Genetic-Specific Effects of Fructose on Liver Lipogenesis

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PROTOCOL TEMPLATE: INTERVENTIONAL STUDY

Complete Title: Genetic-specific effects of fructose on liver lipogenesis

Short Title: Fructose-Liver fat study (FLFS)

Drug or Device Name(s): Sugar-sweetened drinks

FDA IND/IDE (if applicable):

Sponsor: Trans-NORC complementary pilot grant award

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Amendment 3 Date:

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PROTOCOL TITLE: Genetic-specific effects of fructose on liver lipogenesis

Short Title: Fructose-Liver fat study (FLFS)

Lead Investigator:

Saroja Voruganti, PhD

University of North Carolina at Chapel Hill

Protocol Version: 3

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I confirm that I have read this protocol and understand it.

Principal Investigator Name: Saroja Voruganti

Principal Investigator Signature: 

Date: 05/15/2021

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ABBREVIATIONS AND DEFINITIONS OF TERMS

Insert and delete terms as relevant

Abbreviation	Definition
NAFLD	Nonalcoholic fatty liver disease
PNPLA3	phospholipase, domain-containing 3
GCKR	Glucokinase regulatory protein
AST	Aspartate transaminase
ALT	Alanine transaminase
GGT	Gamma Glutamyl transferase
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
TG	Triglycerides
VLDL-TG	Very low-density lipoprotein-triglycerides
MRI	Magnetic resonance imaging
DNL	*de novo* lipogenesis
SNP	Single nucleotide polymorphism
LCMS	Liquid chromatography mass spectrometry

PROTOCOL SYNOPSIS

Study Title	Genetic-specific effects of fructose on liver lipogenesis
Funder	Trans-NORC Complementary pilot Grant Program
Clinical Phase	N/A
Study Rationale	Fructose consumption has dramatically increased over the past 20 years in the United States (US) and worldwide[1]. High fructose intake has been shown to increase serum triglycerides, upregulate *de novo* lipogenesis, reduce fatty acid oxidation and decrease the clearance of very low density lipoprotein triglycerides in liver leading to nonalcoholic fatty liver disease (NAFLD)[1]. Nonalcoholic fatty liver disease (NAFLD), the leading cause of chronic liver disease in the US, is characterized by fat accumulation in liver cells not caused by alcohol[2]. A group of disorders including steatosis, nonalcoholic steatohepatitis with fibrosis and cirrhosis, NAFLD has substantially risen in prevalence over the last two decades with recent estimated prevalence being 20% among US adults and 25% in young adults (18-39 years).[2] Although evidence has been presented for the positive relationship between fructose intake and NAFLD, the role of fructose in liver fat accumulation has been unclear. Individuals with specific genotypes seem to be more susceptible to the adverse metabolic effects of fructose. For example Hispanic children with rs738409 (GG) single nucleotide polymorphism (SNP) of patatin-like phospholipase, domain-containing 3 (*PNPLA3*) increased their liver fat by 23% more than CG/CC children exposed to the same amount of dietary fructose[3]. Similarly, adolescents with rs1260326 (TT) of glucokinase regulatory protein (*GCKR*) variant increased their *de novo* lipogenesis by 44% as compared to CC adolescents when given a sugar challenge[4]. Therefore, we hypothesize that when given the same amount of fructose individuals with specific set of genotypes will be more susceptible to increased *de novo* lipogenesis and liver fat accumulation than others.
Study Objective(s)	Our goal for this study is to generate preliminary data that is needed to support an application to NIH for funding of a larger, adequately-powered study to explore whether a set of genotypes, that increase the risk for nonalcoholic fatty liver disease (NAFLD), predispose individuals to increased *de novo* lipogenesis (DNL) and liver fat accumulation when exposed to fructose intake. **Primary Objective:** To determine the impact of acute and prolonged exposure of fructose on hepatic lipid accumulation in adolescents and yound adults (12-40 years) with high and low genetic risk for NAFLD using liver mass resonance imaging (MRI). **Secondary Objective:** To explore the genetic-specific effects of fructose on markers of liver fat accumulation (fibroscan[5] and fatty liver index[6]) and serum biomarkers related to lipids, uric acid and liver function.
Test Article(s) *(If Applicable)*	Sugar-Sweetened bevarages
Study Design	a. We will screen 200 Caucasian children and young adults (12-40 years) to obtain 24 children and 24 young adults who are overweight/obese and drink <14 fructose drinks/week to participate in a 3-week fructose intervention study. The 200 participants will be genotyped for 10 SNPs involved in lipid metabolism and associated with increased NAFLD risk in previously published studies[7,8]. We will select 12 children and young adults with 15 or more risk alleles and 12 children and 12 young adults with 6 or fewer risk alleles and provide them fructose + glucose[9] drinks who will ingest one drink (equal to 2 soft drinks) per day for 3 weeks; b. We will measure liver fat using Liver MRI[9,10] and transient elastography (Fibroscan)[5] at week 0 and 3;

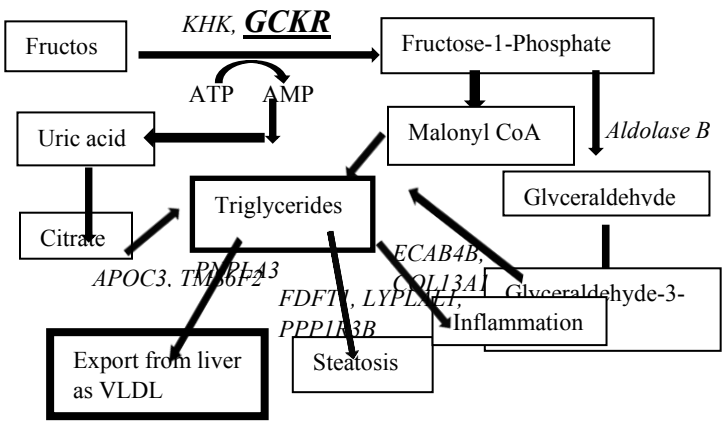
	c. Data will be analyzed using paired and unpaired students T test to differentiate the responses between the time points and between the two groups, respectively. Specific Aim2: Determine the impact of acute exposure of fructose on hepatic *de novo* lipogenesis a. Using the cohort described in Specific Aim1, we will conduct oral fructose tolerance test at week 0 and week 3; b. We will measure liver fat using Fibroscan c. Data will be analyzed using unpaired students T test and repeated measures ANOVA to differentiate the responses between and within the two groups, respectively. Specific Aim3: Determine the relationship between markers of DNL, liver fat accumulation and serum concentrations of lipids, uric acid and liver function markers before and after the fructose challenge a. We will measure serum concentrations of liver function markers, lipids and uric acid at week 0 and week 3; b. As in previous aims, data will be analyzed using students T test, and Pearsons correlations.
Subject Population key criteria for Inclusion and Exclusion:	**Inclusion Criteria** 1. Subjects between 12 and 40 years 2. No history of alcohol abuse (> 7 drinks per week) 3. History of fructose intake of < 14 drinks per week 4. Caucasian ethnicity 5. BMI > 25kg/m^2 – 32kg/m^2 or 85^{th} -99^{th} percentile but otherwise healthy **Exclusion Criteria** 1. ages < 12 and > 40 years 2. Pregnant/lactating 3. known alcohol abuse or fructose intake > 14 drinks per week 4. not of Caucasian ethnicity 5. glucose levels > 100 mg/dl if fasting, > 140mg/dl if within 2 hours post meal and > 200 mg/dl if random sample 6. taking anti-hypertensive, anti-diabetic, uric acid and/or lipid-lowering medications 7. known diagnosis of diabetes, fructose intolerance, chronic kidney disease, NAFLD or any liver-related disease, hypertriglyceridemia, polycystic overy syndrome, hypothyroidism, obstructive sleep apnea, hypopituitarism and hypogandism 8. BMI < 25kg/m^2 or > 32 kg/m2 or < 85^{th} or > 99^{th} percentile 9. Liver fat fraction >5% as per baseline MRI scan
Number Of Subjects	200 for first phase and 48 for the second and third phases
Study Duration	The first phase will be about 30 minutes and the second and third phase will be divided into two visits. Both visits in both phases will be about 3.5 hours each.
Study Phases **Screening** **Study Treatment** **Follow-Up**	(1) Screening: We will screen participants for eligibility to participate in the study based on the inclusionary/exclusionary criteria specified above. The 200 children and young adults selected to participate will provide saliva which will be used to extract DNA and then genotype 10 SNPs. These SNPs are selected based on their association with *de novo* lipogenesis (DNL) or liver fat accumulation in previously published studies in Caucasians. We will select 12 children and 12 young adults with more than 15 risk alleles and 12 children and 12 young adults with less than six number of risk alleles. (2) Intervention: This part is divided into two phases. Each phase entails two visits. Phase2-visit 1: The 48 enrolled (24 in each of the high and low genetic risk group) will

	visit the UNC NRI clinical suite run by the Human Research Core. They will be taken to the WakeForest imaging center for their baseline MRS scan. Visit 2: Acute fructose challenge: The next day, they will have their initial anthropometrics and Fibroscan measurements. They will then undergo an oral fructose challenge using a protocol used by Hudgins et al[13]. The participants will be provided with a drink consisting of fructose (0.75g/kg body weight) + glucose (0.45g/kg body weight) dissolved in 24 Oz of water (equal to 2 soda cans) to be consumed within 15 minutes. Blood and urine will be sampled at baseline and 1 and 3 hours after sugar drink ingestion. During the challenge period, no other beverage (except water) or food will be allowed. 3-week intervention: The 48 participants will be provided with sugar drinks for the three week period. The drinks that need to be taken per day will contain fructose (0.75g/kg body weight) + glucose (0.45g/kg body weight) dissolved in 24 Oz of water (equal to 2 soda cans) which is similar to that provided during the acute fructose challenge[7]. Addition of glucose to fructose in the challenge and 3-week intervention induces the lipogenic effects of fructose, alleviates gastrointestinal effects of fructose and mimics the real-life situation[14]. They will visit at the end of each week to collect drinks for the next week. In addition, participants will be given standardized meals for the day prior to the baseline fructose challenge. They will also be instructed to avoid alcohol, juices and sodas the previous evening.
Efficacy Evaluations	Changes in liver fat accumulation through MRI scans and fibroscan measurements, and serum concentration of other lipids, liver function markers and uric acid.
Pharmacokinetic Evaluations	N/A
Safety Evaluations	At screening, to check for blood glucose values, we will conduct a spot glucose check using a glucometer (Accu-Chek, Roche Diagnostics, CH) to check that the glucose levels of the participants are in the normal range (fasting < 100mg/dl, post meal < 140 mg/dl and random < 200 mg/dl). Secondly, we will have a physician who will be available if needed. We will also conduct pregnancy test if needed. Also, we will exclude participants for further participation if we find that the participant has high liver fat fraction (>5%) during our baseline MRI scan[10]. The participant will be given a report that they can show to their physician for treatment.
Statistical And Analytic Plan	Our main outcome measure is change in liver fat via MRI scan, fatty liver index equation and through fibroscan measurements. We will also measure serum concentrations of other lipids, liver function markers and uric acid**.** Data will be assessed using descriptive measures and confidence intervals. Paired t-tests and repeated measures regression analysis will be performed to explore the significant differences between week 0 and week 3.
DATA AND SAFETY MONITORING PLAN	A data and safety monitoring plan will be instituted for this study that will ensure adherence to the protocol approved by UNC IRB. Dr. Voruganti (PI) and co-investigators will review the phenotype and genotype data as they are generated. Any adverse event that is reported to either the principal investigator or her designated research associates by the subject or medical staff caring for the subject and which meets the criteria will be documented as such. Any event that is reported will then generate an adverse event report, which will be submitted to the IRB. The report will include a description of the event, when and how it was reported, as well as any official chart records or documentation to corroborate the event or the reporting of the event

1 BACKGROUND AND RATIONALE

NAFLD is a significant health burden in the US: The leading cause of chronic liver disease in the US, NAFLD is characterized by fat accumulation in liver cells not caused by alcohol. NAFLD, representing a group of disorders including steatosis, nonalcoholic steatohepatitis with fibrosis, has substantially risen in prevalence over the last two

decades with the estimated prevalence being 20% among US adults and 25% in young adults (18-39 years)[2]. Over 64 million individuals are believed to have NAFLD with annual medical costs rising to more $100 billion. More common in individuals who are obese or diabetic and/or have metabolic syndrome, NAFLD has been associated with increased cirrhosis, liver-related mortality and hepatocellular carcinoma[2].



Gene	SNP	Major/Minor	MAF (%)
PNPLA3	rs738409	C/G	23
GCKR	rs1260326	C/T	45
TMS6F2	rs58542926	T/C	07
APOC3	rs2854116	T/C	39
EFCAB4B	rs887304	G/T	49
NCAN	rs2228603	C/T	07
COL13A1	rs1227756	A/G	48
FDFT1	rs2645424	G/A	42
LYPLAL1	rs12137855	C/T	21
PPP1R3B	rs4240624	A/G	7

Potential genes (italicized and bold) which modulate the effect of fructose on lipogenesis in the liver. **Figure 1** shows genes in the fructose-induced lipogenic pathway. **Table 1** shows the genes and variants that have been associated with liver lipogenesis through genome-wide association studies. *PNPLA3*- patatin-like phospholipase, domain-containing 3, *GCKR*- glucokinase regulatory protein, *APOB* and *APOCIII* - apolipoprotein B and CIII (*APOB and APOC3*), *TM6SF2* - Ttransmembrane 6 superfamily member 2, *EFCAB4B* - EF-hand calcium-binding domain-containing protein 4B, *COL13A1* - Collagen type XIII alpha 1chain, *FDFT1* - farnesyl-diphosphate farnesyltransferase 1, *NCAN*- Neurocan, *LYPLAL1*-Lysophospholipase like1, *PPP1R3B*- Protein phosphatase 1 regulatory subunit 3B.

Dietary factors spur the development of NAFLD: Unhealthy diet plays a major role in the development of NAFLD. Most of the attention has been on increased calorie intake. However, an important but underappreciated dietary change in US society for the past few decades has been increase in fructose intake. Contained in soft drinks, fruit juices and energy drinks, fructose affects many metabolic processes, foremost being an increase in fat accumulation in liver and contributing to the onset and progression of NAFLD (**Figure 1**). Fructose, which is almost entirely metabolized in liver, is rapidly phosphorylated to fructose 1 phosphate by fructokinase (ketohexokinase) with ATP depletion in parallel. The fructose 1 phosphate gets metabolized to dihydroxyacetone-phosphate and glyceraldehyde and finally to triglycerides[15-17] which gets deposited in liver and leads to NAFLD.

Fructose is a more potent stimulator of DNL than glucose: In population studies, it has been shown that fructose, and not glucose, is associated with increased visceral adiposity, insulin resistance and hyperuricemia[18-20]. Fructose is unique in its effects that it stimulates KHK and thus potentiates its own metabolism. Secondly, the rate of phosphorylation of fructose by KHK is 10 times higher than the phosphorylation of glucose by glucokinase. Third, fructose is directly absorbed into portal vein and delivered to liver without entering the systemic circulation. Liver, thus, is exposed to a much higher fructose load than rest of the tissues. Fourth, fructose activates the lipogenic transcriptional factors, SREBP1c and ChREBP in the liver, promoting DNL[21]. And finally, fructose tends to deplete liver ATP levels and one of the outcomes is generation of more AMP which get converted to uric acid. Uric acid has been shown to stimulate fat synthesis in the hepatocyte thus pointing to an additional pathway through which fructose can increase liver fat[22,23].

Genes and gene by nutrient interactions play a major role in the regulation of NAFLD: Both genetic and environmental factors contribute to the onset and progression of NAFLD. NAFLD is a complex and heritable phenotype. Family-based studies have reported heritability estimates for NAFLD to be between 20% and 70%[7,8]. Genome-wide and candidate gene studies have identified several genes associated with NAFLD[8]. Representative genes that were implicated in lipid metabolism and in the development and progression of NAFLD are shown in **Table1**. Few studies have shown that fructose affects lipogenesis in liver in a genotype-specific manner. Davis and colleagues found that Hispanic children with GG genotypes of *PNPLA3* SNP rs738409 were more inclined to accumulate fat in the liver as compared to children with CC or CG genotypes[3]. Similarly, another study investigating the effects of added sugars on liver fat found that individuals with TT of rs1260326 of *GCKR* increased their *de novo* lipogenesis by 44% during an oral fructose + glucose challenge[4]. However, none of the studies have shown the cumulative effects of SNPs on change in liver fat when exposed to fructose.

The genetic-specific effects of fructose on liver lipogenesis need to be studied as a first step towards individualized nutrition: This study assumes significance given that fructose affects liver lipogenesis differentially based on genotypes. It also paves the way towards individualized or personalized nutrition. Previous studies investigating the metabolic effects of fructose have been controversial. Some have shown significant effects of fructose on insulin sensitivity and de novo lipogenesis and triglycerides while others have shown no effects[23-27]. The range of these studies is wide, with the duration of the intervention ranging from 7 to 665 days, sample size ranging between 8 and 64 per group and amount of fructose per day ranging from 40 to 250g/d. For this exploratory and feasibility study we have selected the duration of 21 days, sample size as 12 per group and amount of fructose a ~0.75g/kg/day (equivalent to two sodas/per day).

1.1 Introduction

This study is a combination of acute and long-term investigation of the effects of fructose on liver lipogenesis and whether genetics modify this relationship. It is similar to a case-control study based on the number of alleles that increase the risk for liver lipogenesis which further increases the risk for NAFLD. There is an increasing concern about the effects of fructose on metabolic diseases. Some studies, as shown in the background section, have also suggested that effects of fructose may be modified by some genotpes. However, all these studies have focused on only one gene or genotype. We fill the gap by conducting the study with a set of genotypes.

1.2 Name and Description of Investigational Product or Intervention

Acute fructose challenge: We will be using a fructose drink which will be prepared by dissolving 0.75g/kg fructose + 0.45g/kg glucose (NOW Foods, Bloomingdale IL), in 24oz water. This composition of fructose and glucose mimics the composition of fructose and glucose in 2 regular sodas[28] based on an approximate adolescent/adult body weight of ~65kg.

3-week intervention: We will provide each participant the same drink as described in the acute fructose challenge to be taken one per day.

1.3 Non-Clinical and Clinical Study Findings

There are no immediate benefits. In long-term, we can provide genetic risk-guided diet plans to individuals who may be at high risk.

Risks: We don't anticipate major risk since we will exclude individuals who are diabetic, pre-diabetic, have high liver fat at baseline and are at high risk for these and other metabolic diseases. The only risk can be weight gain. We will advise on cutting calories elsewhere in their daily diet. We will also advise the participants to avoid fruit juices and other sodas for the duration of 3 weeks.

1.4 Relevant Literature and Data

The studies similar to that being conducted here have been conducted before (Table 1, Figure 1). The only difference is that we are using a set of 10 genotypes instead of individual genotypes that were used in studies before**.**

2 STUDY OBJECTIVE

2.1 Our goal for this study is to generate preliminary data that is needed to support an application to NIH for funding of a larger, adequately-powered study to explore whether a set of genotypes, that increase the risk for nonalcoholic fatty liver disease (NAFLD), predispose individuals to increased *de novo* lipogenesis (DNL) and liver fat accumulation when exposed to fructose intake.

2.2 **Primary Objective:** To explore the genetic-specific effects of fructose on liver fat accumulation using MRI

2.3 **Secondary Objective:** To explore the genetic-specific effects of fructose on markers of liver fat accumulation (fibroscan[5] and fatty liver index[6]) and serum biomarkers related to lipids, uric acid and liver function.

3 INVESTIGATIONAL PLAN (brief overview)

3.1 Study Design

- This exploratory and feasibility study is an intervention study assessing the effects of fructose on liver fat accumulation. The study design is described below.

3.2 Allocation to Treatment Groups and Blinding (if applicable)

- We wil allocate participants to high and low genetic risk groups

3.3 Study Duration, Enrollment and Number of Subjects

- The study has three phases. First phase will have 200 participants who will visit Human Research Core clinical suite and spend about 30 minutes providing saliva sample and completing a brief questionnaire.
- Second and third phases will have 48 participants. They will first undergo a MRI scan and next day a 3 hour acute fructose challenge (total time 3.5 hours) and then will consume sugar drink as given to them. They will visit again at the end of week 3 for further biomarker, MRI scan and fibroscan measurements.

3.4 Study Population

- Inclusion criteria: Causasian children and young adults**,** overweight/obese, ages between 12 and 40 years and no history of alcohol abuse (> 7 drinks per week for a year) and no history of excessive fructose intake (> 14 drinks per week for a year). We will conduct a spot glucose check using a glucometer (Accu-Chek, Roche Diagnostics, CH) to check that the glucose levels of the participants are in the normal range (fasting < 100mg/dl, post meal < 140 mg/dl and random < 200 mg/dl).
- Exclusion criteria includes ages < 12 and > 40 years, healthy weight or morbidly obese, known alcohol abuse, not of Caucasian ethnicity, pregnancy, lactation, glucose levels > 100 mg/dl if fasting, > 140mg/dl if within 2 hours post meal and > 200 mg/dl if random, baseline MRI scan of liver fat fraction > 5%[27], and known diagnosis of diabetes, fructose intolerance and chronic kidney disease, use of lipid or uric acid lowering medications.

4 STUDY PROCEDURES (what will be done)

4.1 Screening/Baseline Visit procedures

Phase 1: Recruitment and screening of participants:

Screening: We will recruit 200 Caucasian children and young adults fulfilling our inclusionary criteria: ages between 12 and 40 years, overweight or obese, no history of fructose intake > 14 drinks per week and alcohol abuse (> 7 drinks per week for a year). Height will be measured to the nearest 0.1 cm by a stadiometer situated against the wall in an upright standing position. Height and weight recorded will be used to calculate BMI as kg/m^2. We will match all participants for BMI ($25kg/m^2$ -$32kg/m^2$ or 85^{th}-99^{th} percentile).

We will conduct a spot glucose check using a glucometer (Accu-Chek, Roche Diagnostics, CH) to check that the glucose levels of the participants are in the normal range (fasting < 100mg/dl, post meal < 140 mg/dl and random < 200 mg/dl). The exclusion criteria includes ages < 12 and > 19 years, known alcohol abuse or fructose intake > 14 drinks per week, not of Caucasian ethnicity, glucose levels > 100 mg/dl if fasting, > 140mg/dl if within 2 hours post meal and > 200 mg/dl if random, and known diagnosis of diabetes, fructose intolerance and chronic kidney disease.

We will collect saliva from the eligible 200 children and young adults, extract DNA and genotype 10 SNPs shown in Table1.

Genotyping: SNPs will be genotyped from DNA extracted from saliva. Genotyping will be conducted in Voruganti lab using TaqManSNP genotyping assays (Applied Biosystems, Foster City, CA). The minor allele frequencies (MAFs) of these SNPs range between 7 and 49% based on Caucasian ethnic background. Therefore we are confident about finding 48 individuals with all 10 SNPs. If we don't find individuals with the 10 SNPs, we will mine the literature and find more SNPs that have a role in liver lipogenesis.

4.2 Intervention/Treatment procedures (by visits)

Phase2: Selection of 48 participants for the acute fructose challenge and 3-week intervention study:
The risk allele will be defined as the allele that is associated with higher levels of liver lipogenesis or fat as per previously published studies in Caucasians.The number of risk alleles will range from 0 to 20. The SNP will get a score of 2 with two risk alleles, 1 with one risk allele and 0 with no risk alleles. All the score will be added to get a cumulative score between 0 and 20 for 10 SNPs. We will select participants having > 15 risk alleles and < 6 risk alleles for our acute challenge and 3-week intervention study.

Phase 2&3- visit 1- MRI scan: The 48 individuals enrolled will visit the UNC NRI clinical suite run by the Human Research Core. After obtaining consent, they will be taken to the WakeForest Imaging center for baseline MRI scan. Studies have shown that 5-6% of liver fat is considered normal and anything more than that can reflect increased risk for fatty liver. If the MRI report of the participants shows liver fat > 6%, we will consult with the participant (if > 18years) and/or their parents in confidence and explain why they (or their child) cannot continue to

participate and that they should consult with their primary care physician. We will also advise them on diet and lifestyle that they can follow to minimize the risk. If non-liver adverse findings are reported during MRI, we will provide the participant with a letter that they can show to their physician.

Phase 2&3- visit 2- Oral fructose challenge:. After their initial anthropometrics and Fibroscan measurements, they will undergo an oral fructose challenge using a protocol used by Hudgins et al[13]. The participants will be provided with a drink consisting of 0.75g/kg fructose + 0.45g/kg glucose dissolved in 24 Oz of water to be consumed within 15 minutes. An average overweight adolescent of 65 kg will consume 48.75g (65 x 0.75g/kg) fructose and 29.25g (65 x 0.45g/kg) of glucose which is similar equivalent to the sugars in two soda cans[28].

Blood and urine will be sampled at baseline and 1 and 3 hours after fructose ingestion. During the challenge period, no other beverage (except water) or food will be allowed.

Anthropometric measurements: Height will be measured using a portable stadiometer. Body composition and weight will be measured by bioelectric impedence technology using the Tanita Dual Frequency total Body composition analyzer (Tanita Inc., Arlington Heights, IL). Measurements will be conducted in a standing position, with subjects wearing light clothing and without shoes. Height will be measured to the nearest 0.1 cm by a stadiometer situated against the wall in an upright standing position. Height and weight recorded will be used to calculate BMI as kg/m^2. Waist circumference (WC) will be measured using a stretch-resistance tape at the midpoint between the lower margin of the least rib and the top of the iliac crest to the nearest 0.1 inch. Waist and hip circumferences will be measured to the nearest cm in a standing position. Sitting blood pressure (systolic and diastolic blood pressure) will be measured three times using the Omron digital blood pressure monitor (HEM907XL, Omron Healthcare Inc, Lake Forest, IL). The means of these measurements will be used for analysis. Well trained personnel in the UNC NRI Human Research Core will administer questionnaires as well as measure anthropometrics, by one staff member to minimize measurement variation and margin of error.

Questionnaires: The following six sets of questionnaires will be administered to the study participants.

- Personal Interview Form: Information related to demographics
- Medical History: Information related to medical conditions, procedures and surgeries
- Modifiable activity form: Information related to physical activity, sedentary time and job activities
- Medications: Information related to prescription, non-prescription and traditional medications
- Dietary data: Combination of diet recall and food frequency questionnaire will be used to obtain detailed dietary data.

Sample preparation: A total of 24 mL of blood will be drawn at fasting at baseline, 1 hour, 3 hours, at weeks 0 and 3 by a trained phlebotomist through venous puncture. After centrifugation, the serum and plasma fractions of the blood samples will be aliquoted and stored at -80°C to measure the biomarkers listed below using standard reference procedures. All samples will be labeled and inventoried as per standard protocols. Urine will be collected at the same points as blood samples collected at baseline, 3 hour at weeks 0 and 3 will be stored at -80°C. The Human Research Core will assist with phlebotomy services

Meals: All participants will be provided standardized meal for the evening prior to participation of part 1 of phase 2.

Assessment of liver fat accumulation and stiffness: Magnetic resonance imaging (MRI): We will assess liver fat accumulation at week 0 and week 3 using the 2012 Siemens MAGNETOM Skyra 3T MRI system available at the WakeForest Translational Imaging program. If the liver fat fraction >5%[10] then we will exclude the participant from further participation in the nutrient challenge.

Transient elastography: We will use transient elastography to ascertain the changes in the amount of fat in the liver. Transient elastography using a **Fibroscan®** is a non-invasive method of assessing fat accumulation in liver and liver stiffness and works on the principle of vibration controlled transient elastography[5]. A vibration of mild amplitude and low frequency is transmitted through the intercostal space using a vibrator at the skin surface. The vibration induces an elastic shear that propagates through the hepatic tissue. Using pulse echo ultrasound acquisition, the velocity of the shear wave can be determined. The shear wave, directly related to tissue stiffness and expressed in kilopascal (kPa), can be a helpful aid to clinically manage patients with liver disease. The UNC Human Research Core houses a Fibroscan and its trained technicians will help with its use for this study.

Medical care: According to the CDC report (cdc.gov/nutrition/data&statistics), ~51% of Americans drink at least one soda or other type of sugar-sweetened beverages on a given day. Higher prevalences have been found in Southern states and in adolescents. Therefore, we don't anticipate any medical issues as three sugar drinks per day is conforming to the present frequency. However, we will refer to our resident clinician in case any participant wants to consult or has concerns. The participant will be free to withdraw anytime during the study with no repercussions.

Dietary compliance: Dietary compliance will be assessed by the use of frequency questionnaires and three 24-hour diet recalls (Phases1,2 and 3).

Dietary guidance: Since each participant will taking daily ~500 Kcal more than their normal daily dietary Kcal, we will provide guidance and options on how to cut down ~500 Kcal from elsewhere in their diet, especially in their consumption of sugary drinks.

Biochemical phenotyping: Liver function markers (alanine transaminase (ALT), aspartate transaminase (AST), gamma glutamyl transferase (GGT)), uric acid and lipids (triglycerides and total cholesterol, high and low density lipoprotein (HDL and LDL) cholesterol will be measured in serum using standard reference methods via clinical chemistry analyzer. Serum lipids, uric acid and liver function markers will be measured in Voruganti lab.

A combination of transient elastography (Fibroscan) and serum liver function markers will be used which will increase the accuracy of non-invasive measurement of fat accumulation in NAFLD; and

Fatty Liver Index (FLI) is a surrogate measure for liver fat estimation. The FLI will be computed based on the following equation which was observed to be 84% accurate in liver fat estimation[6]

Fatty Liver Index (FLI) = $(e^{0.953*\log e(\text{triglycerides}) + 0.139*BMI + 0.718*\log e(ggt) + 0.053*\text{waist circumference} - 15.745}) / (1 + e^{0.953*\log e(\text{triglycerides}) + 0.139*BMI + 0.718*\log e(ggt) + 0.053*\text{waist circumference} - 15.745}) * 100$[6]

4.3 Follow- up procedures (by visits)

There is no follow-up

4.4 Unscheduled visits

- If the participant walks-in or plans to visit due to a health concern, we will refer the participant to our resident clinician or to a healthcare provider

4.5 Concomitant Medication documentation

- Sugary drinks consumption is very common, especially in this age group so we don't anticipate adverse reactions and need for medication. However, if needed, our resident clinician will be available to assist.
- All concomitant medication will be documented in our questionnaire and incorporated as covariates in our data analysis

4.6 Rescue medication administration (if applicable)

4.7 Subject Completion/ Withdrawal procedures

- Participants can voluntarily withdraw anytime during the study if they feel that they cannot continue to consume the drinks. They will be paid according to completion of phases of the study.

4.8 Screen failure procedures

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5 STUDY EVALUATIONS AND MEASUREMENTS (how measurements will be made)

- At baseline and end of week3, we will measure body weight, body composition, blood pressure, waist circumference, liver fat by transient elastography, MRI scan and serum and urinary biomarkers. Blood and urinary biomarkers will also be measured at one and 3 hour timepoints during the acute fructose challenge.
- Body weight and composition (Body weight, body fat, etc) will be measured through a body fat analyzer (Tanita Corporation of America Inc. Arlington Heights, IL)
- Waist circumference (WC) will be measured using a stretch-resistance tape at the midpoint between the lower margin of the least rib and the top of the iliac crest to the nearest 0.1 inch. Waist and hip circumferences will be measured to the nearest cm in a standing position
- Sitting blood pressure and pulse rate will be measured using the Omron digital blood pressure monitor (HEM907XL, Omron Healthcare Inc. Lake Forest, IL)

- Liver fat will be measured using MRI at the Wakeforest Imaging Center and transient elastography (Fibroscan, Echosens, Paris, FR)

***Quality control**:* All phenotypes will be checked for distribution and accuracy of values. Phenotype data collection will be performed on the same equipment with strict quality control regulations. Assay quality is assessed by tracking coefficients of variation for quality control samples that are run with each assay. Appropriate internal standards will be used wherever necessary.

5.1 Efficacy Evaluation (if applicable)

5.2 Pharmacokinetic Evaluation (if applicable)

5.3 Safety Evaluations

6 STATISTICAL CONSIDERATION

6.1 Primary Endpoint

Our main outcome measure is liver fat estimation through MRI scans, transient elastography (Fibroscan) and fatty liver index.

6.2 Secondary Endpoint

Other serum biomarkers such as liver function markers, lipids and uric acid

6.3 Statistical Methods

Each subject will be given sugar drink containing fructose and liver MRI scan with fibroscan measurements will be taken at baseline and end of 3 weeks. Data will be assessed using descriptive measures and confidence intervals. Paired t-tests and repeated measures regression analysis will be performed to explore the significant differences between week 0 and week 3. Also, for each subject, the area under the curve (AUC)[13] will be computed and a linear regression with AUC as the response witll be performed during the acute challenge.

6.4 Sample Size and Power

Although our sample of 48 may not have the power to detect significant effects of fructose on liver fat, this group of 48 well-characterized individuals, focusing only on one ethnicity and one body weight group and specific genotypes will reduce variability and will generate meaningful data. We expect to apply these approaches and use these data to estimate sample size for larger studies with significant power to detect the effects of gene-nutrients interactions on NAFLD and other metabolic disorders.

6.5 Interim Analysis

N/A

7 STUDY INTERVENTION (drug, device or other intervention details)

- Phase 2 &3- visit 2: Acute fructose challenge – each participant will be given a drink made of 24 oz water and fructose (0.75g/kg) and gluose (0.45g/kg).
- 3-week intervention study- each participant will be given a drink to be taken one daily for three weeks starting the next day after acute challenge. These sugar drinks contain the same amount of sugars that are provided as part of the acute fructose challenge

8 STUDY INTERVENTION ADMINISTRATION(if applicable)

N/A

9 SAFETY MANAGEMENT

Data and Safety monitoring plan will be instituted for the project. As per the plan, PI, co-investigators and physician will ensure that the proper protocol is being followed. Any adverse event that is reported to either the principal investigator or her designated research associates by the subject or medical staff caring for the subject and which meets the criteria will be documented as such. Any event that is reported will then generate an adverse event report, which will be submitted to the IRB. The report will include a description of the event, when and how it was reported, as well as any official chart records or documentation to corroborate the event or the

reporting of the event. All adverse events will be graded on a scale from 0 to 5. Any severe and/or unanticipated adverse event will be immediately reported to the safety officer and IRB. All other adverse events will be reported in a timely fashion to IRB, preferably within 2 weeks of the date of the event. All adverse events will be summarized annually and submitted to the IRB. The annual report will address: (1) whether adverse event rates are consistent with pre-study assumptions; (2) reason for dropouts from the study; (3) whether all participants met entry criteria; (4) whether continuation of the study is justified on the basis that additional data are needed to accomplish the stated aims of the study; and (5) conditions whereby the study might be terminated prematurely.

10 DATA COLLECTION AND MANAGMENT

All personnel with access to data collected for the study are required to sign a Confidentiality Pledge form which states that they understand the sensitive and confidential nature of the data and that divulgence of any information will result in disciplinary action. The pledge is co-signed and kept by the PI. Only authorized staff members have the key to the office and access to the data forms. Passwords and/or encryption keys will be known only to authorized personnel safeguard data on computers at UNC-NRI.

Any adverse event that is reported to either the principal investigator or her designated research associates by the subject or medical staff caring for the subject and which meets the criteria will be documented as such. Any event that is reported will then generate an adverse event report, which will be submitted to the IRB. The report will include a description of the event, when and how it was reported, as well as any official chart records or documentation to corroborate the event or the reporting of the event

11 RECRUITMENT STRATEGY

- Subjects will be recruited through our website https://www.uncnri.or/index.php/volunteer/ and other community announcements and events

12 CONSENT PROCESS

- All participants will provide informed consent. Each participant will be consented in private. The PI and/or other project investigators will explain the study to the participant and answer questions that the participant might have. If the participant agrees to participate after reviewing the risks/benefits, they will be asked to sign the consent form. For participants who are not adults, their parents permission will also be obtained. We will try our best to explain in a lay person's language so that the participant is completely aware of the risks associated with participation.

13 PLANS FOR PUBLICATION

- We do plan to publish at least one manuscript based on the results of this study.

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