominal obesity, defined as waist circumference > 102 cm for men or > 88 cm for women
        ii. Elevated triglycerides (> 150 mg/dL) or the use of medication to lower triglycerides
        iii. Reduced HDL cholesterol (< 40 mg/DL for men or < 50 mg/dL for women)
        iv. Elevated blood pressure (> 135/80 mmHg) or use of medication for hypertension
        v. Elevated fasting glucose levels (> 100 mg/dL) or use of anti-diabetic medication

    For the purpose of inclusion, the presence of overt diabetes mellitus type 2 will be considered equivalent to the presence of the metabolic syndrome, even if the other criteria are absent.
2. Estimated average alcohol consumption < 30 g/d for men or < 20 g/d for women in the 6 months prior to enrollment and no binge-drinking behavior.
3. Age > 18 years at enrollment
4. Willingness to participate in the study

Exclusion criteria

1. Chronic infection with hepatitis C virus (HCV) or hepatitis B virus (HBV). Patients who were treated successfully for HCV and achieved sustained virological response can be eligible for enrollment > 18 months after treatment cessation. Patients who are inactive carriers of HBV (HBV DNA < 1000 copies/mL, HBeAg negative, Anti HDV negative) for at least 12 months prior to enrollment are also eligible. Patients receiving antiviral therapy are ineligible.
2. Concomitant liver disease such as autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, Wilson's disease, alpha-1 antitrypsin deficiency.
3. Presence of definite or probable drug-induced liver injury. In the case of lipid-lowering, anti-hypertensive or anti-diabetic medications that are suspected to cause elevation of aminotransferases, patients will be eligible if treatment is associated with stable enzyme levels for at least 6 months and inclusion criteria 1a. and 1c. are both present.
4. Treatment with medications known to cause fatty liver disease such as atypical neuroleptics, tetracycline, methotrexate or tamoxifen
5. Uncontrolled hypo- or hyperthyroidism.
6. Decompensated advanced liver disease, defined as direct bilirubin > 0.5 g/dL, PT > 18", albumin < 3 g/dL, or history of ascites, encephalopathy, variceal bleeding, spontaneous bacterial peritonitis or liver transplant.
7. Active coronary artery disease, defined as persistent angina pectoris, reversible ischemia on cardiac stress test or imaging, or the presence of significant coronary artery disease on imaging or catheterization. Patients with coronary artery disease that was treated by angioplasty or bypass surgery may be eligible if they have no evidence of active disease >= 1 year after intervention, can safely stop antiplatelet and anticoagulant medications before the performance of invasive procedures, and have adequate ventricular function as assessed by echocardiography or cardiology consultation. These patients will require cardiology consultation and clearance prior to enrollment.
8. Congestive heart failure.
9. Chronic kidney disease, with creatinine clearance < 60 ml/min or eGFR < 60/ml/min/sq. m.
10. Uncontrolled diabetes mellitus. Patients may be enrolled if they have been on stable therapy with any anti-diabetic agent for at least 3 months prior to enrollment, are not foreseen to require antidiabetic medication or dose changes during the trial, and have an HbA1c <= 7.5% on enrollment.
11. Treatment with vitamin E. Patients who are currently taking vitamin E as a supplement will be requested to stop for at least 3 months before becoming eligible for enrollment. Patients who are taking vitamin E for a medical indication other than NAFLD will not be eligible.
12. Contraindication or inability to perform a liver biopsy.

13. Patients who had a liver biopsy performed <= 2 years before enrollment, unless they are willing to undergo all of the trial biopsies, knowing that these biopsies are purely for research and are not clinically indicated. This will be clearly documented in the patients' charts prior to enrollment.
14. Patients with coagulopathy (PT/PTT values that are prolonged >= 3 seconds from the upper limit of the normal, including treatment with oral and parenteral anticoagulants), thrombocytopenia (<70,000), abnormal bleeding time or platelet dysfunction will not be enrolled because of potential increase in risk of bleeding with vitamin E treatment. Antiplatelet agents taken for cardiovascular prevention will not exclude patients, unless they cannot be stopped safely for the performance of a liver biopsy.
15. Maldigestion or malabsorption that can interfere with absorption of vitamin E including: steatorrhea of all causes, chronic pancreatitis, cystic fibrosis, short bowel syndrome, severe cholestasis, orlistat treatment and similar conditions
16. Inability to swallow vitamin E capsules
17. Allergy to vitamin E
18. Alcohol or substance abuse within the past 12 months. Patients will be required to have an AUDIT score of 7 or less[18], and drink no more than 14 drinks/week (for men) or 7 drinks/week (for women).
19. For women of childbearing age, pregnancy or inability (or unwillingness) to practice contraception for the duration of the study or breast feeding.
20. Hemoglobin level < 11 g/dL
21. History of past or present significant gastrointestinal bleeding, unless treated definitively (i.e. resection, eradication of H. pylori with documentation of ulcer healing, etc).
22. Known history of past or present condition that according poses a significant risk for significant gastrointestinal bleeding (i.e. peptic ulcer) unless definitively treated.
23. Inability to understand and give informed consent for participation

Monitoring subjects and criteria for withdrawal of subject from the study

Patients will be monitored every 2 weeks during the run-in phase. At this stage, the only intervention is lifestyle modification and no related serious adverse events are anticipated. At this stage, visits that are within 1 week before or after the planned visit date will not be considered a protocol violation.

After the run-in phase, patients will be admitted for up to 5 days, for the initial biopsies and extended tests as detailed above. At the LDB, it is our standard practice to keep all patients for observation overnight after a biopsy, and to perform a routine complete blood count 6 hours after the procedure. Following discharge, patients will be seen in the

outpatient clinic for the first 3 weeks of treatment, and will be subsequently admitted during the 4th week of treatment, for the second biopsy. At this stage, visits that are within 2 days before or after the scheduled visit date will not be considered a protocol violation. Following the second admission, the patients will be followed in clinic every 4 weeks. From this time point onwards, visits that are within 1 week before or after the planned visit date will not be considered a protocol violation. Patients will be admitted again at the end of the 24 week period for another extensive evaluation. During the extended treatment phase, patients will be seen every 12 weeks.

Patients will also be monitored as appropriate for patients with liver disease. Specifically, patients with liver cirrhosis or high-risk for hepatocellular carcinoma (HCC) will undergo surveillance for HCC by ultrasound or cross sectional imaging every 6-12 months, or according to most current guidelines. Patients with cirrhosis will be monitored by endoscopy as appropriate for the development of esophageal varices.

Patients who become pregnant during the study will be withdrawn, as pregnancy and associated hormonal and metabolic changes may affect hepatic fat metabolism and will confound the analysis. Patients who do not respond to treatment by week 48 will be withdrawn for futility. There are no other pre-specified criteria for withdrawal of patients from the study. Patients will be withdrawn if, according to the best judgment of the treating physician or the principal investigator, continuing treatment poses an unacceptable risk to the patient.

Patients with hemoglobin < 10 g/dL will not undergo the FSIVGTT test and/or collection of blood for NK/NKT cells. Patients with hemoglobin < 10 g/dL will not undergo the first (baseline) liver biopsy. That biopsy may be deferred for up to 4 weeks if the anemia can be treated sufficiently by that time. Patients who cannot undergo the baseline liver biopsy will be withdrawn from the study. Patients with hemoglobin < 10 g/dL will not undergo the second or third liver biopsy; these biopsies will not be deferred but the patients may continue on the study.

Table 1 – Monitoring and investigation schedule

Phase	Visit week	Vit E dose	Nurse[1]	MD	Nutritionist[2]	Routine labs[3]	Extended labs[4]	NK/NKT cell studies[5]	Liver & Adipose Tissue Biopsy	FSIVGTT[6]	Imaging	Breath Test	Questionaires
Enrollment	(-4) – (-1)		+	+		+							DHQ II, IPAQ, 3-day food
Run-in	0		+		++	+	+	+			MRS, MRI		Symptom, quality of life
	2				++, +								
	4		+		+	+							
	6				+								
	8		+		+	+							
	10				+								
Admission	12		+	+	++	+	+	+	+	+	MRS, MRI, DEXA	+	3-day food, IPAQ, symptom, quality of life
Randomized Rx	0 (1-2 wk later)	200/400/800	+	+		+							
	1	200/400/800	+			+							
	2	200/400/800	+			+							
	3	200/400/800	+			+							
2nd Admission	4	200/400/800	+			+	+	+	+	+	MRS		
	8	200/400/800	+	+	++	+							
	12	200/400/800	+			+	+						symptom
	16	200/400/800	+	+	++	+							
	20	200/400/800	+			+							
3rd Admission	24	800	+	+	++	+	+	+		+	MRS, MRI, DEXA	+	3-day food, IPAQ, symptom, quality of life
Extended Rx	36	800	+	+	++	+							
	48	800 (or lower[7])	+	+	++	+	+				MRS		3-day food, IPAQ, symptom, quality of life
	60-132 (q12 wks)	800 (or lower[7])	+	+	++	+	+ (q24 wks)						3-day food, IPAQ, symptom, quality of life (q 24 wks)

Exit Admission	144	Stop	+	+	++	+	+	+[5]	+	+	MRS, MRI, DEXA,	+	3-day food, IPAQ, symptom, quality of life

[1] Nursing visit – includes vital signs, weight, list of current study and non-study medications and nursing assessment

[2] Nutrition visit - + marks group session, ++ marks individual session

[3] Routine labs – complete blood count, Chem-20, serum insulin, c-peptide, free fatty acids, lipid panel, vitamin E level, research blood (total volume 31.5 ml)

[4] Extended labs – GGT, PT, PTT, HbA1c, CRP, Thyroid panel, apolipoproteins, ferritin, iron, transferrin saturation, immunoglobulins, PSA, research plasma (total volume 52.5 ml with Routine labs), urinalysis

[5] NK/NKT cell analyses – On Run-in phase, first admission and week 144 will be drawn for all patients. On weeks 4 and 24 these will be performed only on the 200 IU/d and 800 IU/d arms (total volume 48 ml). On week 144 only 30 ml will be obtained. Will not be performed if Hb < 10 g/dL.

[6] FSIVGTT – total blood volume drawn 120-150 ml. Will not be performed if Hb < 10 g/dL.

[7] Possible lowering of vitamin E dose based on week 48 results (see Figure 4).

Analysis of the study

End points

Primary end points

There are two primary clinical end points in the study. The primary biochemical response is defined in a binary manner as an end-of-treatment AST <=32 or ALT <=35 U/L. The selection of these cut-off values is based on a pilot analysis by us that examined in an unbiased manner several rules for biochemical response and found this to be the one most strongly associated with histological response to treatment, irrespective of treatment modality[31]. We will also analyze the absolute change in ALT and AST from pre-treatment to end-of treatment as continuous parameters of the effect of treatment on liver enzymes. Patients who at the time of starting vitamin E treatment have normal liver enzymes will be excluded from this analysis. The primary physiological response is the absolute change in liver fat content as determined by comparing the ^{1}H-MRS results from pre-treatment to end of treatment.

Secondary end points

Secondary end points include: absolute and percent decrease in liver enzymes, decrease in GGT levels, percent reduction in liver fat.

A secondary analysis will analyze the effect of the lifestyle modification by comparing the end points between enrollment and the end of the lifestyle modification phase. Another analysis will determine the effect of increasing the dose of vitamin E by comparing the end points between the end of the randomized phase (week 24) to weeks 24 and 144 of the extended phase.

The kinetics of response will be determined by analyzing sequential ALT and AST measurements. Current data suggest biphasic response pattern to vitamin E and mathematical modeling will be applied to the results and will be compared between doses. Similar modeling will be applied to changes in liver fat content by ^{1}H-MRS.

Basic scientific end points

Gene expression in liver and adipose tissue will be analyzed using microarray and quantitative PCR (qPCR) of tissue samples. Treatment-induced changes or comparisons between groups of patient (i.e. comparison of baseline expression between responders and non-responders) will be expressed as fold-induction.

NK and NKT cell changes in the serum and liver tissue will be analyzed using phenotyping and functional assays.

Oxidative stress will be measured in the serum, urine, liver and adipose tissue using TBARS assay, F_2-isoprostanes, 9-HODE, 13-HODE, SOD activity or similar measures. In women of child-bearing age, the phase of the menstrual cycle will be recorded on extended visits, as F_2-isoprostane levels can be affected by it.

Statistical analyses

Parameters of response to treatment will be analyzed by matched-pair comparison between pre-treatment and end of treatment values. Student's t-test will be used for values that are distributed normally while Wilcoxon's sign-ranked test will be used for non-parametric values. Categorical comparisons will be performed using X^2 or Fisher's exact test, as appropriate. The Cochran-Armitage test for trend will be used to test for a dose effect on the primary clinical end points.

To determine the effect of genetic variants, genotype-based analyses will be preferred to allelic analyses, with an additive model used primarily, unless other (dominant or recessive) models seem more appropriate. Linear or ordinal logistic regression will be used.

All analyses will be done with and without stratification for the presence of diabetes mellitus.

An $\alpha \leq 0.05$ will be used to determine statistical significance.

Sample size calculation

The main goal of the study is to understand and characterize the mechanism of response to vitamin E. An important goal of the study is to simultaneously collect liver and adipose tissue to determine the site of action of the vitamin E and the relative contribution of each tissue to the disease. For most of the planned end points, including changes in liver fat content by ^{1}H-MRS and the basic scientific end points, there is not enough pre-existing data to allow for accurate sample size calculation.

For the comparison of effectiveness between different doses of vitamin E (primary aim 1), the primary biochemical end point will be used. We assume a response rate of 45% with the 800 IU/d dose (similar to that seen at the PIVENS trial), a 10% response rate to the 200 mg/d dose and a 20% response to the 400 mg/d dose. Under these assumptions, a sample size of 25 patients per group will give a power of 80% to detect a linear trend with statistical significance of 5% (Cochran-Armitage test). To account for possible drop-out and loss to follow-up, we are aiming to recruit 30 patients per group or an overall of 90 patients in the study.

As the sample size calculation is limited by the absence of published data, we will re-assess our calculations after 10 patients have been enrolled in each arm, and again after 20 have been enrolled.

Human subject protections

Rationale for subject selection

The study is designed to enroll patients with NAFLD that can be potentially benefit from treatment. Currently, there are no reliable non-invasive markers that accurately distinguish NASH from steatosis only, although the latter seems to carry a more favorable prognosis. For patients with steatosis only, it is unclear whether treatment can improve their disease and prevent progression to NASH. In most clinical trials with histological end points, patients with steatosis only were excluded as they cannot show significant histological

improvement. In this study, with a primarily scientific outcome, we are interested in studying the effect of vitamin E on these patients as well and we consider the intervention to be low-risk enough to allow us to recruit all patients with NAFLD.

The exclusion criteria for the study are mainly geared towards limiting enrollment to patients for whom treatment is considered safe and that have no confounders that would limit the analysis of the results. There are no limitations based on race or ethnicity. However, we anticipate that very few patients of African ancestry will be enrolled, because of the scarcity of NAFLD in these patients and their apparent "protection" from liver fat accumulation.

We intend to enroll women as well as men and do not foresee a marked difference by gender. We exclude pregnant women and require stable contraception from participants, because of the interventions, blood draws and imaging using radiation. Furthermore, pregnancy has an effect on metabolic parameters and fat handling and will not allow analysis of the data. Similarly, we choose not to enroll breast-feeding women as the dietary recommendations for them are different from those given to the general population and will limit the ability to analyze the data.

Enrollment in this study is limited to adult patients as the pediatric form of NAFLD has unique clinical and histological features and the choice of therapies may be different.

Recruitment strategy

Patients with NAFLD who have been followed at the LDB clinic will be offered participation based on eligibility. New patients are continuously evaluated at the clinic and will be enrolled as well. We will inform local primary care providers, gastroenterologists, hepatologists and free clinics of the availability of the protocol and eligibility criteria. Patients seen by the Liver Service as consult cases will be offered participation in the protocol if deemed eligible and only with the agreement of the referring physician and the principal investigator for the referring protocol. We aim to recruit 90 patients for the study within 3 years.

Informed consent process

The initial screening visit to assess eligibility for the protocol will be done under protocol 91-DK-0214, "Evaluation of Patients with Liver Disease"; only eligible patients will then be consented into this protocol. Written informed consent will be obtained from the participant prior to any study procedures or treatments. The Principal Investigator or other designated qualified protocol investigators will explain the study in language understandable to the subject. Sufficient time and opportunity will be given for discussion of the research as well as to answer any questions they may have, taking care to minimize or eliminate the perception of coercion or undue influence. The participant and the investigator will sign the current IRB-approved informed consent documentA copy of the consent will be given to the subject for future reference. The signed documents will be sent to the Medical Records Department for placement in the subject's permanent CC medical record. The consent process will additionally be documented in the electronic medical record (CRIS).

We anticipate the enrollment of Spanish speaking subjects into this study for which English consents have been fully translated.

Evaluation of benefits and risks

Benefits

Most patients with NAFLD are overweight and have some degree of insulin resistance. For these patients lifestyle modification with dietary consultation aiming at weight loss and a moderate exercise regimen is of proven benefit, is considered standard medical care[32] and conforms to the recommendations for the population as a whole[33, 34]. The target level of exercise, 150 minutes of moderate intensity per week, is the minimum recommended for all adults.

Currently, there are no reliable non-invasive markers that accurately distinguish NASH from steatosis only, which carries a much more favorable prognosis. Even the presence of elevated liver enzymes in patients with liver fat predicts the presence of NASH in only 55%[35] to 62% (NASH-CRN data, unpublished) of cases. Thus, a liver biopsy is essential for proper staging, as well as to provide tissue for the scientific studies.

Patients with NAFLD who did not previously have a liver biopsy can benefit from one; however, a liver biopsy is not considered essential in routine management of NAFLD. Repeat liver biopsies are not routinely performed on patients with fatty liver disease outside of the research context but in this study are crucial to define the histology and to allow evaluation of response mechanism.

Vitamin E therapy for NASH has been shown to improve fat deposition in the liver, decrease liver enzymes and reduce histological evidence of cellular injury, without affecting insulin sensitivity[3]. Thus, although it is not yet approved as standard medical treatment for NASH, there is clearly a potential benefit to the patients.

Risks and Discomforts

Physical exercise. Adults who exercise are inherently at a higher risk for exercise-induced musculoskeletal injury, but lower risk for non-leisure time injuries; however, the risk of injury with a supervised program and moderate intensity is not much different for that of sedentary adults[36]. The risk of a major cardiac event during exercise is small with moderate-intensity activity and there is no clear evidence to suggest a need for screening of asymptomatic individuals. In this study, we adopt the screening recommendations of the American Heart Association for diabetic patients who start an exercise program[37] and apply them to the whole study population.

Exercise induced hypoglycemia can occur in diabetic patients treated by insulin or insulin secretagogues, especially those maintaining tight glycemic control. An endocrine consult will be requested on these patients to assess the need for dose modification and the patients will be encouraged to monitor their blood sugar levels routinely.

Diet. The dietary intervention in this study is aimed at achieving a balanced diet with mild caloric deficit and gradual weight reduction, and conforms with the general

recommendations for obese or diabetic patients. No significant complications or risks are anticipated from this diet.

Liver biopsy. Patients will undergo 3 liver biopsies in this protocol; one after the lifestyle modification phase, a second after 4 weeks of vitamin E therapy, and an exit biopsy at the end of the study. The liver biopsy will be performed when the patients are admitted as inpatients to the Clinical Center for the extensive evaluation detailed above.

The major side effects of liver biopsy are pain, bacteremia, puncture of another organ and bleeding[38]. Local pain and discomfort at the liver biopsy site occur in about 20% of persons undergoing percutaneous liver biopsies. This is transient (lasting one to twelve hours) and usually mild, rarely requiring analgesics. Bacteremia occurs in 1-2% of persons undergoing liver biopsy. In the absence of bile duct obstruction, this is almost always self-limited and is rarely symptomatic. Inadvertent puncture of another organ such as lung, colon, gall bladder, kidney and adrenal gland can occur during liver biopsy. All biopsies will be performed after a bedside ultrasound examination by the performing physician to identify internal organs and select and optimal site and direction for the biopsy. Significant bleeding after liver biopsy is the most serious side effect of this procedure. In the absence of blood coagulation defect or hepatic malignancy, significant bleeding is rare, occurring in less than one in a thousand cases of liver biopsy. Death due to bleeding after liver biopsy has been reported to occur in less than 1/10,000 cases. At the NIH Clinical Center, the Liver Diseases Branch has performed approximately 150 liver biopsies each year for the past 25 years. During this time, two patients have died as a result of liver biopsy. One patient had cirrhosis and advanced hepatocellular carcinoma. The second patient had Gaucher's disease with hepatic involvement and severe coagulopathy. Both patients died after surgical attempts to stop the bleeding were unsuccessful.

Adipose Tissue Biopsy. The major risks/discomforts are represented by pain, bruising, hematoma, infection, scarring, and localized lipodystrophy. The procedure will be performed under sterile technique to minimize the chances of infection. Ice will be applied to the site immediately after the procedure to limit bruising, swelling and tenderness. After subcutaneous tissue biopsy, patients will be monitored by nurses. Post-biopsy, the needle site will be cleaned and covered with gauze and translucent dressing tape. Study participants will be instructed to report to the clinical staff any changes at the biopsy site including bleeding, secretion, erythema, pain, and signs and symptoms of infection. Study participants will be instructed to self-monitor the puncture site after discharge from the Clinical Center.

Frequent phlebotomy. To document the baseline and to monitor the effects and toxicities of treatment, frequent blood sampling will be required. Patients will have 15-20 venipunctures during the period of evaluation and the first 6 months of therapy and 4 venipunctures per year in the extended treatment phase. Each venipuncture will be for 32 to 150 ml of blood. However, no more than 550 ml or 10.5 ml per kg (the lower of the two) will be drawn from any one person during any eight-week period.

Imaging studies.

1) ***DEXA scan***. Patients will undergo 2 DEXA scans in the first year of treatment and another test at the end of the extended treatment phase, to assess whole body composition. The effective dose of radiation that patients will receive

from a single study is 0.5 mrem; thus the total effective dose from DEXA for the duration of the study is 1.5 mrem.

2) ***MRI scans***. Patients will undergo 5 MRI scans in the first year of treatment and another test at the end of the extended treatment phase, to determine the amount of liver fat, liver volume and subcutaneous and visceral fat. While serial MRI scanning is thought to be safe, the procedure may cause anxiety in some patients since current equipment used at the Clinical Center uses a closed tube. Patients will be offered sedatives such as Valium if they express worry about being in a closed space.

All female patients with childbearing potential will have a pregnancy test prior to any imaging test involving ionizing radiation.

IVGTT. Patients will undergo 3 IVGTT tests in the first year of the study and another one at the end of the extended treatment phase. Patients will have two intravenous catheters placed for the procedure, one for the infusion of glucose and insulin and the other for blood drawing. Patients may experience pain from intravenous catheter insertion or may feel a burning sensation during the rapid injection of glucose. There is a small risk of hypoglycemia from the short period of insulin infusion, although it is extremely rare and blood glucose will be monitored frequently during the test. With mildly low blood sugars (<50 mg/dl), subjects may get hungry, may become drowsy or sweaty. With blood sugars below 30 mg/dl, subjects may be difficult to arouse or have seizures. We do not anticipate significant hypoglycemia as most or all of the patients are predicted to have some degree of insulin resistance. Nevertheless, a physician or nurse will be present during this test for the first hour of the test (including the first thirty minutes following insulin administration) to monitor for signs of significant hypoglycemia. If significant hypoglycemia occurs it will be treated with intravenous dextrose or glucagon according to clinical evaluation. The total amount of blood that will be drawn during this test will be 120 to 150 ml.

Vitamin E treatment. The dose of vitamin E used in this study is lower than the recommended upper limit of supplement intake (1500 IU/d)[9]. Some studies of vitamin E supplementation for primary or secondary prevention of disease suggested increased mortality or risk of hemorrhagic stroke[12]. Two meta-analyses[39, 40] suggested slight increased mortality in patients treated with high-dose vitamin E. However, these works were criticized for several reasons and the studies selected for the meta-analyses included mostly patients with chronic diseases. A recent meta-analysis using all trials found no excess mortality risk[41]. Furthermore, these studies focused on the use of vitamin E for disease prevention (a scenario where little or no risk would be tolerable), whereas our trial uses vitamin E as a proven treatment for an existing disease. In the PIVENS trial[3], no excess adverse events were noted in the vitamin E in comparison to the placebo arm.

As some studies suggest an effect of vitamin E on platelet aggregation[42], and given the possible association with hemorrhagic stroke, we exclude patients with high bleeding risk, such as those with coagulopathies, use of anticoagulants, thrombocytopenia or known platelet dysfunction. Furthermore, due to the possibility of increased risk of gastrointestinal bleeding, patients with a pre-existing history of iron deficiency anemia secondary to gastrointestinal sources are excluded. On the other hand, vitamin E supplementation is not associated with changes in standard parameters of bleeding tendency, including PT, PTT,

platelet count and bleeding time[43, 44], rendering these useless as a tool for safety monitoring during vitamin E treatment. However, if during the study the platelet count or PT/PTT cross the thresholds for study inclusion (i.e. platelets <70,000, PT/PTT prolonged by >= 3 seconds), treatment will be stopped and patients withdrawn from the study.

Recently, data from extended follow-up of the SELECT trial[45] suggested increased risk for the diagnosis of prostate cancer in patients treated with vitamin E. This is in contrast to the findings of two other similar studies[46, 47] which did not show such an effect. Our patients are anticipated to be significantly younger than the study population in SELECT (median age 62); furthermore, risk for prostate cancer appeared to increase numerically only after more than 3 years of treatment. We will monitor PSA levels every 12 months on male patients in our study. Screening for prostate cancer may lead to further evaluation of abnormal results, including referrals, blood tests and possible invasive procedures. There is also a risk of detecting prostate cancers that are not destined to become clinically significant. The risks and benefits of screening with annual PSA testing will be discussed with all male participants prior to enrollment and patients will have the option to elect not to be screened.

Adverse event reporting and data monitoring

Reportable events will be tracked and submitted to the IRB as outlined in Policy 801.

The PI will immediately report SAEs to the Sponsor according to the requirements of 21 CFR 312.64(b) and as agreed upon with the sponsor. The PI will record nonserious AEs and report them to the Sponsor according to the timetable for reporting specified in the protocol (21 CFR 312.64(b).

All serious, unexpected, suspected adverse reactions (SUSAR) will be reported to the sponsor within 15 calendar days. All unexpected suspected adverse reactions resulting in death or life-threatening events will be reported to the sponsor within 7 calendar days. The investigator may also be responsible for completing an FDA MedWatch 3500 form and additional information for a follow-up SAE report as information becomes available.

Summary of adverse events and efficacy data will be provided in annual reports to the FDA.

Monitoring

Safety monitoring will be maintained by weekly meetings of the investigators to review clinical data on patients seen in the outpatient clinic and maintenance of individual flow sheets on each patient. Laboratory and imaging test results will be reviewed routinely by the treating physician, the principal investigator and the study nurse.

As required by FDA 21 CFR 312.50, this study will be monitored to ensure compliance with the protocol and applicable regulatory requirements. Monitoring results will be reported to the Principal Investigator/Sponsor for further reporting to the FDA consistent with applicable regulations. The specific monitoring plan will be developed with the Principal Investigator and frequency of monitoring visits will be determined by such factors as study enrollment, data collection status and regulatory obligations. Study

documents and pertinent hospital or clinical records will be reviewed to verify that the conduct of the study is consistent with the protocol plan.

Collection and storage of human specimens or data

Patients will have serum stored from selected time points during this study. These specimens will be used for repeat testing (in case of missing samples) or for special tests as needed (such as for serum cytokines or adipokines). Serum samples will be stored at the NIDDK clinical core laboratory inside locked freezers in locked rooms and will be labeled by coded unique identifiers without personally identifiable information. The code to these samples will be maintained by the clinical core personnel.

Liver and adipose tissue biopsy samples may be stored if residual tissue is available after routine samples are taken for routine histological staining and evaluation. PBMCs will be stored until they are used for NK/NKT cell and gene expression analysis. RNA or protein that is extracted from these samples will be stored as well. The samples will be stored in a locked freezer in a locked room at the LDB laboratory and will be labeled by a coded unique identifier without personally identifiable information. The code to these samples and their location will be maintained by the primary investigator and the laboratory manager.

Access to stored samples and the identifiers will only be provided to the principal or associate investigators, and only with the written approval of the principal investigator and the branch chief. If samples are to be evaluated by outside collaborators in the future, they will be coded and all identifying data removed from the samples. The code to identify samples and associate them with patient identity will be maintained by the principal investigator and not shared with outside collaborators.

Research records and data as well as liver biopsy slides, biopsy reports, liver tissue and sera will be stored indefinitely in our locked offices and freezers, the medical record department and the pathology department. These materials will be protected and tracked by standard operating procedures in the medical record and pathology departments as well as a compulsive filing system in our locked offices and freezers. There will be redundant storage of clinical information in the medical record department and our offices. Likewise, there will be redundant storage of biopsy information and materials in the pathology department and our offices. This should minimize the risk of loss or destruction of information and specimens. If that were to occur we would report it to the IRB. We do not plan to destroy this personal medical information or the liver biopsy specimens or research subject sera after completion of the study because it may be critically important for physicians (here or elsewhere) to have access to this information when caring for these patients in the future.

Remuneration/compensation

Compensation will be provided to subjects for participation in some of the research procedures and tests in the study, according to the following schedule:

For the baseline (week 0 of run-in phase) MRI ……$ 60.00

For the 1st inpatient hospital admission including:

DEXA scan…….$ 10.00

MRI…….$ 60.00

FSIVGTT ……..…….$ 70.00

Adipose tissue biopsy……..$ 50.00

Resting Energy Expenditure……....$ 25.00

Time ……$ 120.00 (3 nights/$40.00 per night)

Total compensation for the admission…… $ 335.00

For the 2nd (week 4) inpatient hospital admission including:

MRI…….$ 60.00

FSIVGTT…….$ 70.00

Liver Biopsy…….$ 280.00

Adipose tissue biopsy……$ 50.00

Resting Energy Expenditure……..$ 25.00

Time ……..$ 80.00 (2 nights/$ 40.00 per night)

Total compensation for the admission …… $ 565.00

For the 3rd (week 24) inpatient hospital admission including:

DEXA scan…….$ 10.00

MRI…….$ 60.00

FSIVGTT ……..…….$ 70.00

Resting Energy Expenditure……$ 25.00

Time …….. $ 40.00 (1 overnight/$40.00 per night)

Total compensation for the admission…… $ 205.00

For the final (Week 144) inpatient hospital admission including:

DEXA scan ……$ 10.00

MRI ……... $ 60.00

FSIVGTT …… $ 70.00

Liver biopsy …… $ 280.00

Adipose Tissue biopsy ……	$ 50.00
Resting Energy Expenditure……	$ 25.00
Time ……	$ 120.00 (3 nights/$ 40.00 per night)

Total compensation for the admission ……$ 615.00

Total overall (maximal) study compensation…….$ 1,780.00

No remuneration is offered for taking vitamin E, outpatient visits, blood tests, nutritional consults and study questionnaires.

Patients may also be eligible for reimbursement of travel expenses based on standard Clinical Center and LDB criteria.

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