Study Title:

Hepatic fat content and mitochondrial flux in obese youth before and after bariatric surgery (MANGO Study)

Protocol Version Date: 2/14/2022

NCT03587727

COMIRB Protocol

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Project Title: **Hepatic fat content and mitochondrial flux in obese youth before and after bariatric surgery**

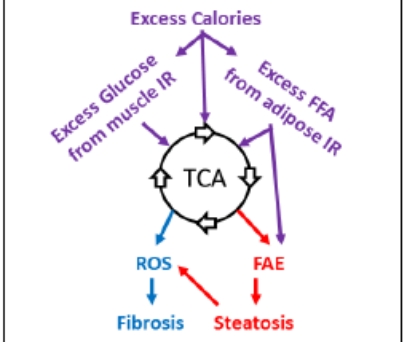
Version Date: **2/14/2022**

Principal Investigator: Melanie Cree Green, MD, PhD

I. Hypotheses and Specific Aims:

NAFLD has become a major public health problem in the United States because of its high prevalence, association with serious cardiometabolic complications and progression to cirrhosis and liver failure. NALFD is characterized by increased intrahepatic triglyceride content (>5.5% of the liver volume or more than 5% of hepatocytes contain triglyceride), and progression to inflammation and fibrosis.[1-3] NAFLD is associated with increased risk of developing type 2 diabetes (T2D), hypertension, and coronary heart disease.[2,4] Obesity is an important risk factor for NAFLD; NAFLD occurs in ~70% of adults with obesity and in ~35% of children with obesity.[5-7] However, data from several studies suggest the histological manifestations and progression to fibrosis are different in adults and children with NAFLD[7]. Differences between the ages suggest distinct pathology as i) Youth with NAFLD may progress to liver failure fairly rapidly, by early adolescence, as compared to a more indolent course in adults and ii) biopsy criteria are distinct with periportal steatosis and fibrosis in youth and sinusoidal ballooning and fibrosis in adults.[7] Alterations in mitochondria function, secondary to an influx of substrates from the diet and dysfunction muscle and adipose tissue are thought to contribute to NAFLD[6,8]. We hypothesize that alterations in mitochondrial energy and substrate metabolism are involved in the pathogenesis and pathophysiology of NAFLD and these factors differ in children and adults. A better understanding of intrahepatic regulation of energy and intermediary metabolism in children and adults with NAFLD is needed to provide a framework for future studies designed to prevent and treat NAFLD across the lifespan.

Fig 1: Obesity induced NAFLD



Excess calories lead to early NAFLD via ↑ mitochondria TCA flux and products

Nonalcoholic fatty liver disease (NAFLD), characterized by an increase in intrahepatic triglyceride (IHTG) content (i.e. steatosis) with or without inflammation and fibrosis (i.e. steatohepatitis), is a common complication of obesity. It is estimated that 70% of adults and 35% of children with simple obesity have NAFLD, with rates of 50% in polycystic ovarian disease and even higher in diabetes[7,9]. In particular, youth with severe obesity who are candidates for bariatric surgery have even high rates of NAFLD, in excess of 50%. Further it is not clear if bariatric surgery decreases the risk of progression of NAFLD, as the influx of free fatty acids during rapid weight loss may actually lead to worsening of NAFLD, if the liver cannot metabolize these increased substrates.

Data from a series of studies conducted in animal models and in people suggest hepatic mitochondrial metabolism is directly involved in the pathogenesis and pathophysiology of NAFLD. Animal and human studies have demonstrated that both the anaplerotic and cataplerotic cycles of hepatic mitochondria are upregulated in NAFLD.[10,11] Excess carbon from

surplus calories (glucose or free fatty acids (FFA)) are directed to the TCA cycle in the mitochondria, but the TCA cycle cannot serve as a carbon sink, and thus cataplerosis is also increased, providing substrates for fatty acid esterification (FAE) and *de novo* lipogenesis.[12,13] In addition, increased TCA cycle activity increases the production of reactive oxygen species (ROS), which cause inflammation and subsequent fibrosis[1] (Fig. 1). Pediatric and adult NAFLD are not the same; hepatic steatosis is located primarily in the periportal areas of the liver in youth but in perisinusodal areas in adults, and youth typically have more rapid progression to fibrosis. The mechanism(s) responsible for these differences is not known, but could be related to differences in mitochondrial dysfunction. Together, these studies underscore the need for sophisticated metabolic studies in both children and adults to better understand the factors involved in the pathogenesis and pathophysiology of NAFLD.

Methods used to assess hepatic mitochondrial energy metabolism include: i) obtaining liver tissue and assessing *ex vivo* mitochondrial ATP production and ii) ^{31}phosphorus magnetic resonance spectroscopy (^{31}P-MRS) to measure phosphate concentrations.[14] The existing ^{31}P-MRS procedure is limited because it assesses non-dynamic concentrations of ^{31}P metabolites or the response to a specific dietary challenge, but does not provide a direct measure of global hepatic mitochondrial energy metabolism.[14-16] In addition, this procedure requires excessive participant time in the magnet which is burdensome, particularly for children, and expensive. *Therefore, there is an unmet need for a non-invasive research method that can assess global dynamic hepatic mitochondrial energy metabolism*.

New methods that magnetically remove the γ-phosphate of ATP via inversion transferase sequences allow for dynamic measurement of phosphate kinetics.[17] This methodology has been successfully used to assess ATP production *in vivo* in skeletal muscle tissue, but has not yet been applied to the liver.[17] This cross-institution project will allow the development of a non-invasive *in vivo* methods to study hepatic energy metabolism. *We will combine the novel ^{31}P-MRS method with concomitant ex vivo analysis of mitochondrial functional capacity by using the Oroboros analysis of liver tissue obtained during bariatric surgery to provide a unique and comprehensive analysis of hepatic energy and substrate metabolism in human participants across the lifespan. Metabolic status will also be categorized at that time. A follow-up study in individuals a year later will allow for evaluation of the long-term effects of bariatric surgery on NAFLD risk and progression and or regression in this youth population.*

In the wake of our national obesity epidemic, bariatric surgery has become a critical option for many youth with severe obesity for management of obesity and related complications such as risk for type 2 diabetes and NAFLD. Bariatric surgery is very effective in inducing weight loss, although the effects on NAFLD are unclear, and may vary by time post-operatively. Following bariatric surgery, there is adipose tissue breakdown in the negative energy state, with very large influxes of free fatty acids to the liver. These FFA can be oxidized in the mitochondria for ATP generation, or stored in the liver[18]. Especially in individuals who have underlying mitochondrial defects in the liver, as is known to occur in NAFLD[19], this can lead to excess deposition of FFA in the liver, and potential worsening on NAFLD state prior to resolution. One limited study in 20 youth with repeat biopsies showed 100 percent improvement in glucose and NAFLD markers and 90% improvement in fibrosis, although fill assessment of diabetes risk was not performed[20]. NAFLD status is the best predictor for development of type 2 diabetes in youth and thus it is important to assess the 2 components of diabetes risk- insulin resistance and insulin secretion. Predictors for improvement of NAFLD beyond weight loss are not clear, especially in youth. A better understanding of the post-prandial nutrient handling following bariatric surgery is needed to further optimize post-surgery therapies to improve NAFLD.

HYPOTHESIS:

1) NAFLD status will be improved by 1-year post-bariatric surgery and this may relate to post-prandial glucose and FFA concentrations and hepatic mitochondrial metabolism.

2) ^{31}P-MRS, blood and liver isotopomer analysis and metabolomics can be used as surrogate measures to assess hepatic function in NAFLD

Specific Aims:
Aim 1: Determine the effects of bariatric surgery on NAFLD status, substrates available for the development of NAFLD (glucose and FFA) and surrogate markers of NAFLD status, 1-year post-surgery
Hypothesis: NAFLD status will be better 1-year post-surgery.
Rationale: Data from adults indicates that NAFLD status can worsen with bariatric surgery. This is thought to relate to the excessive free fatty acid delivery to the liver, however, less is known in youth with severe obesity, and clinical measures such as ALT, which are not very accurate have been used for follow-up testing in youth.
Methods: A study identical to the one performed pre-surgery will be performed, with the exception of the liver biopsy. Alterations in glucose and fat metabolism will be assessed with a mixed meal tolerance test (MMT).
^{31}P-MRS will be used to assess hepatic oxidative phosphorylation, MRI for hepatic fat fraction and MR elastography for liver stiffness. Serum for metabolomics for preidentified markers will be performed.

Aim 2: Utilize resting ^{31}P-MRS method and stable istopomer analysis to measure hepatic mitochondrial metabolism
Hypothesis: Markers of mitochondrial function can be assessed with ^{31} Phosphorus MR spectroscopy (^{31} P-MRS) and stable isotopomer analysis
Rationale: A resting ^{31} P-MRS can give surrogate measures of mitochondrial function. Administration of a glycerol drink with stable isotope tracers and subsequent blood and liver analysis of serum glucose and glycerol labelling and liver TCA cycle intermediates can give direct results on hepatic metabolism. The MR methodology is non-invasive and the blood analysis is finally invasive, as compared to the current need for an invasive liver biopsy.
Methods: The standard pulse/acquire hepatic ^{31} P-MRS protocol is already in use in a similar patient population (14-0542 and 16-2399) and well tolerated. The oral glycerol tracer has/is being used in a similar patient population (16-2399, 18-0803, 19-0636) with and identical blood sampling timeline in 18-0803 and 19-0636 .

Aim 3: Determine hepatic mitochondrial activity in vivo using ^{31}P-MRS, isotopomer and plasma metabolomic analysis and ex vivo using Oroboros in youth with severe obesity. NAFLD features will be further characterized using liver histology to assess steatosis and NASH and by using MR elastography to assess global hepatic fibrosis. Alterations in glucose and fat metabolism will be assessed with a mixed meal tolerance test (MMT). Comparisons between these surrogate markers and biopsy samples will help identify minimally invasive measures to predict NAFLD severity without the need for future biopsies.
Hypothesis: We hypothesize that alterations in mitochondrial energy and substrate metabolism are involved in the pathogenesis and pathophysiology of NAFLD. A better understanding of intrahepatic regulation of energy and intermediary metabolism in children with NAFLD is needed to provide a framework and identify minimally invasive biomarkers for future studies designed to prevent and treat NAFLD.
Rationale: NAFLD is unfortunately an increasing common complication of pediatric obesity. Youth onset metabolic disease confers an early and more severe morbidity and earlier mortality than adult onset disease, emphasizing the urgency of treating this population[21].
Methods: We will compare multiple methods of non-invasive and minimally invasive measure of hepatic metabolism with the gold standard but invasive liver biopsy. ^{31}P-MRS and isotopomer analysis will be used to assess hepatic oxidative phosphorylation, MRI for hepatic fat fraction

and MR elastography for liver stiffness. Serum for metabolomics analysis will be collected and metabolites which related to MR and biopsy measure identified (targeted analysis for fatty acids, acycarnitines and bile acids). Ex vivo measures of mitochondrial function will be measured in liver biopsy samples (already being collected during bariatric surgery for clinical NALFD staging) with oroboros technologies. NAFLD staging data from the pathology lab will also be collected. Alterations in glucose and fat metabolism will be assessed with a mixed meal tolerance test (MMT).

III. Research Methods

A. Outcome Measure(s):

Primary Outcome Measure(s): Post-prandial glucose, insulin, C-peptide, GLP-1 and glucagon dynamics as assessed with an MMTT.
Secondary Outcome Measure(s): Hepatic fat content per MR, and biopsy, Stiffness per MR elastography,and fibrosis per biopsy. . Exploratory Aims: ex vivo hepatic mitochondrial activity per biopsy, isotopomer analysis and Hepatic mitochondrial activity per ^{31}P-MRS. Likely contributors to above measures: Lipid/glucose markers:(fasting C-peptide and lipid panel, HbA1c); hepatic markers:(c-reactive protein, glucagon, GLP-1 and leptin, adiponectin, AST, ALT, GGT) Body size and composition: (BMI, waist/hip ratio, Bodpod, hepatic visceral fat via MRI[22-25]), Whole body fat oxidation at rest as measured with a metabolic cart; Physical activity/ diet:(accelerometer, activity survey (3DPAR); Food frequency survey). Questionnaires for presence of obstructive sleep apnea. Questionnaires for perceived mental strengths and difficulty (note there is no assessment of suicidality on this tool).

B. Description of Population to be Enrolled:

Study staff aims to enroll 16 obese boys and girls 13-20 years old.

Ethnic Categories	**Gender**		
	Females	Males	Total
Hispanic or Latino	7	6	**13**
Not Hispanic or Latino	6	6	**12**
Ethnic Categories: Total of All Participants	**13**	**12**	**25**
Racial Categories			
American Indian/Alaska Native			
Asian			
Native Hawaiian or Other Pacific Islander	2	2	
Black or African American	2	2	
White	9	8	
Racial Categories: Total of All Participants *	**13**	**12**	**25**

Inclusion Criteria:

1. Obese youth (>95th percentile for age and sex) ages 13-20 years, scheduled for bariatric surgery
2. BMI 35-60 kg/m^2
3. Largest body circumference <200cm (in order to fit into MRI machine)

Exclusion Criteria:

1. Use of medications known to affect insulin sensitivity or hepatic outcomes: oral glucocorticoids within 10 days, atypical antipsychotics,

immunosuppressant agents, HIV medications, PPAR-γ or PPAR-α, metformin.
2. Infectious hepatitis
3. Alcohol abuse, (defined by the America Association for the Study of Liver Diseases as >21 drinks/week for males >14 drinks/week for females, with a drink considered 14 grams of alcohol)
4. Mitochondrial disease
5. Currently pregnant or breastfeeding women. Development of pregnancy during the study period will necessitate withdrawal from the study.
6. Severe illness requiring hospitalization within 60 days
7. Diabetes, defined as Hemoglobin A1C > 6.4%
8. Anemia, defined as Hemoglobin < 10 mg/dL
9. Diagnosed major psychiatric or developmental disorder limiting informed consent
10. Implanted metal devices that are not compatible with MRI

C. Study Design and Research Methods

Study Calendar	Screening Visit	Baseline Visit: MMTT and MRI	Post-Surgery Visit: MMTT and MRI
Consenting and Eligibility Assessment	X		
History & Physical		X	X
Urine Pregnancy Test (females only)		X	X
Questionnaires- SEARCH Food frequency, Activity 3DPAR, Strengths and Difficulties and Sleep assessments		X	X
Bodpod		X	X
Mixed Meal Tolerance Test (MMTT)		X	X
MRI of abdomen and liver		X	X
^{31}P-MRS of Liver		X	X
Metabolic Cart		X	X
Glycerol drink - optional		X*	X
Total Time of visit (approximately)	30 minutes	6-8 Hours	6-8 Hours
Location of Visit	CHCO CTRC Outpatient	CHCO CTRC Outpatient, and Brain Imaging Center	CHCO CTRC Outpatient, and Brain Imaging Center

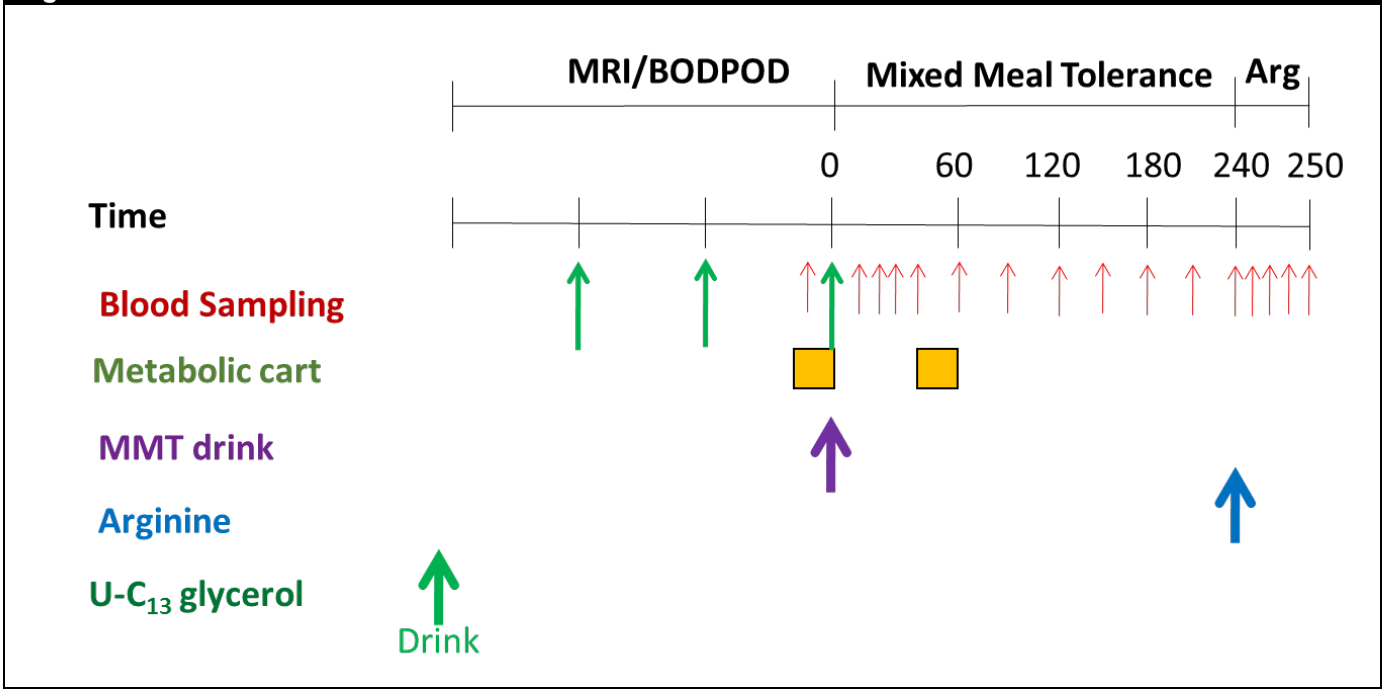
The study consists of 3 outpatient visits. The Baseline Visit would be completed within two months prior to bariatric surgery, and the Post-surgery visit will be scheduled approximately 1-year post-surgery.
*The glycerol drink will be taken the day of the Baseline visit, and in the morning of operation, then 1 year later
NOTE: Depending on scheduling and availability, the MRI scans can be completed on a separate visit within 10 days of the MMTT.

SCREENING VISIT
Pediatric CTRC Outpatient Unit: Chart review of bariatric patients will be conducted for inclusion/exclusion criteria evaluation. Participants whom appear to meet eligibility criteria would then be approached about a screening visit. During this visit, patients will review and complete consent documents, have demographics and medical history confirmed, assess allergies and further evaluation of inclusion/exclusion criteria, and have anthropometrics completed for eligibility assessment.

BASELINE VISIT 2 (FIGURE 2: MMTT AND MRI)

Overall Plan:

Figure 2: Baseline Visit



MRI at UCD Research (Brain Imaging Center):

1. The imaging will be of the liver. Participants will undergo a standard MRI of the mid-abdomen to assess the amount of subcutaneous fat, visceral fat and percent liver fat. MR elastography will be performed to assess liver stiffness. Finally, ^{31}P spectroscopy to measure concentrations of phosphate molecules including PDE, PME and PCr.

Abdominal/Hepatic Imaging and Elastography

Imaging will be performed on a Siemens 3 Tesla MRI magnet. Hepatic fat fraction will be performed using modification of the Dixon method as in our previous studies[9,26]. Visceral adiposity will be measured using the gold standard of an MRI slice at L4-L5[9]. Hepatic fibrosis will be measured with a standard clinical elastography sequence.

^{31}P MRS of the Liver

MRS Data acquisition: MRS will be performed on a Siemens 3 Tesla MRI magnet with research capabilities. A custom ^{1}H/^{31}P abdominal coil will be used for imaging and MRS (Clinical MR Solutions, Brookfield, WI) as in our previous ^{31}P work[27]. The coil has a concentric probe with an inner coil 16-18 cm in diameter (for ^{31}P) and a 20 cm outer coil (for ^{1}H scout imaging and shimming). Resting phosphate concentrations can be measured with a pulse/acquire sequence.

MRS Data Analysis: For the ^{31}P data, peak positions and areas of interest [phosphocreatine (PCr), inorganic free phosphate (Pi), β-ATP(3 peaks), α-ATP(2 peaks), γ-ATP(2 peaks), and PME] will be determined by time domain fitting with jMRUi [28,29], utilizing AMARES (A Method of Accurate, Robust and Efficient Spectral fitting), a nonlinear least-square-fitting algorithm using our previously built prior knowledge files [30].

We have utilized this method for muscle ^{31}P analysis for the previous 6 years, and have extensive experience with this analysis. Percent PME relative to all other phosphate peaks will be calculated.

2. MMTT at Pediatric CTRC Outpatient unit: Participants will be asked not to have caffeine or exercise for 3 days prior to this visit. After an overnight fast, participants will ingest a UC13 glycerol tracer, then 3 hours later a liquid meal consisting of (45g carbohydrates,14g fat, and 14g protein). The metabolic response to the tracer will be measured for 3 hours fasting, and then the MMT will be measured for 4 hours. Body composition will be measured by BodPod.

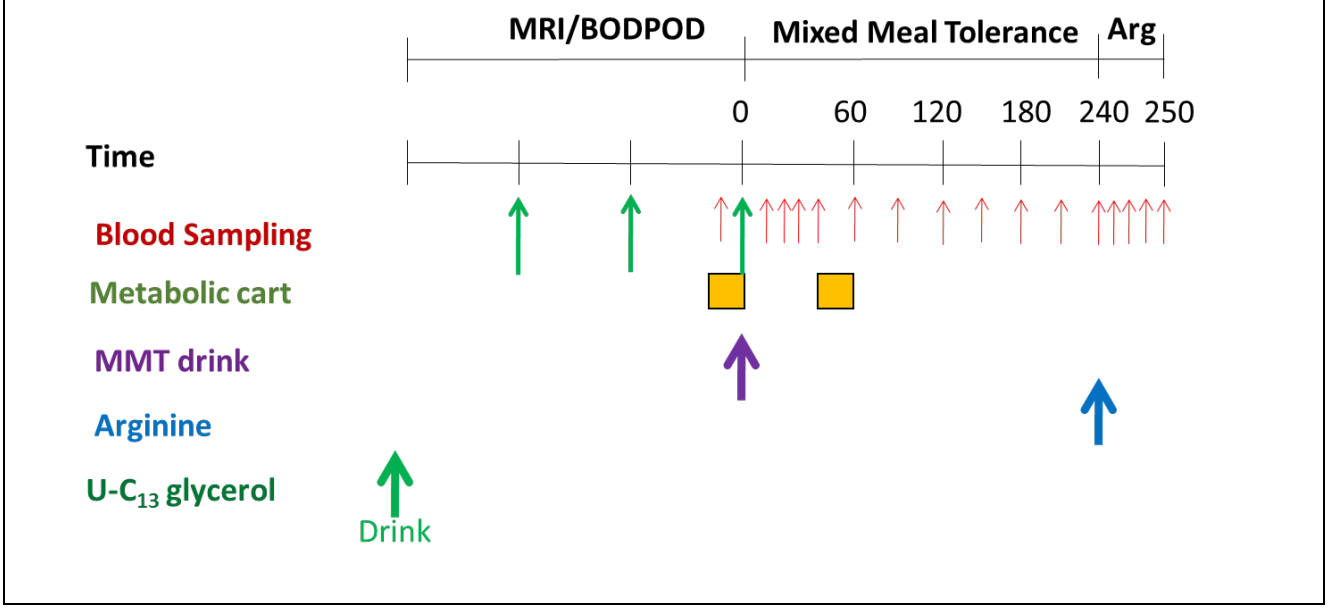
Accelerometer: One week prior to the MMTT visits, the participant will be provided two accelerometers (GT3X BT by Actigraph and ActiWatch by Philips Respironics) to be worn for seven days to measure level of habitual physical activity, which affects insulin sensitivity, and sleep patterns. Accelerometers are effective tools for the objective measurement of physical activity [31] because they have the ability to continuously record physical activity data and such data can be used to estimate METs of activity. They provide more detailed information than pedometers, which only measure walking steps, and help get around the recall bias of questionnaires. We are currently using the GT3X BT Actigraph in adolescents in our other diabetes studies; therefore, we are familiar with their use in this population and have the necessary computer software and interpretation skills. The Actiwatch is being used as a tool for objective measurement of sleep patterns. The Actiwatch is fitted with a LED monitor that detects multiple spectrums of light to better assess sleep patterns in this population.

*One week prior the MMTT, participant will receive via mail the accelerometer, sleep watch, sleep diary, and food log.

*Note: The day of the surgery, participants will injest the UC13 glycerol (small volume 30 ml, clear) 2 hours prior to anesthesia time. This is within the standard pre-surgical patient recommendations for small volume of clear liquids up to 2 hours pre-anesthesia. Participants will have a liver biopsy during their bariatric surgery as part of their clinical standard of care. Participants will be asked to give their consent for this study to use a small sample of the liver tissue for research purposes. We will also collect approximately 7 teaspoons of blood during surgery to measure glucose, insulin glycerol, FFA, triglycerides and isotopomer analysis at the time of the liver biopsy.

POST-SURGERY VISIT 3 (FIGURE 3: MMTT AND MRI):
Same procedures as Baseline Visit will be repeated 1-year post-surgery (with exception of liver biopsy sample).

Figure 3: Post-Surgery Visit



Purpose for lab test to be drawn:

MMTT Labs	Purpose
Glucose	Determination of IR
Insulin	Determination IR and beta cell function
FFA	Measure of lipolysis
Glycerol	Measure of lipolysis
Glucagon	Gut hormone known to influence meal response glycemia
GLP-1	Gut hormone known to influence meal response glycemia
GGT	Marker of hepatic disease
C-peptide	Measure of beta cell function, insulin clearance
CMP	Markers of hepatic disease (Alk phos, ALT, AST, albumin)
Lipid panel	Markers of serum lipids, hepatic function
HbA1c	Long term marker of glycemia
Stored blood	Future markers of glucose and fat metabolism, CVD or hormones related to NAFLD
Isotopomer analysis	Hepatic mitochondrial function

Purpose for questionnaires being done:

Questionnaire	Description	Study Validity
Adolescent Sleep Hygiene Scale	Measurement of sleep patterns/habits	Cronbach's alpha ranges from .46-.74; total scale alpha = .80 LeBourgeois et al. 2005. *The Relationship Between Reported Sleep Quality and Sleep Hygiene in Italian and American Adolescents*
Center for Epidemiological Studies Depression (CES-D)	Measuring for depression	See Table 3 below, adapted from: Stockings et al. 2015. *Symptom screening scales for detecting major depressive disorder in children and adolescents: A systematic review and meta-analysis of reliability validity and diagnostic utility*
Cleveland Adolescent Sleepiness Questionnaire	Measurement of sleepiness during a typical week	alpha = .89 Spilsbury et al. 2007. *The Cleveland Adolescent Sleepiness Questionnaire: A New Measure to Assess Excessive Daytime Sleepiness in Adolescents*
SDQ: Strengths and Difficulties Questionnaire	Behavioral screening questionnaire	The internal reliability of the various self-report scales was assessed using Cronbach's alpha coefficient. This was 0.82 for the total difficulties, 0.75 for emotional symptoms, 0.72 for conduct problems, 0.69 for hyperactivity, 0.65 for prosocial behavior, and 0.61 for peer problems. Goodman et al. 2003. *The Strengths and Difficulties Questionnaire: a pilot study on the validity of the self-report version*
Sleep Disturbances Scale for Children	Gain understanding of sleep-wake rhythm and any problems in sleep behavior	Internal consistency ranged from .71-.79l test-retest reliability r = .71 Bruni et al. 1996. *The Sleep Disturbance Scale for Children (SDSC)Construct ion and validation of an instrument to evaluate sleep disturbances in childhood and adolescence*
3DPAR: Activities Scale	Measuring activity in the 3 days previous as a typical activity score	Interrater and test-retest reliability was 0.99 and 0.98, respectively ($P < 0.01$). The correlation between relative energy expenditure from the PDPAR (kcal.kg-1.l.d-1) and pedometer and Caltrac counts was 0.88 ($P < 0.01$) and 0.77 ($P < 0.01$), respectively. The correlation between percentage heart rate range (HRmax-HR-rest) and mean energy expenditure from the PDPAR was 0.53 ($P < 0.01$). The correlation between 1-min heart rates > 50% HRR sustained for 20 min and the number of 30-min blocks with a relative energy expenditure of at least four metabolic equivalent tasks (MET) was 0.63 ($P < 0.01$). The PDPAR provides valid and reliable estimates of physical activity and also accurately identifies bouts of moderate to vigorous activity. Weston et al. 1997 *Validation of an instrument for measurement of physical activity in youth*

Food Frequency Questionnaire	Measuring typical food intake over previous seven days.	The mean correlations, adjusted for measurement error, of food groups and nutrients between the FFQ and true usual intake were 0·41 and 0·38, respectively, with 57 % of food groups and 70 % of nutrients exhibiting correlations >0·35. Correlations were high for low-fat dairy (0·80), sugar-sweetened beverages (0·54), cholesterol (0·59) and saturated fat (0·51), while correlations were poor for high-fibre bread and cereal (0·16) and folate (0·11). Reliability of FFQ intake based on two FFQ administrations was also reasonable, with 54 % of Pearson correlation coefficients ≥0·5. Reliability was high for low-fat dairy (0·7), vegetables (0·6), carbohydrates, fibre, folate and vitamin C (all 0·5), but less than desirable for low-fat poultry and high-fibre bread, cereal, rice and pasta (0·2-0·3). Liese et al. 2015 *Relative validity and reliability of an FFQ in youth with type 1 diabetes* First described in 2006: Mayer-Davis et al. 2006. *Search FFQ Dietary Intake among Youth with Diabetes: The SEARCH for Diabetes in Youth Study*
Optional 7 Day Food Log	Optional record of 7-day diet to aid in food recall. Added per participant request to help fill out food frequency questionnaire.	N/A
Actigraphy Daily Sleep Diary	Recording bedtime/wake time during actigraphy. Needed to corroborate watch collected data	N/A

PARTICIPANT RECRUITMENT/CONSENT/PAYMENT

1. Participant Recruitment Plan

Participants will be recruited from the bariatric surgery clinic at Children's Hospital Colorado. The PI and Co-I's have a treatment relationship with the bariatric surgery patients from clinic or participants can call from study advertisements. Protected health information will only be accessible by study investigators. The initial patient contact will be made by personnel who have a treatment relationship with the participant if a chart review by treating physician reveals that they may qualify for the study.

2. Informed Consent Plan

Appropriately qualified and informed personnel who have completed the COMIRB and HIPPAA course requirements will fully explain the study protocol and consent form to the participant and guardian verbally in the language they understand. The explanation will be conducted in a quiet

environment with adequate time given for the participant and guardian to review the study procedure before the commencement of the study. Asking the participant to explain the study in their own words will assess the participant's understanding. If non-English speaking participants are enrolled in the study, the investigators will adhere to Section 10C of the COMIRB Instructions for Clinical Investigators regarding the consent of these participants. The consent form will also be translated into Spanish. The qualified personnel mentioned above will then obtain written consent from the guardian and assent from the participant, co-signed on the consent form, or in participants who are 18 years old, direct consent. The participant and guardian will be provided a copy of the consent form for better understanding and record purposes.

Special Consent/Assent Plan

Consent will be obtained from all participants in the study. Following explanation, all participants under 18 years old will co-sign the consent form in addition to the parent/legal guardian signing the consent form. All participants age 18 years old will sign the standard consent form.

Participant Compensation, Incentives and Rewards

Participants will be compensated with Target gift cards. They will receive a total of $100 for completing Baseline Visit: MRI/BodPod ($50) and MMTT/questionnaires ($50). Post-surgery visit is also a total of $100: MRI/BodPod ($50) and MMTT/questionnaires ($50).

Total paid to participants for completing the full study is $200, but there may be additional payments listed below (max payment to one participant is $600).

Participants may be paid an additional $50, if they have to come for an extra visit (ie MRI). For participants who live 50-99 miles from Children's Hospital Colorado, some travel expenses (mileage) may be eligible for reimbursement up to a maximum of $50. If they live 100 or more miles from Children's Hospital Colorado, some travel expenses (hotel stay, mileage, or a plane ticket) may be eligible for reimbursement up to a maximum of $300.

D. Description, Risks and Justification of Procedures and Data Collection Tools:

Please note: with the emergence of COVID-19 we must follow hospital policies for screening hospital patients and visitors. This may include testing for COVID-19. This may involve a nasal swab, and the risks of this are discomfort and potentially bleeding from nose. To limit participant time in our clinics, portions of study visits may be done virtually (phone or Telehealth).

1. IV Risks and Blood Sampling

Description: One peripheral IV will be placed to draw blood during the MMTT. Blood will be drawn for labs relating to glucose and fat metabolism.

Risk: Risk of pain, bruising at site of blood draw. There is temporary discomfort when the needle goes in and 10% of the time there is a small amount of bleeding under the skin that may produce a bruise. Rarely, there is a risk of a blood clot forming or infection. We will use a low dose of a medication called heparin to try to prevent blood clotting.

Justification/Minimization: Our MMTT visit is within the NIH Clinical Center guidelines of 9 ml/kg in 6-8 weeks and within Children's Hospital Colorado's institutional guidelines of 5 ml/kg. These studies involve sampling blood at multiple time points. Thus, an IV is needed, so as to avoid multiple needle sticks. These studies are focused on measured rates of change which necessitates the sampling of the same test over time. Proper sterile technique will be used with blood draws and IV placement to decrease the infection risk. EMLA cream will be offered if participant desires to minimize pain of IV. If the patient is being admitted that same day for

bariatric surgery this IV can be left in place, so that when patient is admitted for their bariatric surgery, a new IV does not need to be started.

2. Mixed Meal Tolerance Test (MMTT):
Description: An MMTT will be performed with multiple blood draws. The purpose of the MMTT is to provide a controlled oral stimulus to effect changes in glycemia, pancreatic and gut hormones and lipolysis.
Risk: There are no known risks for an MMTT, but participants may not like the taste of the drink and may rarely experience nausea after consuming the drink.
Justification/Minimization: Our team of investigators, CTRC pediatric research nursing staff and physicians are well experienced with similar blood draw procedures. A CTRC nurse will be available during our outpatient visits and patients will be distracted by TV or other similar means during the MMTT, to minimize queasiness.

3. Magnetic Resonance Imaging (MRI) and MR elastography and MR spectroscopy
Description: The MRI will usually be obtained the day of MMTT at the UCD Brain Imaging Center on the Fitzsimmons campus. A trained research radiographer who is supervised by Dr. Mark Brown, of UCD radiology, will perform an abdominal MRI to obtain hepatic, visceral and subcutaneous fat on a 3.0 T whole–body MRI scanner (Siemens MAGNETROM, Malverne, PA). Participants will lie supine while these measurements are obtained, need to hold reasonably still during the scans of approximately 1 hour and cannot weigh >550 lbs or have a largest body circumference > 200 cm. A standard research sequence to measure hepatic fat is performed (approx. 10-15 min). A standard clinical MR elastography sequence to measure the amount of fibrosis (if any present) in the liver will be performed (approximately 5 min). A specialized phosphorus coil will be utilized to measure the concentration of ^{31}P via MR Spectroscopy to assess mitochondrial function (approximately 20-30 min).
Risks: Minimal. Participants may develop claustrophobia in the magnet.
Minimizing Risk: The participant is provided with audio protection and optional television to help increase comfort. Some participants might feel claustrophobic while having an MRI and the scan will be stopped if it cannot be tolerated. In addition, any participants with implanted metal that is not cleared by the MRI technician may not be able to have the MRI due to the type of magnet involved.
Justification/Minimization: MRI is a non-invasive and non-radiation method to assess body fat, and mitochondrial function. The risks are minimized by assuring patient comfort prior to starting the scan, placing eye goggles that plays movies on the participants. Further, per standard protocol, no patient will be placed into the scanner if they do not meet the rigorous safety standards for the MRI, including the absence of non-compatible implanted metal.

4. Body Composition
Description: Participants will also undergo body composition analysis by BodPod by standard methods. BodPod is a noninvasive air displacement plethysmograph which uses whole-body densitometry to determine fat and fat-free mass. Participants sit inside of the BodPod for approximately 2 minutes and rest while measurements are taken.
Risk: Minimal. Participants may feel claustrophobic while in the BodPod.
Justification/Minimization: There is a large glass window in which the participant can see out of and there is also a button located inside the chamber that can be pressed if the participant wishes for the procedure to stop and exit the BodPod.

5. Body fat distribution

Description: Height, weight, waist circumference, and hip circumference will be measured. Body fat distribution will be determined using the waist-to-hip ratio where the waist circumference is measured 1/2 the distance from the xiphoid process to the navel and the hip circumference is measured at the level of the greater trochanter.
Risk: None
Justification/Minimization: IR has been associated with central obesity. Whereas we are measuring central obesity with MRI, it is important to see if this simple non-invasive measure matches the MRI results, as it is a much simpler measure to follow clinically.

6. Accelerometer:
Description: Participants will be provided two accelerometers (GT3X BT by Actigraph and ActiWatch by Philips Respironics) to be worn for seven days to measure level of habitual physical activity, which affects insulin sensitivity, and sleep patterns. The accelerometers will be worn on the participant's wrist. The wrist position has been validated to hip position actigraphy in this population.
Risk: There is no risk involved with the accelerometer.
Justification/Minimization: Accelerometers are effective tools for the objective measurement of physical activity [31] because they have the ability to continuously record physical activity data and such data can be used to estimate METs of activity. They provide more detailed information than pedometers, which only measure walking steps, and help get around the recall bias of questionnaires. We are currently using the GT3X BT Actigraph in adolescents in our other diabetes studies; therefore, we are familiar with their use in this population and have the necessary computer software and interpretation skills. The Actiwatch is being used as a tool for objective measurement of sleep patterns. The Actiwatch is fitted with a LED monitor that records multiple spectrums of light to better assess sleep patterns in this population.

7. Indirect Calorimetry with a metabolic cart:
Description: The metabolic cart measures the amount of air that the participant breathes in and out. The machine attaches to the participant's mouth through a tube, or a plastic bubble that is placed over the participant's head. There is the potential for experiencing claustrophobia from having the plastic bubble over the participant's head. A metabolic cart will be utilized during the MMTT study day to measure rates of oxygen consumption and carbon dioxide release. These rates can be utilized to calculate rates of carbohydrate and fat oxidation and resting energy expenditure.
Risk: Minimal risk of claustrophobia.
Justification/Minimization: These studies are well tolerated by youth, and involve placing a clear plastic hood over the participant's head for approximately 20 minutes. This procedure has been well tolerated in the youths we have studied.

8. Liver biopsy.
Description: A liver biopsy will be collected at the time of bariatric surgery. This tissue will be used for pathology scoring, isotopomer analysis and for oroboros.
Risk: The biopsy will only be collected as part of standard medical care and therefore no additional risk is posed by the newly proposed study. Risks of this procedure will be reviewed with participants by the surgical team.
Justification: NALFD is high in severe obesity, and the liver biopsy is collected for clinical purposes. The amount of tissue required for oroboros is minimal, and does not change the biopsy procedure.

9. Food Frequency Questionnaire (SEARCH FFQ)
Description: Customary macronutrient pattern will be ascertained by diet interview at the time of admission using a SEARCH FFQ, modified to incorporate common food choices among

ethnically and regionally diverse youth aged 10-19 participating in another large childhood diabetes study, SEARCH (48). The instrument is self-administered with staff support to provide instructions, answer questions, and to review the form after completion, and captures the last week of dietary intake.
Risk: None
Justification/Minimization: Several of the measurements being assed are affected by prior nutritional intake.

10. 3DPar Questionnaire
Description: A questionnaire (3DPAR) recalling the physical activity levels of the three previous days will be completed at visit 3.
Risk: None
Justification/Minimization: Physical activity can directly affect insulin sensitivity, our primary outcome measure. The 3DPar is a well validated measure to assess 3 days of physical activity in youth, and includes a variety of youth centric activities.

11. Strengths and Difficulties Questionnaire:
Description: This is a survey which identifies areas in a youth's life that they believe they are strong or weak in dealing with, as a measure of coping skills. Low coping skills have been associated with the development of depression.
Risk: None
Justification/Minimization: This survey can help identify youth at risk for depression or anxiety, and identify poor coping skills. It does not directly assess depression or suicidality.

12. Stable Isotope Studies Glycerol:
Description: Oral stable isotope tracer of glycerol will be utilized to determine rates of intrahepatic substrate flux. These are substances normally present or produced in the body, and thus pose no more risk than typical glucose infusions. Measurements of these metabolic processes are only able to be made with the utilization of stable isotope tracers.
Risk: We are utilizing an isotope which already exists in all humans, but are simply increasing the percentage. We are only giving this medication orally. These are NOT radioactive substances.
Justification/Minimization: Pyrogen-free $^{2}C13$ glycerol will be obtained from the manufacturer and delivered to CHCO IDS. The IDS pharmacist will deliver the tracer to the pediatric CTRC (inpatient unit or 3rd floor outpatient) once ordered by the physician.

13. Violation of Privacy and Loss of Confidentiality
Description: These are both risks to which research participants are exposed. The possibility of these risks increases when protected health information is collected. Every effort will be made to decrease this risk by limiting access to protected health information, storing this information in a password protected database, and identifying participants only by a unique identifier that is kept in a separate location in a locked container, traceable only by study personnel. All of the tests involve the risk of identifying asymptomatic abnormalities. The study may include risks that are unknown at this time.
Justification/Minimization: Every effort will be made to decrease the risk of loss of confidentiality by limiting access to protected health information, storing this information in a password protected database, and de-identifying study specimens.

E. Benefits of the study:

Benefits to Society:

This study has several potential benefits to society. We will be comparing less reliable non-invasive clinical measures of NAFLD or invasive clinical NAFLD measures with multiple research methods of assessing NAFLD. Whereas we are utilizing a bariatric population for this study to take advantage of the clinical indication for a liver biopsy, the would could potentially be translated into numerous other populations which experience higher rates of NAFLD, such as those with obesity, polycystic ovarian syndrome, obstructive sleep apnea, and of Hispanic heritage. We will also be obtaining data on the post-bariatric surgery meal response in youth, to better understand how this procedure affects multiple aspects of metabolism and potential diabetes risk.

Knowledge to be gained:

A better understanding of how NAFLD in severe obesity could lead to more effective treatment strategies. This understanding could ameliorate development of diabetes and heart disease for these individuals. Since severe obesity is becoming more common, improving severe obesity care could have major health implications. The data to be generated from this project will be utilized to inform R01 grant applications of treatment trials with new medications.

Benefits to participant:

The participant will undergo a metabolic cart which will determine basal metabolic rate. This information can be used by the medical team post-surgery to help determine the optimal caloric intake to assist with weight loss post-surgery.

The participant will have a thorough evaluation of their hepatic function. If abnormalities are noted, we will let the post-bariatric surgery team know, as this could influence their post-bariatric surgical care.

With the MMTT, we can identify if the participant has pre-diabetes. This will not occur otherwise clinically, and this again may change the post-surgical screening, and would be brought to the attention of the clinical team.

Evidence of Direct Benefit

We believe that this protocol is in the 405 risk category for pediatric research. Participants can benefit from the above study measurements.

Adolescents with severe obesity are at increased risk for diabetes, reduced exercise capacity, cardiovascular dysfunction, and increased visceral and liver fat. Therefore, obese non- severe obesity participants may benefit from the results of body composition, basal metabolic rate testing, MRI testing, glucose testing, insulin resistance assessment during baseline and 1-year post bariatric surgery. The metabolic cart testing can detect the basal metabolic rate, which can determine a personalized goal for target caloric intake following surgery. The MRI of the liver can detect early evidence of fatty liver disease or fibrosis. The blood tests done for screening can detect alterations in blood glucose and prediabetes. The risk of all of these endpoints is increased in sedentary participants, especially those who are also obese, and if left untreated can increase long term health risk, thus the benefit of detecting any of these would directly impact both the health and the longevity of the individual participant.

F. Alternative Treatment

The alternative is for participants to not participate in the study

G. Consideration of Specific Participant Categories

1. Inclusion of Women

Every effort will be made to include a diverse gender distribution.

2. Inclusion of Minorities

Every effort will be made to include a diverse participant distribution. Severe obesity affects Caucasians, Hispanics and African Americans equally.

3. Inclusion of Children

All participants will be between ages 13 and 20. Insulin sensitivity needs to be studied in the adolescent age group as available is scarce in this age group and it is critical to understand the pathophysiology of NAFLD in its developing stages.

IV. Potential Scientific Problems:

Limitation of Method Development:

The protocol as described includes an OSTT with an oral tracer will be conducted in some of the participants. The specific points that are to be evaluated are listed below:

1) Measurement of Hepatic Substrate flux in youth during fasting.
   a. Purpose: Asses hepatic substrate flux in youth looking at shifts from pentose phosphate pathway (PEP), TCA cycle and fatty acid synthesis
   i. Rational: utilizing and oral glycerol model with NMR isotopmer analysis is a newer technique. We have validated the timing with an OSTT in youth. However, this relies on consistent OSTT responses, and in this study we will be using an MMTT. When we anticipate that the response to the MMTT may change pre/post surgery, and the timing and thus results would vary, and we are thus better off using a fasting model. This has been done in adults, but not in our youth.
   ii. Measurement: NMR isotopomer analysis with pathway flux calculations and modeling in the fasting state
   iii. Revision plan if model not correct: 1) increase the amount of tracer given 2) increase the frequency of blood draws in the later sampling period.

Overall Project Limitations:

1) Participant recruitment is always a potential concern, but is minimized by a streamlined recruitment system and experience with this population; the previous, more intensive study is well tolerated in youth and enrolled faster than projected. The bariatric surgery clinic is becoming established, and referrals are increasing rapidly, now more than 10 a month. Of the surgeries completed in the last 5 months since the start of the clinic, 15 of the youth would have potentially qualified for this protocol.

2) Participant drop-out: We expect reasonably good completion rates due to the minimally-invasive nature of the study visits and our low dropout rate in our previous studies in control and severe obesity adolescents using similar procedures.

3) Underpowered: This is a pilot study and is likely underpowered to find associations between changes in metabolism and NAFLD status. However, the data generated from this protocol could then be used to apply for funding to continue the study in a larger cohort of youth, to fully answer some more of the mechanistic questions. If we can obtain additional funding we will revise the protocol to include more participants.

V. Data Management and Security Plan

Data Entry
Data will be entered from paper forms. Once forms are completed, verified and corrected for inconsistencies, they will be manually entered at our site using a computerized data management system (Redcap).

Edit Checks
Computerized data validation routines will be used to enhance data quality and verify the accuracy of data within predefined value ranges. These checks include, but are not limited to: (a) initial screening of data, using logic and range checks built into data entry screens; (b) cross-form functional and consistency checks; and (c) edits assessing the serial integrity of data.

Disaster Recovery
Routine data backup will occur on data in conjunction with the Children's Hospital secure server and Redcap.

Security and Confidentiality
All hard copy forms will be de-identified with a study number and filed in a locked in a place in which only the investigators and qualified study team will have access. Standard protection against computer hackers is implemented. Recovery from natural disasters (water, fire, or electrical) can occur through the ability to reconstruct both the database management system and the data from nightly backups.

VI. Data and Safety Monitoring Plan

The principal investigator and study coordinator will monitor the protocol and the safety of the research participants. The PI will review all laboratory data and report any abnormal values to the patient and guardian and instruct the participant to follow-up with their PCP. If an abnormal result from a research procedure exists, the PI will notify the family, the bariatric surgery team and their PCP and refer the participant to the appropriate clinic for further evaluation. The PI may also share research results in a reasonable and prudent manner with appropriate medical professionals if the participant was seriously injured as a result of a procedure or if follow-up of the result of the procedure is in the best interest of the participant's health as determined by a medical professional. If immediate medical follow-up of participant required, the PI will share the research results via EPIC when clinically relevant. The PI will report adverse events, and any decision to suspend or halt the protocol to CTRC and COMIRB immediately. The PI will also prepare a written report for the yearly continuing review required by COMIRB and the CTRC. There are no other entities that require notification about this protocol.

No protected health information will be collected until the appropriate HIPAA forms are completed. The protected health information that will be collected will include: Name and phone number, demographic information (DOB, sex, ethnicity, address, etc.), diagnosis(es), history and physical, laboratory or tissue studies, radiology studies, procedure results, survey/questionnaire results, research visit records, and portions of previous Medical Records that are relevant to this study. This information will be accessible only by the study investigators, Federal agencies overseeing human participant research, the Colorado Multiple Institutional Review Board, regulatory officials from the institution where the research is being conducted to monitor safety and compliance with policies.

A. Adverse Events (AE)

The MMTT is a standard procedure used in a large number of research studies and settings. Adverse events are uncommon when the procedure is done by experienced personnel in an appropriate setting.

MRI procedures are considered safe as long as the participant does not have implanted metal. All participants undergoing screening by trained MR personnel prior to any scanning procedures.

1. Adverse Event Definition

For the purposes of this study, an Adverse Event (AE) is defined as any significantly abnormal physical finding identified on examination and any significantly abnormal laboratory result obtained on the patient between visits or at the time of the visit. Questions answered YES and any new abnormal physical findings are pursued by the study staff in order to determine the seriousness of the event and the need for further evaluation, follow-up, or referral. If follow-up or referral of abnormal research results or procedure is required, the PI will place a referral in EPIC, update the PCP on patient condition and reason for referral and contact family to discuss follow-up treatment options.

a) Adverse Event Reporting

AEs are reported on a standard form that is completed by the study staff at each regular follow-up visit and phone interview. Adverse events reported or ascertained between clinic visits are captured and reported at the time of the next scheduled visit.

Pre-existing conditions (that is, conditions present prior to study enrollment) are not considered or recorded as AEs or SAEs unless the condition worsens in intensity or frequency after enrollment. Likewise, continuing adverse events are not reported as AEs at subsequent visits unless they increase in severity or frequency between the visits, they result in criteria for an SAE, and/or they resolve between visits.

B. Serious Adverse Events (SAE)

1. Serious Adverse Event Definition

Events are divided into those that are not serious (AE) and those that are serious (SAE). The distinction between an SAE and an AE is a regulatory definition established by the FDA, not a clinical definition. The definition of SAE is not always related to clinical severity of the event. For the purposes of this study an AE is considered a Serious (SAE) when it satisfies any one of the following criteria:

- The event results in an inpatient hospitalization (any overnight stay associated with an admission).
- The event results in the prolongation of a hospital stay.
- The event results in permanent or severe disability.
- The event results in death.
- The event is life-threatening.
- Treatment is required to prevent a serious event.

There have been no SAE’s in the research groups experience in the Pediatric CTRC. We do not anticipate encountering SAE’s; however, we have identified the following as possible SAE’s for the purposes of monitoring:

- Infection related to blood draw or IV placement

a) Serious Adverse Event Reporting

Study patients are instructed to contact the clinic with any serious adverse event meeting the above criteria. Each SAE is recorded on the study form and the PI is informed as soon as possible after they occur and preferably within 24 hours of the notification of the clinic staff. This notification should occur even if data are incomplete. Additional data and follow-up information are documented and sent subsequently as an update to the original report. The PI immediately forwards SAE reports to COMIRB and any other required institutional monitoring committee.

C. Participant Discontinuation Criteria

If a participant experiences any of the following, the participant will be withdrawn from the study.

1. Inability to complete study procedures
2. Abnormal clinical labs (HbA1C>6.4%, Hg <10 mg/dL)
3. Participant becomes pregnant during study

D. Protocol Stopping Criteria

If one or more participants experience any of the SAE's listed above, the PI will consult with the study staff prior to continuing study visits with participants. The PI and RSA will consult about the significance of the SAE's and make a recommendation about participant continuation in the study.

VII. Data Analysis Plan:

Mathematical plans:

MMTT: The metabolic response to the MMTT will be measured for 4 hours. Blood will be collected for glucose, insulin, C-peptide, GLP-1 and glucagon at -10, 0, 10, 20, 30, 45, 60, 90, 120, 150,180 and 240 min after the start of the liquid meal ingestion. β-cell function will be quantitated by mathematical modeling of the plasma C-peptide response to the MTTT [32,33]. The MMTT allows for calculating an estimate of the principal parameters of β-cell function after a physiological stimulus and is better tolerated than oral glucose post-bariatric surgery. The main parameter of the model is β-cell glucose sensitivity (ß-GS), which is calculated as the mean slope of the dose-response function (i.e., the relationship between insulin secretion and plasma glucose concentrations during corresponding times of the MMT). The model also yields a measure of total insulin release over the 3 hr [34]. Intestinal GLP-1 and α-cell glucagon peak responses and area under the curve will also be calculated. The primary model outcome is *β-cell glucose sensitivity (β-GS),* a measure of β cell function calculated as the mean slope of the dose-response function i.e., relationship between insulin secretion rates and glucose concentration during the MMTT. The model also provides an estimate of glucose rate sensitivity (insulin secretory response to rate of change in glucose), and total insulin output (total amount of insulin released over the 4 h of the MMTT)[34]. The metabolic response to the MMTT can also be calculated using the using the trapezoid method for plasma glucose, insulin, and C-peptide AUCs[35].

Overall Project Statistical Plan:

SA1: Changes in hepatic fat will be compared to changes in insulin resistance, beta cell function and 31P-MRS and metabolomics score. Results will be adjusted for absolute and percent weight loss.
SA3: Pearson correlations between 31P-MRS rate of ATP recovery, metabolomics outcomes, hepatic fat fraction, MRE, Oroboros outcomes and biopsy scores will be performed to see which

minimally invasive measures relate most to biopsy markers. Liver measures will also be compared to insulin sensitivity and insulin secretion from the MMT.

Overall Power analysis:
SA1: The data collected for SA3 is to be preliminary data to provide background data for future additional studies in youth.
SA2/SA3: Relation of metabolic outcomes for this type of protocol typically requires 6-8 participants, especially with such a homogenous participant group. However, as it is known that African American tend to have different hepatic fat metabolism patterns compared to Hispanics, we have allowed for 10 participants.

VIII. Summarize Knowledge to be Gained:

Overall Expected Results*:* We anticipate that we will identify several minimally invasive biomarkers in severely obese patients that can be used to predict NAFLD status. We also anticipate that we will generate preliminary data as to the relationship between type 2 diabetes risk and liver health in these patients, and how these both change following bariatric surgery. This data would then provide the background evidence for additional post-bariatric mechanistic studies and future therapeutic trials.

Significance: Recent evidence in adults with severe obesity indicates that 60-70% have NAFLD[37,38]. And youth may have a similar prevalence. NAFLD has been described as the primary driver of worsening metabolic syndrome and CVD in obesity across populations, and can progress to cirrhosis and liver failure[39,40]. Thus, understanding the relative contributions of NAFLD to metabolic disease critical to designing strategies to improve the long-term health of these youth and reduce their risk of T2D, CVD and liver failure. Further as bariatric surgery is now being advocated for treatment of NAFLD, T2D and severe obesity, understanding the effects and mechanism on metabolic disease are critical.

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