HRP-503B – BIOMEDICAL RESEARCH PROTOCOL (2017-1)

Protocol Title: Mechanisms of Obesity and Its Metabolic Complications in Youth.

Principal Investigator: Nicola Santoro

Version Date: Amendments 10/30/2023

(*If applicable*) Clinicaltrials.gov Registration #: NCT03454828

INSTRUCTIONS
This template is intended to help investigators prepare a protocol that includes all of the necessary information needed by the IRB to determine whether a study meets approval criteria. Read the following instructions before proceeding: 1. Use this protocol template for a PI initiated study that includes direct interactions with research subjects. Additional templates for other types of research protocols are available in the system Library. 2. If a section or question does not apply to your research study, type "Not Applicable" underneath. 3. Once completed, upload your protocol in the "Basic Information" screen in IRES IRB system.

SECTION I: RESEARCH PLAN

1. Statement of Purpose: State the scientific aim(s) of the study, or the hypotheses to be tested.

The overarching goal of this project is to determine whether the effect of gut microbiota on human metabolism might be mediated by the Short Chain Fatty Acid (SCFA) and whether the SCFA might modulate lipid metabolism.
In fact, despite the evidence provided by animal studies, such studies have not been performed in humans. Moreover, this would be the first study determining the effect of SCFA synthesis on hepatic de novo lipogenesis and to assess whether and how isocaloric dietary changes (namely low carbohydrates) might modify the composition of the gut microbiota and reduce the synthesis of SCFA during adolescence, a sensitive period for the development of obesity.

Aim 1: Determine whether the gut microbiota of obese adolescents have greater ability to ferment carbohydrates (CHO) and synthetize SCFA compared to the microbiota of lean subjects, therefore obtaining a higher metabolizable energy yield from an equal amount of dietary CHO. Hypothesis: For an equal amount of dietary CHO, obese youth will synthesize SCFA at higher rates than lean youth.

Aim 2: Determine whether the SCFA synthetic rates modulate hepatic de novo lipogenesis. Hypothesis: High rates of SCFA synthesis enhance hepatic de novo lipogenesis.

Aim 3: Test the effect of an isocaloric low carbohydrate (CHO) diet on the bacterial synthesis of SCFA. Hypothesis: Lowering the amount of dietary CHO consumed will shift the composition of the gut microbiota towards species with a lower ability to ferment CHO and synthetize SCFA.

Aim 4: To explore how COVID-19 lockdown has changed the dietary habits of participants enrolled in this study. In order to collect this information, we will ask for the participant to complete an online 3-day food record. This part of the study is non-invasive and there will be no in-person contact.

Aim 5: To explore whether the colonic fermentation modulate gluconeogenesis.

2. Probable Duration of Project: State the expected duration of the project, including all follow-up and data analysis activities. 7 years

3. Background: Describe the background information that led to the plan for this project. Provide references to support the expectation of obtaining useful scientific data.

Pediatric obesity: the growing epidemic.

The prevalence of childhood obesity has progressively increased in the last four decades[2]. In 2010, approximately 43 million children were overweight worldwide and this number is expected to escalate up to 60 million by 2020[1]. Obese children and adolescents are at high risk of developing obesity complications (e.g. cardiovascular disorders and type 2 diabetes[10-16]) early in life.

Furthermore, obesity overburdens the healthcare system by the high costs of such comorbidities[17]. Early onset obesity is the key risk factor for adult obesity[2,18], but the only current treatment for it is represented by physical exercise and caloric restriction. Weight loss programs in children are especially challenging and show a very high attrition rate, approaching 30% in the first 12 months and 70% after 24 months[19,20]. Moreover, although some of these programs can be successful, a clinically significant weight loss is achieved for a short period of time given that only 1 in 6 overweight and obese report ever having maintained weight loss of at least 10% for 1 year[21]. As long-term weight loss remains largely unachievable with current interventions, children who become obese are trapped in this phenotype for years, if not for life, therefore accumulating years of increased risk of cardio-metabolic complications. Why children and adolescents become obese and why the obese phenotype is so stable (irreversible in most cases) remain unanswered questions. This lack in knowledge represents a critical barrier towards our understanding of the pathophysiology underlying childhood obesity.

Role of the gut microbiota in the development of obesity.

The human gut microbiota is a complex community of 100 trillion archaeal and bacterial cells distributed over more than 1000 species[5,22]. The combined genomes of the gut microbiota – the microbiome – contain 100-fold

more genes than the human genome[23] with significant biochemical capabilities to modulate human physiology[5,24]. Animal studies demonstrated that the gut microbiota may modulate both sides of the energy balance equation, namely energy harvest from the diet, energy storage and energy expenditure, therefore mediating diet induced obesity[6,25]. In fact, germ free animals show about 40% lower body fat than conventionalized animals[26]. Although most of the studies related to the gut microbiota and obesity has been carried out in animals, in the last few years it has been shown that obese individuals have a "predisposing" microbiota environment. Notably, studies in human twins discordant for obesity have shown that by transplanting fecal microbiota from adult obese human twin into germ-free mice caused an increase of total body fat mass[3], while when mice harboring a lean twin's microbiota were housed with mice containing an obese co-twin's microbiota, the development of obesity and its complications were prevented in the cage mates[3]. More recently a human study has shown that subjects undergoing fecal microbiota transplantation for the treatment of Clostridium difficile develop new onset obesity if the transplant is received by a healthy but obese donor[27]. These data indicate that the composition of the gut microbiota may play a pivotal role in the development of the obese phenotype in humans, but the mechanisms by which the gut microbiota maybe causing weight gain remain unclear.

Short Chain Fatty Acids as a link between gut microbiota and energy accumulation.

Microbiota dysbiosis may influence the development of obesity through several mechanisms, including the production of bio-active metabolites[5,28], in particular, the short chain fatty acids (SCFA) (acetate, butyrate and propionate)[29]. The SCFA are among the most abundant microbiota-derived metabolites and are produced through the fermentation of dietary carbohydrates not digested by the host. Direct evidence of the role of gut microbiota in energy harvest and fat deposition comes from germ-free rats, which have reduced intestinal levels of SCFA[30], and twice as much urinary and fecal excretion of calories as that of conventional ones fed the same polysaccharide-rich diet[31]. Microbial energy harvest in obesity has been investigated in conventional genetically obese ob/ob mice, revealing increased amounts of SCFA in their caecum and reduced energy content in their feces compared to their lean littermates[32]. In vitro and animal studies suggest that, once produced, SCFA may enter the circulation and may be utilized for de novo lipid or glucose synthesis[33,34]. While the role of gut microbiota in promoting energy harvest has been shown in animal studies, the only evidence in humans derives from indirect investigations. For instance, people who are obese have higher levels of ethanol in their breath than lean[35] and show greater amounts of fecal SCFA[36], which suggests altered fermentation and increased microbial energy harvest.

Mechanisms by which the SCFA can modulate the human metabolism.

Microbial fermentation of polysaccharides may affect host adiposity through several complementary mechanisms. The main mechanism is related to the ability of the SCFA to be taken up by the liver and used as substrates for lipogenesis and gluconeogenesis, in states where their synthesis exceeds the intestinal metabolic needs. In fact, the liver metabolism of germ-free and colonized mice differs considerably, possibly because of the increased influx of SCFA into the liver of colonized mice. As a consequence, colonized mice have higher levels of stored triglycerides (TG) in the liver [37]. Increased TG production in the liver of colonized mice is associated with reduced expression in the small intestine of the fasting-induced adipose factor, or ANGPTL[4,6] which is a potent inhibitor of the enzyme lipoprotein lipase and mediates cellular uptake of TG. Germ-free Angptl4-deficient mice gain as much fat mass and body weight during high-fat feeding as colonized mice, indicating that ANGPTL4 may directly mediate microbial regulation of adiposity in mice6. In addition, SCFA can regulate gene expression by binding to the G-protein-coupled receptors (GPCRs) GPR41 and GPR4328. Signaling through these receptors affects several different functions depending on the cellular type. For example, SCFA suppress inflammation through GPR43 signaling and modulate the expression of PYY through a GPR41- dependent

mechanism[38]. Conventional Gpr41-deficient mice have reduced adiposity compared with conventional wild-type mice, whereas germ-free wild-type and Gpr41-deficient mice had similar adiposity, clearly indicating that the effect of the microbiota on fat deposition might be dependent on this SCFA receptor[38]. Altogether these data suggest that gut microbiota function as a metabolic organ fully integrated with the human metabolic pathways.

Colonic fermentation and glucose metabolism. Human, in vivo and in vitro studies have shown that colonic fermentation has an effect on gluconeogenesis[42-46]. Although in vitro studies suggest that some SCFA deriving from colonic fermentation (such as propionate) might increase gluconeogenesis [42], studies in humans consistently suggest that colonic fermentation is associated with a better glycemic control[44-46]. In fact, previous studies on this topic have focused on adults and adolescents with type 1 and type 2 diabetes and had as main outcome postprandial changes of glucose or doses of insulin needed to maintain a good glycemic control[45-47]. Despite this evidence, to date there are no studies testing the direct effect of colonic fermentation on gluconeogenesis.

References

1. de Onis M, Blossner M, Borghi E: Global prevalence and trends of overweight and obesity amongpreschool children. The American journal of clinical nutrition 2010;92:1257-1264
2. Boelsen-Robinson T, Gearon E, Peeters A: Incidence of childhood obesity in the United States. The New England journal of medicine 2014;370:1659-1660
3. Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, Griffin NW, Lombard V, Henrissat B, Bain JR, Muehlbauer MJ, Ilkayeva O, Semenkovich CF, Funai K, Hayashi DK, Lyle BJ, Martini MC, Ursell LK, Clemente JC, Van Treuren W, Walters WA, Knight R, Newgard CB, Heath AC, Gordon JI: Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science (New York, NY) 2013;341:1241214
4. Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI: The gutmicrobiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A 2004;101:15718-15723
5. Tremaroli V, Backhed F: Functional interactions between the gut microbiota and host metabolism. Nature 2012;489:242-249
6. Backhed F, Manchester JK, Semenkovich CF, Gordon JI: Mechanisms underlying the resistance todiet-induced obesity in germ-free mice. Proceedings of the National Academy of Sciences of the United States of America 2007;104:979-984
7. Bergman EN: Energy contributions of volatile fatty acids from the gastrointestinal tract in variousspecies. Physiol Rev 1990;70:567-590
8. Royall D, Wolever TM, Jeejeebhoy KN: Clinical significance of colonic fermentation. The Americanjournal of gastroenterology 1990;85:1307-1312
9. Goffredo M, Mass K, Parks EJ, Wagner DA, McClure EA, Graf J, Savoye M, Pierpont B, Cline G, Santoro N: Role of Gut Microbiota and Short Chain Fatty Acids in Modulating Energy Harvest and Fat Partitioning in Youth. The Journal of clinical endocrinology and metabolism 2016:jc20161797
10. Goran MI, Dumke K, Bouret SG, Kayser B, Walker RW, Blumberg B: The obesogenic effect of highfructose exposure during early development. Nat Rev Endocrinol 2013;9:494-500
11. Nobili V, Alkhouri N, Alisi A, Della Corte C, Fitzpatrick E, Raponi M, Dhawan A: Nonalcoholic fatty liverdisease: a challenge for pediatricians. JAMA Pediatr 2015;169:170-176

12. Tryggestad JB, Willi SM: Complications and comorbidities of T2DM in adolescents: findings from the
TODAY clinical trial. J Diabetes Complications 2015;29:307-312
13. McCrindle BW: Cardiovascular consequences of childhood obesity. Can J Cardiol 2015;31:124-130
14. D'Adamo E, Guardamagna O, Chiarelli F, Bartuli A: Atherogenic dyslipidemia and cardiovascular risk factors in obese children. 2015;2015:912047
15. Herouvi D, Karanasios E, Karayianni C, Karavanaki K: Cardiovascular disease in childhood: the roleof obesity. Eur J Pediatr 2013;172:721-732
16. Weigensberg MJ, Goran MI: Type 2 diabetes in children and adolescents. Lancet 2009;373:17431744
17. Attard SM, Herring AH, Howard AG, Gordon-Larsen P: Longitudinal trajectories of BMI andcardiovascular disease risk: the national longitudinal study of adolescent health. Obesity (Silver
Spring) 2013;21:2180-2188
18. Taveras EM, Rifas-Shiman SL, Sherry B, Oken E, Haines J, Kleinman K, Rich-Edwards JW, GillmanMW: Crossing growth percentiles in infancy and risk of obesity in childhood. Arch Pediatr Adolesc
Med 2011;165:993-998
19. Cali AM, Pierpont BM, Taksali SE, Allen K, Shaw MM, Savoye M, Caprio S: Rosiglitazone improvesglucose metabolism in obese adolescents with impaired glucose tolerance: a pilot study. Obesity 2011;19:94-99
20. Savoye M, Shaw M, Dziura J, Tamborlane WV, Rose P, Guandalini C, Goldberg-Gell R, Burgert TS,Cali AM, Weiss R, Caprio S: Effects of a weight management program on body composition and metabolic parameters in overweight children: a randomized controlled trial. JAMA : the journal of the
American Medical Association 2007;297:2697-2704
21. Kraschnewski JL, Boan J, Esposito J, Sherwood NE, Lehman EB, Kephart DK, Sciamanna CN: Longtermweight loss maintenance in the United States. Int J Obes (Lond) 2010;34:1644-1654
22. Chalasani N, Guo X, Loomba R, Goodarzi MO, Haritunians T, Kwon S, Cui J, Taylor KD, Wilson L, Cummings OW, Chen YD, Rotter JI: Genome-wide association study identifies variants associated with histologic features of nonalcoholic Fatty liver disease. Gastroenterology 2010;139:1567-1576, 1576.e1561-1566.
23. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende DR, Li J, Xu J, Li S, Li D, Cao J, Wang B, Liang H, Zheng H, Xie Y, Tap J, Lepage P, Bertalan M, Batto JM, Hansen T, Le Paslier D, Linneberg A, Nielsen HB, Pelletier E, Renault P, Sicheritz-Ponten T, Turner K, Zhu H, Yu C, Li S, Jian M, Zhou Y, Li Y, Zhang X, Li S, Qin N, Yang H, Wang J, Brunak S, Dore J, Guarner F, Kristiansen K, Pedersen O, Parkhill J, Weissenbach J, Bork P, Ehrlich SD, Wang J: A human gut microbial gene catalogue established by metagenomic sequencing. Nature 2010;464:59-65
24. Sommer F, Backhed F: The gut microbiota--masters of host development and physiology. Naturereviews Microbiology 2013;11:227-238
25. Larsson E, Tremaroli V, Lee YS, Koren O, Nookaew I, Fricker A, Nielsen J, Ley RE, Backhed F: Analysis of gut microbial regulation of host gene expression along the length of the gut and regulation of gut microbial ecology through MyD88. Gut 2012;61:1124-1131
26. Dumas ME, Barton RH, Toye A, Cloarec O, Blancher C, Rothwell A, Fearnside J, Tatoud R, Blanc V,Lindon JC, Mitchell SC, Holmes E, McCarthy MI, Scott J, Gauguier D, Nicholson JK: Metabolic

profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice. Proc Natl Acad Sci U S A 2006;103:12511-12516
27. Alang N, Kelly CR: Weight Gain After Fecal MIcrobiota Transplantation. Open Forum of InfectiousDisease 2014;
28. Kuwahara A: Contributions of colonic short-chain Fatty Acid receptors in energy homeostasis. Front
Endocrinol (Lausanne) 2014;5:144
29. Macfarlane S, Macfarlane GT: Regulation of short-chain fatty acid production. Proc Nutr Soc2003;62:67-72
30. Hoverstad T, Midtvedt T: Short-chain fatty acids in germfree mice and rats. J Nutr 1986;116:17721776
31. Wostmann BS, Larkin C, Moriarty A, Bruckner-Kardoss E: Dietary intake, energy metabolism, andexcretory losses of adult male germfree Wistar rats. Lab Anim Sci 1983;33:46-50
32. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI: Obesity alters gut microbialecology. Proceedings of the National Academy of Sciences of the United States of America 2005;102:11070-11075
33. Ley RE, Turnbaugh PJ, Klein S, Gordon JI: Microbial ecology: human gut microbes associated with obesity. Nature 2006;444:1022-1023
34. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ,Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI: A core gut microbiome in obese and lean twins. Nature 2009;457:480-484
35. Nair S, Cope K, Risby TH, Diehl AM: Obesity and female gender increase breath ethanolconcentration: potential implications for the pathogenesis of nonalcoholic steatohepatitis. Am J
Gastroenterol 2001;96:1200-1204
36. Schwiertz A, Taras D, Schafer K, Beijer S, Bos NA, Donus C, Hardt PD: Microbiota and SCFA in leanand overweight healthy subjects. Obesity 2010;18:190-195
37. Velagapudi VR, Hezaveh R, Reigstad CS, Gopalacharyulu P, Yetukuri L, Islam S, Felin J, Perkins R,Boren J, Oresic M, Backhed F: The gut microbiota modulates host energy and lipid metabolism in mice. J Lipid Res 2010;51:1101-1112
38. Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, Hammer RE, Williams SC,Crowley J, Yanagisawa M, Gordon JI: Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. Proc Natl Acad Sci U S A
2008;105:16767-16772
39. Hellman B, Larsson S, Westman S: Acetate metabolism in isolated epididymal adipost tissue fromobese-hyperglycemic mice of different ages. Acta Physiol Scand 1962;56:189-198
40. Villee C: The effect of insulin on the incorporation of C14-labeled pyruvate and acetate into lipid andprotein. Anat Rec 1948;101:680
41. Reshef L, Niv J, Shapiro B: Effect of propionate on lipogenesis in adipose tissue. J Lipid Res1967;8:682-687
42. Boillot J, Alamowitch C, Berger AM, Luo J, Bruzzo F, Bornet FR, Slama G: Effects of dietarypropionate on hepatic glucose production, whole-body glucose utilization, carbohydrate and lipid metabolism in normal rats. Br J Nutr 1995;73:241-251

43. Gao Z, Yin J, Zhang J, Ward RE, Martin RJ, Lefevre M, Cefalu WT, Ye J: Butyrate improves insulinsensitivity and increases energy expenditure in mice. Diabetes 2009;58:1509-1517
44. den Besten G, Bleeker A, Gerding A, van Eunen K, Havinga R, van Dijk TH, Oosterveer MH, Jonker JW, Groen AK, Reijngoud DJ, Bakker BM: Short-Chain Fatty Acids Protect Against High-Fat DietInduced Obesity via a PPARgamma-Dependent Switch From Lipogenesis to Fat Oxidation. Diabetes 2015;64:2398-2408.
45. Cavagnuolo L, Bozzetto L, Franco L, Costabile G, Riccardi G, Rivellese AA, Annuzzi G. Pizza Leavening Technique Influences Postprandial Glucose Response: A Randomized Controlled Trial in Patients With Type 1 Diabetes. Diabetes Care. 2019 Oct;42(10):e157-e158
46. Giacco R, Brighenti F, Parillo M, Capuano M, Ciardullo AV, Rivieccio A, Rivellese AA, Riccardi G. Characteristics of some wheat-based foods of the Italian diet in relation to their influence on postprandial glucose metabolism in patients with type 2 diabetes. Br J Nutr. 2001 Jan;85(1):33-40.
47. Zanfardino A, Confetto S, Curto S, Cocca A, Rollato AS, Zanfardino F, Troise AD, Testa V, Bologna O, Stanco M, Piscopo A, Cohen O, Miraglia Del Giudice E, Vitaglione P, Iafusco D. Demystifying the Pizza Bolus: The Effect of Dough Fermentation on Glycemic Response-A Sensor-Augmented Pump Intervention Trial in Children with Type 1 Diabetes Mellitus. Diabetes Technol Ther. 2019 Dec;21(12):721-726.

4. Research Plan: Summarize the study design and research procedures using non-technical language that can be readily understood by someone outside the discipline. Be sure to distinguish between standard of care vs. research procedures when applicable, and include any flowcharts of visits specifying their individual times and lengths. Describe the setting in which the research will take place.

OGTT Visit

During this visit the patients will undergo the OGTT

Assessment of Glucose Tolerance Status

Oral Glucose Tolerance Test (OGTT). The study nurse will do a nursing assessment, including measuring the patient's height, weight, waist circumference, hip circumference, blood pressure, and pulse, along with evaluation of acanthosis nigricans and striae rubrae. The subject's percent body fat, fat mass and lean mass will also be measured using a Tanita scale. The nurse will obtain a family and medical history from the patient and/or the patient's parent/guardian. In addition, before starting the OGTT, the nurse will request a urine sample from the patient for analysis of microalbumin and creatinine, and to test for pregnancy. A consult may be provided by the YCCI bionutritionist at the time of the OGTT.

The patient will receive 1.75 g/kg to a maximum of 75g of a sugar drink, orally (Glucola). The patient will have one intravenous line. "Emla" or a local anesthetic (0.1cc buffered lidocaine) will be applied before the placement of the IV catheter. Blood will be drawn 10 times over three hours. Should abnormal glucose results be found, appropriate referrals will be made. We will draw approximately 120 cc of blood during this study. The blood will be analyzed for metabolic parameters, including glucose, lactate, insulin, proinsulin, c-peptide, IL-6, TNF-α, GLP-1, glucagon, lipoproteins free fatty acids (FFAs), leptin, and adiponectin.

As part of the 120 cc of blood drawn for the study, samples will be obtained via the IV line for genetic testing for the variants in the GCKR, TCF7L2, and PNPLA3 gene. This will be clearly explained in the Adolescent assent, Adult Consent and Parental Consent.

Stool Specimen: After study consenting, the subject (or subject's parent/guardian) will be provided with a kit to collect stool. If the subject cannot provide the sample at the given time, they can complete it at home. Once collected, the container will remain at 4□C and be brought back to researchers within 12 hours. The bacterial DNA will be extracted and the bacterial gene coding for the 16S RNA subunit will be characterized by Next Generation sequencing to assess the bacterial diversity.

MRI VISIT

Assessment of Body Composition: Imaging Studies

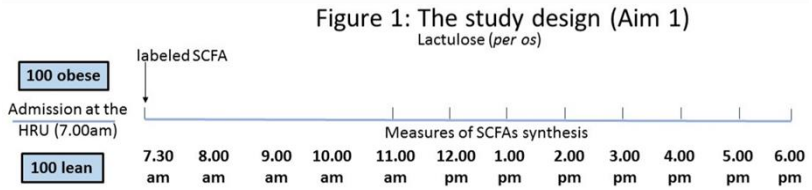
Tanita. Percent body fat, fat mass, and fat-free mass will be measured via bioelectrical impedance analysis (BIA) using a Tanita scale. This method is non-invasive and, if found to be as accurate as the DEXA scan, may prove to be a more practical approach to measuring percent body fat.

Abdominal Magnetic Resonance Imaging (MRI). MRI of the abdomen will be used to directly assess intra-abdominal fat deposition on a 1.5 Tesla magnet (Siemens). During the same session, we will acquire liver images, from which we will calculate the hepatic fat fraction and hepatic iron concentration. All patients will be screened for MRI safety before undergoing imaging of their abdomen. A localizing gradient echo sequence will be performed to allow accurate slice selection. Five true axial Fast Gradient Echo sections will be obtained with respiratory compensation through the abdomen, with the 3rd section at the L4/5 disc space. Saturation bands will be placed above and below the field of view in order to negate high signal in blood vessels. The largest field of view will be used to encompass the abdomen, although in larger patients this will be inadequate, and the sequence will need to be performed twice-once for the right side of the abdomen and once for the left. Each sequence should take approximately 6 minutes to acquire, and the entire scan should take no more than 45 minutes. The images will be analyzed for the amount of subcutaneous and visceral fat present. The hepatic fat fraction will be calculated using a modified Dixon technique. This will be performed using a single breath-hold scan of about 15 seconds to obtain a section through the liver. A gradient echo pulse sequence is used with varying TR and TE parameters to provide in- and out-of-phase images, with TR kept constant. The signal from an appropriate area of hepatic tissue will be used to calculate the fat fraction. Importantly, an excellent correlation was reported between the liver fat content measured by FAST-MRI and liver biopsy ($r2 = 0.853$, $p<0.001$). The hepatic fat fraction will also be calculated using a three-point Dixon technique in combination with the iterative least-squares estimation method. As this method is implemented in a steady-state free precession sequence, it allows one to obtain multiple sections (typically 10-14 slices) through the liver within a single breath-hold scan of about 25-30 seconds. A scout image requiring a breath-hold of about 12 seconds will also be obtained. The hepatic iron concentration will be calculated using a T2 weighted and intermediate weighted gradient echo sequence obtained during two breath-hold sequences of about 26 seconds each. The signal from an appropriate area of hepatic tissue will be used to calculate the hepatic iron concentration. Subjects may be asked to repeat a scan if a sub-optimal image is obtained on the first attempt.

When possible, the MRI scan will be on the same day as the OGTT as noted above, however it can be scheduled on a different day (either prior to or following completion of OGTT) based on ease of scheduling for the patient.

Aim 1: Determine whether the gut microbiota of obese adolescents have greater ability to ferment carbohydrates
(CHO) and synthetize SCFA compared to the microbiota of lean subjects, therefore obtaining a higher metabolizable energy yield from an equal amount of dietary CHO. *Hypothesis: For an equal amount of dietary CHO, obese youth will synthesize SCFA at higher rates than lean youth.*

All subjects will be instructed about the benefit of a healthy diet and will meet with the dietician who will advise on healthy feeding behavior and lifestyle and will be given the option of enrolling, free of charge, in the Yale Pediatric Weight Management Program called "Bright Bodies." The program includes the following components: exercise, nutrition education, behavior modification, and psychosocial support.
We will enroll an equal number of obese (100) and lean (100). Lean adolescents will be recruited if at risk of developing obesity, prediabetes or diabetes due to their family history. The dietitian will instruct the subjects to follow a 3-day pre-intervention meal plan to ensure standardized calories and macronutrients intake up to the day of investigation in amounts to maintain weight in both groups. The pre-intervention diet will be based on the subjects' age, weight and usual intake. The subjects will be admitted at the Hospital Research Unit (HRU) of the
Yale New Haven Hospital at 7.00 AM. To determine SCFA synthetic rates we will give orally 20g of lactulose, an oligosaccharide naturally present in nature that is extracted from chicory. To account for hepatic uptake, isotope exchange in the TCA cycle in the liver and endogenous production by the liver, deuterated-SCFA will be infused from 7.30 AM, this will allow us to reach the quasi steady state for 2H-SCFA after ~1 hour. We will infuse 0.7 micromol/kg/min of labeled acetate after a labeled acetate priming dose of 1 mg/Kg. To assess SCFA synthesis blood samples will be collected every hour until 6.00 pm for a total of 150 ml (Figure 1). The subjects will be allowed to drink during the study, a meal will be served at the end of the study.



We will also collect the breath hourly hydrogen breath test during the study to assess colonic fermentation by measuring hydrogen in the breath. Subjects blow into a small box to collect the breath. There are no risks related to this test.

Rationale for using lactulose as substrate to measure SCFA synthesis. In order to study the ability of the gut microbiota to ferment carbohydrates, we have chosen lactulose because the SCFA synthesis and kinetics arising from lactulose ingestion has been widely studied and is well known. Due to the structural conformation of its linkages, lactulose resists hydrolysis in the human small intestine and is almost completely fermented by the colonic microbiota. Lactulose has low osmotic activity, however it may cause uncomfortable bowel movements, flatulence, loose stools, bloating, cramps, and diarrhea.

Aim 1 Follow-up: After the completion of Aim 1, participants will be invited back for a one-year follow-up to measure height, weight, body mass composition with the Tanita scale, and to collect a 3-day food log. This follow-up is to assess if acetate production predicts change in BMI and body composition one year post study.

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

Aim 2: Determine whether the SCFA synthetic rates modulate hepatic de novo lipogenesis. Hypothesis: High rates of SCFA synthesis enhance hepatic de novo lipogenesis. Subjects will assume 3ml/ Kg of body water of D20 in three doses *per os* (at 7:00 PM, 10:00 PM and 3:00 AM) the night before the study. At the test day at 7:30 AM they will consume 2g of 13C Inulin, 13g of unlabeled inulin. Blood draws will be taken every hour until 8.00 PM. The total blood to be drawn is about 150 ml . The subjects will be allowed to drink during the study, a meal will be served at the end of the study.

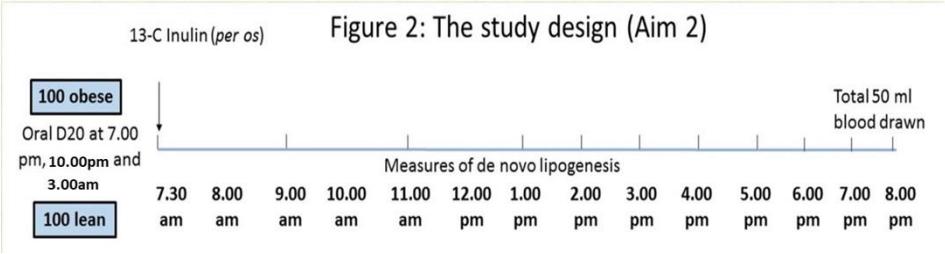


Figure 2: The study design (Aim 2)

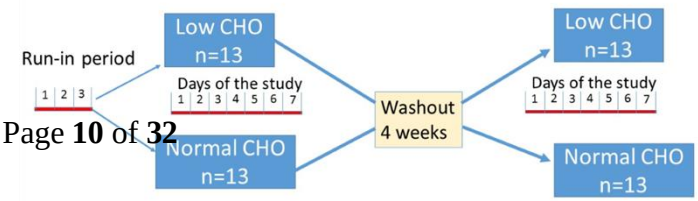
We will also collect the breath hourly hydrogen breath test during the study to assess colonic fermentation by measuring hydrogen in the breath. Subjects blow into a small box to collect the breath. There are no risks related to this test.

Aim 3: Test the effect of an isocaloric low carbohydrate (CHO) diet on the bacterial synthesis of SCFA. Hypothesis: Lowering the amount of dietary CHO will shift the composition of the gut microbiota towards species with a lower ability to ferment carbohydrates and synthetize SCFA. Twenty-six obese subjects will be enrolled in a cross-over study and challenged with a low CHO or a normal CHO diet for a week. The subjects will switch diet after 4 weeks washout. Stool samples will be collected before and after the intervention to evaluate the gut microbiota. SCFA synthesis (as described in aim 1) will be measured at the beginning and at the end of the diet. Subjects will be enrolled after a 3 days run in period, in which they will try whether they could follow a low carbohydrates diet.

Dietary intervention.

a) Dietary Composition: The control "standard" diet composition will follow the American Dietary Guidelines of 55% CHO, 15% pro, and 30% fat. CHO content will be primarily complex CHO of high quality (14 g fiber/1,000 kcals and <10% of total kcals in the form of sugar). Fat content will be <7% saturated fats and primarily consist of monounsaturated fats. The experimental diet will consist of 30% CHO, 35% pro, and 35% fat. Since the experimental diet is lower in CHO, the fiber and sugar content will be calculated based on total CHO in the same percentage as the control (0.25 g fiber per each kcal of CHO and 18.2% sugar for total CHO). This will ensure the same quality of CHO in both diets (for example, <10% of total kcals from sugar would be a higher sugar intake in the low CHO diet). Kcal levels will be determined based on subject's usual food intake. The subjects will be instructed to keep a 3-day food record (2 weekdays and 1 weekend day) prior to the study and the average kcal intake will be calculated using the Nutrition Data Systems for Research (NDSR) (University of Minnesota, 2016 version). Kcal levels will be assigned to the closest 200 kcals (1800, 2000, 2200, 2400, 2600, 2800, 3000). Utilizing the formulas to derive the same quality of CHO in both the standard- and low-CHO diets, the 2000 kcal standard diet, for example, will consist of 1100 kcals of CHO (55% total kcals), 27 g fiber (.025 g fiber per each kcal of CHO), and 50 g sugar (18.2% total CHO), while the low-CHO diet will consist of 600 kcals of CHO (30% total kcals), 15 g fiber (.025 g fiber per each kcal of CHO), and 27 g sugar (18.2% total CHO). A 3-day rotating menu will be

Figure 3: Study design (Aim 3)



provided for both groups to ensure variety and increase compliance.

b) Run-in period: To screen for adherence, the eligible subjects will undergo a 3 day run-in period. During this period all the subjects will consume a low CHO diet and will complete a food record every day. Participants assessed to be compliant to the diet (defined as following the diet for all the five days without any deviation) will be randomized to the intervention (low CHO) first or the control (standard CHO) diet. During the run in period the food will be provided by the Metabolic Kitchen of Yale New Haven Hospital and subjects will complete a daily food record. Moreover, we will discuss the study expectation at length with the subjects.

c) Instruction of Isocaloric Diets: Four weeks after the run-in period (wash out), the subjects enrolled will undergo each dietary intervention for 1 week. Subjects will be instructed on the meal plan by the dietitian prior to the intervention and will be taught how to keep a simplified food record daily to validate the comprehension of the meal plan and compliance. This simplified food record will allow adolescents to check off foods consumed and write in any foods they may have consumed that are not on the meal plan. Foods will be provided to the subjects twice during the 7 days from the metabolic kitchen. The dietitian will meet with the child and family at mid-week food pick up to obtain the food record, assess compliance, and review the plan.

d) Retention Strategy: Non-compliance of the diet might be a problem in this very challenging population of adolescents, although it is unlikely due to the short nature of the diet intervention period. At Yale we have extensive experience using both dietary interventions and/or pharmacologic interventions as treatments for weight management, insulin resistance and diabetes in obese adolescents. Our retention strategies include developing rapport with each child and family involved in the study, through phone calls, sending holiday greeting cards and birthday cards to the child involved in the study, making sure that test results such as lipids, glucose and insulin levels are well explained to the family, and being appreciative of the time and effort subjects and families give to the study. With a short intervention such as this, we plan to build rapport and have frequent phone calls with the subject/family. The research staff is composed of experienced personnel who have serviced many subjects enrolled in studies through the Yale Pediatric Obesity Clinic. The PI and some of the study personnel live in New Haven and are very familiar with the population. A “hot line” by which study participants may reach the study personnel after work hours will be available. The subjects will be reminded of the visit at least three days before and will be scheduled at the best convenience for the families.

e) Compliance Assessment: To enforce compliance the food for the whole study will be provided by the Metabolic Kitchen of the Yale New Haven Hospital. To assess compliance we will ask the patient to complete a simplified food record daily. Also, should they choose to eat something different from what is given to them by the Metabolic Kitchen, they have the choice of writing the food in the blank space provided for that day or to take a picture of the products and indicate the amount eaten. The latter choice may be of more interest to this technologically savvy age group and will be more accurate for the dietitian to assess. The mid-week (mid intervention) visit for food pick up and food record review will allow the dietitian to problem solve, if necessary, before the final four days of the intervention. Moreover, subjects will also be recruited among a pool of subjects who have been previously recruited in our studies and have shown a high rate of adherence.

Aim 4 – *Exploratory*

To assess how COVID-19 lockdown has changed the dietary habits of participants enrolled in this study. In order to collect this information, we will ask for the participant to complete an online 3-day food record. This part of the study is non-invasive and there will be no in-person contact.

Aim 5 – *Exploratory*

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

To assess whether the colonic fermentation modulate gluconeogenesis. Hypothesis: Glucose fermentation induced by lactulose is associated with a drop in gluconeogenesis. Subjects will assume 3ml/ Kg of body water of D20 in three doses *per os* the night before the study and at 7:30 AM they will consume 20g of lactulose. Blood draws will be taken every hour until 8.00 PM. The total blood to be drawn is about 150 ml. The subjects will be allowed to drink during the study, a meal will be served at the end of the study.

We will also collect the breath hourly hydrogen breath test during the study to assess colonic fermentation by measuring hydrogen in the breath. Subjects blow into a small box to collect the breath. There are no risks related to this test.

Aim 6 - Measurement of TCA cycle in vivo in the brain and in the liver by using Deuterium metabolic imaging.

Deuterium metabolic imaging (DMI) is a simple MR-based technique to map metabolism with high temporal and/or spatial resolution. The metabolic fate of deuterated substrates, including glucose and acetate, can be monitored with deuterium MR methods. Recent preliminary data obtained with this protocol suggest that youth with obesity have higher TCA cycle compounds in plasma than lean. This is probably due to an increased metabolic rate related to insulin resistance. To determine if changes observed in plasma mirror metabolic changes in the tissue (brain and liver), we will use a technique that combines stable isotopes and MRI. To measure TCA cycle in the brain we will use 6,6-2H2-glucose, a compound that has been used for many years in clamp studies, while to measure TCA cycle in the liver we will use D3-Acetate, the compound that we are currently using to measure acetate production (Aim 1 and 3).

TCA cycle measurement in the brain.
To measure TCA cycle measurement in the brain we will infuse 6,6-2H2-glucose for 2 hours and we will detect the changes in TCA derived compounds in the brain during the infusion. A total of 50 ml of blood will be obtained during this study.

The labeled glucose is prepared by the Investigational Drug Service of the Yale Pharmacy, as a solution of 20% (w/v) in water. The labeled glucose is infused following a protocol used for decades in the Yale Magnetic Resonance Research Center (MRRC), consisting of a ramp phase followed by a plateau phase. The amount of glucose infused is 0.75g/kg body weight (BW); the volume of glucose solution infused over a 120 min study is 3.6 ml/kg BW.

TCA cycle measurement in the liver.
To measure TCA cycle measurement in the liver we will infuse d3-acetate for 2 hours and we will detect the changes in TCA derived compounds in the liver during the infusion. A total of 50 ml of blood will be obtained during this study.

The labeled acetate is prepared by the Investigational Drug Service of the Yale Pharmacy, as a solution of 350 mM 2H-labeled sodium-acetate in water. The labeled acetate is infused following a protocol used for decades in the Yale MRRC, consisting of a ramp phase followed by a plateau phase. The amount of acetate infused is 0.73g/kg body weight (BW); the volume of acetate solution infused over a 120 min study is 14.2 ml/kg BW.

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

Calendar of activities for each aim (visits, frequency, assessments, blood draw collection, week number).

Visit number	Frequency	Assessment	Amount of blood to be collected	Week number
AIM 1				
1	once	OGTT	120	1
2	once	Acetate synthesis	94	2
AIM 2				
1	once	DNL assessment	150	3
AIM 3				
1	once	Acetate synthesis	94	4
2	once	Acetate synthesis	94	5
3	once	Acetate synthesis	94	6
4	once	Acetate synthesis	94	7
AIM 4				
1	once	One year follow-up	0	8
AIM 5				
1	once	gluconeogenesis	150	9
AIM 6				
1	once	MRI/Infusion - Brain	50	10
2	once	MRI/Infusion - Liver	50	11

Each visit is one day. If a subject is recruited for more than one aims, then at least 12 weeks will be allowed between the visits

5. Genetic Testing ☒

The genetics portion of the study is optional. Subjects who do not wish to have additional blood drawn for genetic analysis may still have the OGTT. We may wish to contact family members of the subject for the genetic study as well. However, no family member will be contacted without the permission of the index subject, and

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

informed signed consent or assent will be obtained from all participants. Refusal to participate will in no way influence the subjects' relationship with the physicians, nurses or researchers, or with Yale University or Yale-New Haven Hospital, nor will it influence their receipt of treatment. An opportunity will be given to the parent/subject to indicate if the genetic sample results can be used for recruitment for current and future studies. Once a participant turns 18, if he/she has not completed the study, will be reconsented in order to complete the study.

6. Subject Population: Provide a detailed description of the types of human subjects who will be recruited into this study.

We will enroll an equal number of 100 obese and 100 lean, between the age of 15 and 25, lean (BMI <85th percentile) and obese (BMI > 95th percentile). The two groups will be matched by age, gender, pubertal stage and ethnicity. As controls we will recruit only lean adolescents at risk of developing obesity, prediabetes or diabetes due to their family history.

7. Subject classification: Check off all classifications of subjects that will be <u>specifically recruited for enrollment</u> in the research project. Will subjects who may require additional safeguards or other considerations be enrolled in the study? If so, identify the population of subjects requiring special safeguards and provide a justification for their involvement.

☐Children	☐ Healthy	☐Fetal material, placenta, or dead fetus
☐Non-English Speaking	☐ Prisoners	☐Economically disadvantaged persons
☐Decisionally Impaired	☐ Employees	☐Pregnant women and/or fetuses
☐Yale Students	☐ Females of childbearing potential	

NOTE: Is this research proposal designed to enroll children who are wards of the state as potential subjects?
Yes ☐ NoX ☐

8. Inclusion/Exclusion Criteria: What are the criteria used to determine subject inclusion or exclusion?
Inclusion criteria: Good general health, taking no medication on a chronic basis, age 15 to 25 years, in puberty (girls and boys: Tanner stage III – V), BMI >25th and <85th for lean cohort; BMI >95th for obese cohort, girls who begin menstruating must have a negative pregnancy test during the study. Exclusion criteria: hematocrit below 32%, baseline creatinine >1.0 mg, food allergies, pregnancy, presence of endocrinopathies (e.g. Cushing syndrome), significant chronic illness.

The hematocrit will be measured in all the patients at the research unit the day of the visit before the beginning of the study. During the assessment of vital signs the patients will be asked about signs of anemia (fatigue, malaise, weakness, dizziness). The study will be performed only if the hematocrit is above 32% and the patients does not refer any symptoms of anemia. The hematocrit will be measured using the hematocrit analyzer available at the research unit (HemataSTAT II Analyzer Serial).

9. How will eligibility be determined, and by whom?

Before the enrollment, the clinical characteristics will be assessed through a clinical evaluation during a screening visit in which the PI and the study coordinator will assess the eligibility through a comprehensive evaluation (height, weight, disease history, current medications etc.).

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

10. Risks: Describe the reasonably foreseeable risks, including risks to subject privacy, discomforts, or inconveniences associated with subjects participating in the research.

A potential complication of the indwelling catheters used in these studies is thrombophlebitis. The incidence of this complication in our hands is less than 0.1%. Local hematoma is uncommon and on occasion there is short-lived local pain occurring at the site of venipuncture. The risk of stable isotopes appears to be negligible. In fact, 13C inulin and labeled water will be given orally, therefore there will not be any risk related to sterility and pirogenicity. Lactulose is occurring in the normal diet and is benign, though it may cause uncomfortable bowel movements, flatulence, loose stools, bloating, cramps, and diarrhea. The dose of D20 used in this study are in the range of 1-2 mg/Kg of weight, which is what is commonly used in clinical studies. Also, in the past infusions of compounds labeled with stable isotopes have been used to evaluate the SCFA turnover in newborn without any side effect. Acetate infusion is low risk. Acetate is largely present in our body. Labeled acetate has been used for years in medical research all over the world including Yale (Petersen KF Cell Metabolism 2016) without any side effects.

11. Minimizing Risks: Describe the manner in which the above-mentioned risks will be minimized.
As stable isotopes are given orally there is no risk for infection related to stable isotopes infusion. We have been using isotope infusions in conjunction with clamping and lipogenesis studies in children who have not had any adverse events. The risks of acetate infusion are the same as stable isotope infusion.

Magnetic resonance (MR) is a technique that uses magnetism and radio waves, not xrays, to take pictures and measure chemicals of different parts of the body. The United States Food and Drug Administration (FDA) has set guidelines for magnet strength and exposure to radio waves, and we carefully observe those guidelines.

You will be watched closely throughout the MR study. Some people may feel uncomfortable or anxious. If this happens to you, you may ask to stop the study at any time and we will take you out of the MR scanner. On rare occasions, some people might feel dizzy, get an upset stomach, have a metallic taste or feel tingling sensations or muscle twitches. These sensations usually go away quickly but please tell the research staff if you have them.

There are some risks with an MR study for certain people. If you have a pacemaker or some metal objects inside your body, you may not be in this study because the strong magnets in the MR scanner might harm you. Another risk is the possibility of metal objects being pulled into the magnet and hitting you. To lower this risk, all people involved with the study must remove all metal from their clothing and all metal objects from their pockets. We also ask all people involved with the study to walk through a detector designed to detect metal objects. It is important to know that no metal can be brought into the magnet room at any time. Also, once you are in the magnet, the door to the room will be closed so that no one from outside accidentally goes near the magnet.

We want you to read and answer very carefully the questions on the MR Safety Questionnaire related to your personal safety. Take a moment now to be sure that you have read the MR Safety Questionnaire and be sure to tell us any information you think might be important. This MR study is for research purposes only and is not in any way a complete health care imaging examination. The scans performed in this study are not designed to find abnormalities. The principal investigator, the lab, the MR technologist, and the

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

Magnetic Resonance Research Center are not qualified to interpret the MR scans and are not responsible for providing a health care evaluation of the images. If a worrisome finding is seen on your scan, a radiologist or another physician will be asked to review the relevant images. Based on his or her recommendation (if any), the principal investigator or consulting physician will contact you, inform you of the finding, and recommend that you seek medical advice as a precautionary measure. The decision for additional examination or treatment would lie only with you and your physician. The investigators, the consulting physician, the Magnetic Resonance Research Center, and Yale University are not responsible for any examination or treatment that you receive based on these findings. The images collected in this study are not a health care MR exam and for that reason, they will not be routinely made available for health care purposes.

A member from the study staff will accompany the patient to the MRRC and stay for the entire duration of the MRI scan

DNA Analysis. The analysis of blood for genetic research raises special issues of confidentiality. Variation in some genes is known to be directly related to risk for certain illnesses. In some cases, knowledge of genetic information could have negative psychological consequences or could affect access to or retention of certain benefits or entitlements. For example, the information could potentially be used against an individual if it were revealed to insurance companies or potential employers. We will take precautions to ensure that confidentiality is maintained and that the genetic information is not unintentionally disclosed to inappropriate third parties.

12. Data and Safety Monitoring Plan: Include an appropriate Data and Safety Monitoring Plan (DSMP) based on the investigator's risk assessment stated below. (Note: the HIC will make the final determination of the risk to subjects.)
    a. What is the investigator's assessment of the overall risk level for subjects participating in this study? GREATER THAN MINIMAL RISK
    b. If children are involved, what is the investigator's assessment of the overall risk level for the children participating in this study? presenting a minor increment over minimal risk with no prospect of direct benefit to individual subjects, but likely to yield generalizable knowledge about the subject's disorder or condition (per 45 CFR § 46.406), with permission obtained from both parents.
    c. Include an appropriate Data and Safety Monitoring Plan. Examples of DSMPs are available here http://your.yale.edu/policies-procedures/forms/420-fr-01-data-and-safetymonitoring-plans-templates for
        i. Included at the end of the document.

    d. For multi-site studies for which the Yale PI serves as the lead investigator:
        i. How will adverse events and unanticipated problems involving risks to subjects or others be reported, reviewed and managed? *Write here*
        ii. What provisions are in place for management of interim results? *Write here*
        iii. What will the multi-site process be for protocol modifications? *Write here*

13. Statistical Considerations: Describe the statistical analyses that support the study design.
Statistical Plan for aims 1 and 2. Justification of Sample size: Sample size was estimated in PASS

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

V2012 (Kayesville, UT) in order to enroll a sufficient number of obese children to detect differences in SCFA synthesis for aim 3. Based on this sample size, the number of lean children was then determined to detect differences between lean and obese (aim 1). For aim 1, given a standard deviation of 1.0 for SCFA, 100 lean children (compared to 100 obese children) will be required to detect a difference of 0.65 with 90% power at the two-sided 0.05 significance level. This sample size will also provide 80% power to detect an rsquare of 0.07 (aim 2). Statistical analyses: Analysis for Aim 1: the primary outcome will be the peak percent change from fasting in total SCFA estimated by dividing the difference between total SCFA at fasting and the peak total SCFA by the total SCFA at fasting. Total SCFA is calculated by summing the synthetic rates of the single SCFAs. Peak percent change will be compared between the lean and obese groups using a mixed model to account for the pair matching. A fixed effect will be included for group (obese/lean) and a random effect for an indicator of the matched pair. Differences in means along with 95% confidence intervals will be estimated. We will also explore between group differences in SCFA across all post-inulin time-points by estimating the area under the curve following inulin. Since the pre-intervention diet will be based on the subjects' usual intake, we will adjust for dietary intake to try to overcome the possibility that dietary patterns during the study might affect SCFA synthesis. Since the two groups will be matched by age, gender, Tanner stage and ethnicity the analyses will not need to be adjusted for these variables. Nevertheless, we will determine whether differences between lean and obese groups are modified by gender by adding an interaction of gender by group. Analysis for Aim 2: To evaluate the association between total SCFA and de novo lipogenesis, peak percent changes in de novo lipogenesis will be regressed on peak percent changes in total SCFA using a mixed model. The mixed model will include a fixed effect for peak percent total SCFA and a random effect for the matched pair indicator. To explore whether the magnitude of the associations may be dependent on other variables (such as gender), interactions between percent peak total SCFA and these variables will be included to the models in order to describe the degree of effect modification. An additional exploratory analysis using a doubly multivariate repeated measures mixed model will be evaluated to examine the correlation of total SCFA and de novo lipogenesis across all post-inulin time-points.

Statistical Plan for Aim 3. Justification of Sample size: In our preliminary data from obese children we observed a standard deviation for percent peak total SCFA synthesis of 1.0 and an absolute difference of 1.5% between those with the lowest and highest BMI. Given the following: 1) a power of 90%, 2) a standard deviation of 1.0, and 3) a two-sided 0.05 significance level a sample size of 21 obese children receiving both low CHO vs control diet will be required to detect a difference of 0.75 in peak total SCFA synthesis. The conservative 0.75 difference was selected based on being 50% of that estimated between the highest and lowest BMI in our preliminary work. We'll enroll 26 obese children for aim 3 to accommodate up to a 20% dropout. Statistical Analyses: The primary objective of the analysis is to demonstrate that a low CHO diet will lower SCFA synthetic rate more than control at follow-up. The percent change in peak total SCFA rate, calculated as the follow-up peak total SCFA minus the baseline peak total SCFA divided by the baseline peak total SCFA, will be compared between the low CHO period and the control period using a repeated measures mixed model. The mixed model will included fixed effects for treatment and period as well as the baseline covariates of SCFA, age, sex, pubertal stage and ethnicity. A random effect will be included for subject to allow for correlation between repeated measures. The treatment by period interaction will be evaluated and if significant ($p<0.10$) examination of the primary aim will focus on the interventions received during the first period (i.e. to exclude the possibility of carryover). Linear contrasts and two-sided 95% confidence intervals will be estimated comparing mean percent peak total SCFA change for low CHO to control. Sex-specific effects of low CHO will be assessed by inclusion of interactions of treatment with sex. Given the assumption required for missing data, we will evaluate patterns of missing data as well as determine baseline characteristics that are predictive of dropout. If identified, these characteristics will be included as covariates in supportive sensitivity analysis.

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

Statistical Plan for Aim 4. Since aims 4 is an exploratory studies for which we do not have preliminary data it is difficult to determine the sample size needed to detect differences in dietary changes during the lockdown. The main outcome for this arm of the study will be changes in Kcal intake. Secondary aims will be changes in the intake of macro- and micro nutrients. This arm of the study will be limited to ~40 subjects for which we have pre-lockdown dietary information. Data analysis. Descriptive statistics, such as means and standard deviations (or medians and interquartile range, IQR), counts and percent will be used to describe characteristics of subjects before the study. A paired non-parametric test will be used to compare the outcomes before and after the lockdown within groups, while a general linear model will be used to compare changes in the outcomes between groups. Statistical significance will be established at the two-sided alpha of 0.05. Analyses will be performed using SAS 9.4 (Cary, NC), Prism and R statistical software.

Statistical Plan for Aim 5. In our preliminary data, we observed changes in glucose of 89.3 mg/dl at baseline and 78.8 mg/dl at the end of the study (7 hours after lactulose ingestion). Given the following: 1) a power of 95%, 2) a standard deviation of 8.0, and 3) a one-sided 0.05 significance level a sample size of 14 subjects will be required to detect the above shown difference in percent changes with 95% power at the one-sided 0.05 significance level within each group. Since we will explore also whether percent changes in gluconeogenesis are different between lean we will recruit about 20 subjects per group. Statistical Analyses: Median and interquartile ranges will be calculated. The primary outcome will be gluconeogenesis changes after lactulose ingestion within groups (lean and obese). Non-parametric one sided test will be used to compare gluconeogenesis between baseline and the end of the study. We will also explore the possibility that changes in gluconeogenesis might occur to different extents between the groups, therefore we will compare percent changes gluconeogenesis between the groups using a general linear model using basal gluconeogenesis as covariate.

Statistical plan for aim 6. Sample size was estimated with the clinicalc online software (Sample Size Calculator (clincalc.com). Since lactate serves as a precursor of TCA cycle, we based our power calculations on the lactate levels measured during the fasting state in 12 lean and 18 obese children. Based on the mean+/-SD of lactate levels in non-obese group of 0.78+/-0.22 and in the group with obesity 1.023 +/- 0.36, a sample size of 23 subjects per group (46) is needed for an alpha of 0.05 and a power of 95%. Statistical Analyses: Median and interquartile ranges will be calculated. The primary outcome will be TCA cycle after 6,6 D glucose ingestion during the steady state. Non-parametric two sided test will be used to compare TCA cycle between the groups.

SECTION II: RESEARCH INVOLVING DRUGS, BIOLOGICS, RADIOTRACERS, PLACEBOS AND DEVICES

If this section (or one of its parts, A or B) is not applicable, check off N/A and delete the rest of the section.

A. RADIOTRACERS ☒N/A

B. DRUGS/BIOLOGICS ☐ N/A

Does the study do any of the following:

Specify the use of an approved drug or biologic?

Use an unapproved drug or biologic?

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

Use a food or dietary supplement to diagnose, cure, treat, or mitigate a disease or condition? No

This study uses:
13C inulin (labeled) (isotope)
deuterated SCFA/labeled acetate
Deuterium labeled water (heavy water D2o)
6,6-2H2-glucose (isotope)

Lactulose is a dietary supplement that is approved to treat constipation. We will use lactulose to stimulate production of short chain fatty acids by the gut flora.

Cold Isotopes:
FDA allows the use of cold isotopes in research without an IND when certain conditions apply (see below). Confirm that the following are true:

1. *The research is intended to obtain basic information regarding the metabolism (including kinetics, distribution, and localization) of a drug labeled with a cold isotope or regarding human physiology, pathophysiology, or biochemistry.*
2. *The research is not intended for immediate therapeutic, diagnostic, or preventive benefit to the study subject.*
3. *The dose to be administered is known not to cause any clinically detectable pharmacologic effect in humans based on clinical data from published literature or other valid human studies.*
4. *The quality of the cold isotope meets relevant quality standards.*
5. *The investigation [will be] conducted in compliance with the requirements for review by an IRB (21 CFR part 56) and the requirements for informed consent (21 CFR part 50).*

I confirm all of the 5 points above. In fact, the study is intended to obtain basic information regarding the metabolism, not to provide therapeutic or diagnostic benefit.
The dose of isotopes to be used have been shown to be safe. In particular, D20 and 13C inulin have shown to be safe at the dose that we are providing. We and others used D20 at the proposed dose in previous studies without any side effects (Goffredo M et a. JCEM 2017). For the 13C inulin other investigators have used up to 15grams of labeled Inulin without any side effect (Deroover L et al. Nutrients 2017).

1. If an exemption from IND filing requirements is sought for a clinical investigation of a drug product that is lawfully marketed in the United States, review the following categories and complete the category that applies (*and delete the inapplicable categories)*:

Exempt Category 1: The clinical investigation of a drug product that is lawfully marketed in the United States	
can be exempt from IND regulations if all of the following are yes:	
1. The intention of the investigation is NOT to report to the FDA as a well-controlled study in support of a new indication for use or to be used to support any other significant change in the labeling for the drug.	☒

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

2. The drug that is undergoing investigation is lawfully marketed as a prescription drug product, and the intention of the investigation is NOT to support a significant change in the advertising for the product.	☒
3. The investigation does NOT involve a route of administration or dosage level or use in populations or other factor that significantly increases the risks (or decreases the acceptability of the risks) associated with the use of the drug product	☒
4. The investigation will be conducted in compliance with the requirements for institutional (HIC) review and with the requirements for informed consent of the FDA regulations (21 CFR Part 50 and 21 CFR Part 56).	☒
5. The investigation will be conducted in compliance with the requirements regarding promotion and charging for investigational drugs.	☒

Exempt Category 2 (all items i, ii, and iii must be checked to grant a category 2 exemption)

☐ i. The clinical investigation is for an *in vitro* diagnostic biological product that involves one or more of the following (check all that apply):

- ☐ Blood grouping serum
- ☐ Reagent red blood cells
- ☐ Anti-human globulin

☐ ii. The diagnostic test is intended to be used in a diagnostic procedure that confirms the diagnosis made by another, medically established, diagnostic product or procedure; and

☐ iii. The diagnostic test is shipped in compliance with 21 CFR §312.160.

Exempt Category 3

☐ The drug is intended solely for tests in vitro or in laboratory research animals if shipped in accordance with 21 CFR 312.60

Exempt Category 4

☐ A clinical investigation involving use of a placebo if the investigation does not otherwise require submission of an IND.

2. Background Information: Provide a description of previous human use, known risks, and data addressing dosage(s), interval(s), route(s) of administration, and any other factors that might influence risks. If this is the first time this drug is being administered to humans, include relevant data on animal models.

Write here

3. Source: Identify the source of the drug or biologic to be used. The stable isotopes will be purchased from the Cambridge Isotope laboratories Inc. or from the Sigma Aldrich.

   a) Is the drug provided free of charge to subjects? ☐YES ☐NO If yes, by whom? *Write here*

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

4. Storage, Preparation and Use: Describe the method of storage, preparation, stability information, and for parenteral products, method of sterilization and method of testing sterility and pyrogenicity.
   The compounds will be handled from and stored at the Investigational Drug Service. The IDS provides handling and packaging of the stable isotopes.

Check applicable Investigational Drug Service utilized:

☐ YNHH IDS ☐ CMHC Pharmacy ☐ West Haven VA ☐ PET Center ☐ None ☐ Other:

Note: If the YNHH IDS (or comparable service at CMHC or WHVA) will not be utilized, explain in detail how the PI will oversee these aspects of drug accountability, storage, and preparation.

5. Use of Placebo: ☒Not applicable to this research project

6. Continuation of Drug Therapy After Study Closure ☒Not applicable to this project

☒N/A

B. DEVICES

1. Are there any investigational devices used or investigational procedures performed at Yale-New Haven Hospital (YNHH) (e.g., in the YNHH Operating Room or YNHH Heart and Vascular Center)? ☐Yes ☐No

If Yes, please be aware of the following requirements:

A YNHH New Product/Trial Request Form must be completed via EPIC: Pull down the Tools tab in the EPIC Banner, Click on Lawson, Click on "Add new" under the New Technology Request Summary and fill out the forms requested including the "Initial Request Form," "Clinical Evidence Summary", and attach any other pertinent documents. Then select "save and submit" to submit your request; AND

Your request must be reviewed and approved in writing by the appropriate YNHH committee before subjects/subjects may be scheduled to receive the investigational device or investigational procedure.

2. Background Information: Provide a description of previous human use, known risks, and any other factors that might influence risks. If this is the first time this device is being used in humans, include relevant data on animal models. *Write here*
3. Source:
   a) Identify the source of the device to be used. *Write here*
   b) Is the device provided free of charge to subjects? ☐Yes ☐No

4. Investigational device accountability: State how the PI, or named designee, ensures that an investigational device is used only in accordance with the research protocol approved by the HIC, and maintains control of the investigational device as follows:

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

a) Maintains appropriate records, including receipt of shipment, inventory at the site, dispensation or use by each participant, and final disposition and/or the return of the investigational device (or other disposal if applicable): *Write here*

b) Documents pertinent information assigned to the investigational device (e.g., date, quantity, batch or serial number, expiration date if applicable, and unique code number): *Write here*

c) Stores the investigational device according to the manufacturer's recommendations with respect to temperature, humidity, lighting, and other environmental considerations: *Write here*

d) Ensures that the device is stored in a secure area with limited access in accordance with applicable regulatory requirements: *Write here*

e) Distributes the investigational device to subjects enrolled in the IRB-approved protocol: *Write here*

SECTION III: RECRUITMENT/CONSENT AND ASSENT PROCEDURES

1. Targeted Enrollment: Give the number of subjects: 200
   a. Targeted for enrollment at Yale for this protocol: Children and adolescents between 15 and 25 years of age.
   b. If this is a multi-site study, give the total number of subjects targeted across all sites: NA

2. Indicate recruitment methods below. Attach copies of any recruitment materials that will be used.

☒Flyers	☐ Internet/web postings	☐ Radio
☐ Posters	☐ Mass email solicitation	☐ Telephone
☐ Letter	☐ Departmental/Center website	☐ Television
☐ Medical record review*	☐ Departmental/Center research boards	☐ Newspaper
☐ Departmental/Center newsletters	☐ Web-based clinical trial registries	☐ Clinicaltrails.gov
☒ YCCI Recruitment database	☐ Social Media (Twitter/Facebook):	
☒ Other: Yale Pediatric Obesity Clinic		

* Requests for medical records should be made through JDAT as described at http://medicine.yale.edu/ycci/oncore/availableservices/datarequests/datarequests.aspx

3. Recruitment Procedures:

a. Describe how potential subjects will be identified. The subjects will be also recruited from a sample of ~1000 obese children and adolescents, constituting the Yale Pediatric Obesity Cohort, who agreed to being contacted for recruitment purposes . This cohort of lean and obese youth has been carefully phenotyped for fat distribution as well as for lipid and glucose metabolism (including the assessment of insulin sensitivity by the oral glucose tolerance test)70. If needed, more subjects will be recruited through the Yale Pediatric Obesity Clinic as well as from the CTSA-funded Yale Center for Clinical Investigation Database, comprised of subjects in the community.

b. Describe how potential subjects are contacted. Potential subjects will be contacted by the PI or the study personnel by phone, mail or email.

c. Who is recruiting potential subjects? The PI and the research assistant will be recruiting the subjects.

4. Assessment of Current Health Provider Relationship for HIPAA Consideration:

Does the Investigator or any member of the research team have a direct existing clinical relationship with any potential subject?

☐ Yes, all subjects

☒ Yes, some of the subjects

☐ No

If yes, describe the nature of this relationship. Dr Caprio, a co-investigator on this study, is the physician leading the Yale obesity clinic, through which some of the subjects will be recruited.

5. Request for waiver of HIPAA authorization: (When requesting a waiver of HIPAA Authorization for either the entire study, or for recruitment purposes only. Note: if you are collecting PHI as part of a phone or email screen, you must request a HIPAA waiver for recruitment purposes.) Choose one:

   ☒ X☐ For entire study

   ☒ For recruitment/screening purposes only

   ☐ For inclusion of non-English speaking subject if short form is being used and there is no translated HIPAA research authorization form available on the University's HIPAA website at hipaa.yale.edu.

   i. Describe why it would be impracticable to obtain the subject's authorization for use/disclosure of this data: *The PI has moved to a new institution and is asking to transfer samples and data to the new institution, and since recruitment occurred over the past years it would be difficult to reach the subjects and re-consent them to ask permission to transfer data and samples.*

   ii. If requesting a waiver of signed authorization, describe why it would be impracticable to obtain the subject's signed authorization for use/disclosure of this data: Some of the subjects will be responding to a flyer and may call us by phone for a pre-screening.

The investigator assures that the protected health information for which a Waiver of Authorization has been requested will not be reused or disclosed to any person or entity other than those listed in this application, except as required by law, for authorized oversight of this research study, or as specifically approved for use in another study by an IRB.

Researchers are reminded that unauthorized disclosures of PHI to individuals outside of the Yale HIPAA-Covered entity must be accounted for in the "accounting for disclosures log", by subject name, purpose, date, recipients, and a description of information provided. Logs are to be forwarded to the Deputy HIPAA Privacy Officer.

6. Process of Consent/Assent: Describe the setting and conditions under which consent/assent will be obtained, including parental permission or surrogate permission and the steps taken to ensure subjects' independent decision-making.

   Each person will be contacted by the P.I. or member of the research team to determine his or her eligibility for the study. The purpose, nature and potential complications of the study will be explained to each potential subject in detail. The P.I. or designated researcher will set up a screening session during which the study will be reviewed with the subject and she will be asked to read the human consent form which has been previously approved by the Yale Human Investigations Committee. All parties involved will be given time to ask questions. The researcher obtaining consent will ask the subject a series of questions to ensure that they have understood the main procedures involved in the study, along with the risks and commitment. Only after this they will be

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

asked to give informed consent to participate. The signed form (and a copy) will be kept as a permanent record.

7. Evaluation of Subject(s) Capacity to Provide Informed Consent/Assent: Indicate how the personnel obtaining consent will assess the potential subject's ability and capacity to consent to the research being proposed. The researcher obtaining consent will ask the subject a series of questions to ensure that they have understood the main procedures involved in the study, along with the risks and commitment. Only after this they will be asked to give informed consent to participate.
8. Non-English Speaking Subjects: Explain provisions in place to ensure comprehension for research involving non-English speaking subjects. If enrollment of these subjects is anticipated, translated copies of all consent materials must be submitted for approval prior to use. *Write N/A*

As a limited alternative to the above requirement, will you use the short form* for consenting process if you unexpectedly encounter a non-English speaking individual interested in study participation and the translation of the long form is not possible prior to intended enrollment? YES ☐ NO ☐

Note* If more than 2 study participants are enrolled using a short form translated into the same language, then the full consent form should be translated into that language for use the next time a subject speaking that language is to be enrolled.

Several translated short form templates are available on the HRPP website (yale.edu/hrpp) and translated HIPAA Research Authorization Forms are available on the HIPAA website (hipaa.yale.edu). If the translation of the short form is not available on our website, then the translated short form needs to be submitted to the IRB office for approval via modification prior to enrolling the subject. *Please review the guidance and presentation on use of the short form available on the HRPP website.*

If using a short form without a translated HIPAA Research Authorization Form, please request a HIPAA waiver in the section above.

9. Consent Waiver: In certain circumstances, the HIC may grant a waiver of signed consent, or a full waiver of consent, depending on the study. If you will request either a waiver of consent, or a waiver of signed consent for this study, complete the appropriate section below.

☐ Not Requesting any consent waivers

☒ Requesting a waiver of signed consent:

☒ Screening only *(if for recruitment, the questions in the box below will apply to recruitment activities only)*

☐ Entire Study (Note that an information sheet may be required.)

For a waiver of signed consent, address the following:

- Would the signed consent form be the only record linking the subject and the research? YES ☐ NO ☐
- Does a breach of confidentiality constitute the principal risk to subjects? YES ☐ NO ☐

OR

- Does the research pose greater than minimal risk? YES ☐ NO☐

• Does the research include any activities that would require signed consent in a non-research context? YES ☐ NO ☐

☐ Requesting a waiver of consent:

☐ Recruitment/Screening only *(if for recruitment, the questions in the box below will apply to recruitment activities only)*

☐ Entire Study

For a full waiver of consent, please address all of the following:

- Does the research pose greater than minimal risk to subjects? ☐ Yes *If you answered yes, stop. A waiver cannot be granted.*
  ☐ No
- Will the waiver adversely affect subjects' rights and welfare? YES ☐ NO☐
- Why would the research be impracticable to conduct without the waiver? *Write here*
- Where appropriate, how will pertinent information be returned to, or shared with subjects at a later date? *Write here*

SECTION IV: PROTECTION OF RESEARCH SUBJECTS

Confidentiality & Security of Data:

1. What protected health information (medical information along with the HIPAA identifiers) about subjects will be collected and used for the research? Age, gender, height, weight, BMI, previous and current diseases, medications, family history of diseases. *NA*

2. How will the research data be collected, recorded and stored?

De-identified plasma samples will be collected and SCFA and DNL will be measured in plasma.

3. How will the digital data be stored? ☐CD ☐DVD ☐Flash Drive ☐Portable Hard Drive ☐Secured Server ☐Laptop Computer ☒Desktop Computer ☐Other
4. What methods and procedures will be used to safeguard the confidentiality and security of the identifiable study data and the storage media indicated above during and after the subject's participation in the study?

All personal information will be handled in confidence and in accordance with local data protection laws. All research materials will be held in locked cabinets and stored on password-protected computers. Only study personnel will have access to the database to record results as they accrue. While being analyzed samples will be stored in locked areas in the secured laboratories of the investigator. When the results of the research are published or discussed in conferences, no information will be included that would reveal a subject's identity unless specific consent for this is obtained. The subjects will be informed of those parties who may have access to their data as listed on the Research Authorization form. The samples will be stored long term in the facilities of the Core laboratories of the Yale Center for Clinical Investigation.

The research data and specimen will be shared with Kansas University Medical Center.

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

All portable devices must contain encryption software, per University Policy 5100. If there is a technical reason a device cannot be encrypted please submit an exception request to the Information Security, Policy and Compliance Office by clicking on url http://its.yale.edu/egrc or email it.compliance@yale.edu

5. What will be done with the data when the research is completed? Are there plans to destroy the identifiable data? If yes, describe how, by whom and when identifiers will be destroyed. If no, describe how the data and/or identifiers will be secured.

Participant permission is requested to retain research data. This data will be stored for research purposes after the research is completed. This is outlined in the protocol and in all consent/assent forms. Subjects who opt not to store their data long term, data will be anonymized within three years after the end of the study. 6. If appropriate, has a Certificate of Confidentiality been obtained? Yes

Dr. Santoro will be the point of contact should a participant wish to withdraw their samples

SECTION V: POTENTIAL BENEFITS

Potential Benefits: All the subjects will be instructed about the benefit of a healthy diet and will meet with the dietician who will advise on healthy feeding behavior and lifestyle. This will be a direct benefit for those children that are already obese and overweight and for those who are at risk of becoming obese, given their family history. All the subjects enrolled in the study will also be given the option of enrolling, free of charge, in the Yale Pediatric Weight Management Program called “Bright Bodies.” The program includes the following components: exercise, nutrition education, behavior modification, and psychosocial support. Some obese individuals will undergo a low carbohydrates diet during the study. This study will take place under the supervision of Mary Savoye, who is also the director of the Bright Bodies program a weight loss program designed for obese children and adolescents. In addition to the benefits to the subjects, the study will increase our understanding of the role of gut microbiome in the development of obesity in children and adolescents. This is the first study in human exploring the possibility that the products of microbial metabolism in the gut might determine fat accumulation and lead to obesity during childhood. The study about changes in dietary pattern during the lockdown will inform on whether and how adolescents’ diet has changed during that time. If we observe an unhealthy dietary behavior characterized by the consumption of more junk food, our dietician will advise on how to revert this tendency and how to consume a healthier food. Moreover, learning about the effect of colonic fermentation on glucose production will be important to understand if modifying the diet of youth with obesity, that represent a group at high risk for pre-diabetes and diabetes, a better glucose control after a meal of high fermentable food could be reached. In fact, there are studies in children and adults with type 1 and type 2 diabetes that have demonstrated that consuming food made with highly fermented dough improves the glycemic control after the meal so that the patients need lower insulin to control glycemic excursion after the meal (Zanfardino A et al Diabetes Technol Ther. 2019 Dec;21(12):721-726 ; Cavagnuolo L et al Diabetes Care. 2019 Oct;42(10):e157-e158; Giacco R et al. Br J Nutr. 2001 Jan;85(1):33-40).

SECTION VI: RESEARCH ALTERNATIVES AND ECONOMIC CONSIDERATIONS

1. Alternatives: What other alternatives are available to the study subjects outside of the research?
   The study subjects will be given the chance to attend the Yale pediatric Obesity Clinic available at the Department of Pediatrics of Yale University regardless of their decision to participate in the study.
2. Payments for Participation (Economic Considerations): Describe any payments that will be made to subjects, the amount and schedule of payments, and the conditions for receiving this compensation.

   Subjects who will enroll in the study will receive a reimbursement of $150 for every full day study, $150 for each MRI/Infusion study, $60 for the MRI, $50 for the glucose tolerance test, $20 for completion of a 3-day food log, and $20 for a stool sample collection. We will pay for parking for coming to the study visits.

   We will use a pre-paid debit card to provide payment for taking part in the study. We will have to share your name, address, and telephone number with the banking institution issuing the debit card for ePayments. You may receive a card in the mail with the first payment following completion of the first visit. You will need to activate the card over the phone. Payments for additional visits will be automatically added to your card after complete on of each following visit. You are responsible for paying state, federal, or other taxes for the payments you receive for being in this study. Taxes are not withheld from your payments."

   Compensation made to U.S. human research study participants is considered taxable income to the participant, regardless of how the participant is paid, and is reported by Yale University. Jonathan Andrejczyk from Yale Shared Services, will determine which subjects have payments over $600 for whom Yale does not have a Social Security number on file and will work with the coordinator to collect the Social Security numbers that are required. If a coordinator knows in advance that a subject will receive $600 or more in a calendar year for a study, then they should request the Social Security number upfront. For Subjects where social security numbers are required they should complete W-9 Form which the coordinator should submit to Jonathan.

3. Costs for Participation (Economic Considerations): Clearly describe the subject's costs associated with participation in the research, and the interventions or procedures of the study that will be provided at no cost to subjects. There are no costs for the subjects associated with the participation in the study. The low carbohydrates diet will be provided by the Metabolic Kitchen of the HRU free of charge for the subjects.
4. In Case of Injury: This section is required for any research involving more than minimal risk, and for minimal risk research that presents the potential for physical harm (e.g., research involving blood draws).

   a. Will medical treatment be available if research-related injury occurs? *Write here*

   b. Where and from whom may treatment be obtained? *Write here*
   c. Are there any limits to the treatment being provided? *Write here*
   d. Who will pay for this treatment? *Write here*
   e. How will the medical treatment be accessed by subjects? *Write here*

   If injury should occur during any of the procedures, the overseeing physician and the study nurse will attend to the immediate needs of the patient. Should any acute care visit (i.e. emergency room or outpatient clinic), hospital admission or chronic care be necessary, the subject's medical insurance will be responsible for covering any incurred charges.

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

IMPORTANT REMINDERS

Will this study have a billable service? Yes ☐ No☒

A billable service is defined as any service rendered to a study subject that, if he/she was not on a study, would normally generate a bill from either Yale-New Haven Hospital or Yale Medical Group to the patient or the patient's insurer. The service may or may not be performed by the research staff on your study, but may be provided by professionals within either Yale-New Haven Hospital or Yale Medical Group (examples include x-rays, MRIs, CT scans, specimens sent to central labs, or specimens sent to pathology). Notes: 1. There is no distinction made whether the service is paid for by the subject or their insurance (Standard of Care) or by the study's funding mechanism (Research Sponsored). 2. This generally includes new services or orders placed in EPIC for research subjects.

If answered, "yes", this study will need to be set up in OnCore, Yale's clinical research management system, for Epic to appropriately route research related charges. Please contact oncore.support@yale.edu

Are there any procedures involved in this protocol that will be performed at YNHH or one of its affiliated entities?

Yes ☐ No ☐

If Yes, please answer questions a through c and note instructions below.

a. Does your YNHH privilege delineation currently include the specific procedure that you will perform? Yes ☐ No

☐

b. Will you be using any new equipment or equipment that you have not used in the past for this procedure? Yes

☐ No ☐

c. Will a novel approach using existing equipment be applied? Yes ☐ No ☐

If you answered "no" to question 4a, or "yes" to question 4b or c, please contact the YNHH Department of Physician Services (688-2615) for prior approval before commencing with your research protocol.

IMPORTANT REMINDER ABOUT RESEARCH AT YNHH

Please note that if this protocol includes Yale-New Haven Hospital subjects, including subjects at the HRU, the Principal Investigator and any co-investigators who are physicians or mid-level practitioners (includes PAs, APRNs, psychologists and speech pathologists) who may have direct patient contact with subjects on YNHH premises must have medical staff appointment and appropriate clinical privileges at YNHH. If you are uncertain whether the study personnel meet the criteria, please telephone the Physician Services Department at 203-688-2615. By submitting this protocol as a PI, you attest that you and any co-investigator who may have patient contact has a medical staff appointment and appropriate clinical privileges at YNHH.

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

Data and Safety Monitoring Plans (DSMP) 420

FR.1

Greater Than Minimal Risk DSMP

1. Personnel responsible for the safety review and its frequency:

The principal investigator will be responsible for monitoring the data, assuring protocol compliance, and conducting the safety reviews at the specified frequency, which must be conducted at a minimum of every 6 months (including when reapproval of the protocol is sought). During the review process, the principal investigator (monitor) will evaluate whether the study should continue unchanged, require modification/amendment, or close to enrollment. Either the principal investigator, the IRB and Safety Monitoring Committee (DSMC) have the authority to stop or suspend the study or require modifications.

2. The risks associated with the current study are deemed greater than minimal for the following reasons:

1. We do not view the risks associated with the use of stable isotopes as minimal risks.

Although we have assessed the proposed study as one of greater than minimal risk, the potential exists for anticipated and/or unanticipated adverse events, serious or otherwise, to occur since it is not possible to predict with certainty the absolute risk in any given individual or in advance of first-hand experience with the proposed study methods. Therefore, we provide a plan for monitoring the data and safety of the proposed study as follows:

3. Attribution of Adverse Events:
Adverse events will be monitored for each subject participating in the study and attributed to the study procedures / design by the principal investigator (*Nicola Santoro*) according to the following categories:

a.) Definite: Adverse event is clearly related to investigational procedures(s)/agent(s).
b.) Probable: Adverse event is likely related to investigational procedures(s)/agent(s).
c.) Possible: Adverse event may be related to investigational procedures(s)/agent(s).
d.) Unlikely: Adverse event is likely not to be related to the investigational procedures(s)/agent(s).
e.) Unrelated: Adverse event is clearly not related to investigational procedures(s)/agent(s).

4. Plan for Grading Adverse Events:

The following scale will be used in grading the severity of adverse events noted during the study:

1. Mild adverse event
2. Moderate adverse event
3. Severe

5. Plan for Determining Seriousness of Adverse Events:

Serious Adverse Events:
In addition to grading the adverse event, the PI will determine whether the adverse event meets the criteria for a Serious Adverse Event (SAE). An adverse event is considered serious if it results in any of the following outcomes:

1. Death;
2. A life-threatening experience in-patient hospitalization or prolongation of existing hospitalization;
3. A persistent or significant disability or incapacity;
4. A congenital anomaly or birth defect; OR
5. Any other adverse event that, based upon appropriate medical judgment, may jeopardize the subject's health and may require medical or surgical intervention to prevent one of the other outcomes listed in this definition.

An adverse event may be graded as severe but still not meet the criteria for a Serious Adverse Event. Similarly, an adverse event may be graded as moderate but still meet the criteria for an SAE. It is important for the PI to consider the grade of the event as well as its "seriousness" when determining whether reporting to the IRB is necessary.

6. Plan for reporting UPIRSOs (including Adverse Events) to the IRB

The principal investigator will report the following types of events to the IRB:

Any incident, experience or outcome that meets ALL 3 of the following criteria:

1. Is unexpected (in terms of nature, specificity, severity, or frequency) given (a) the research procedures described in the protocol-related documents, such as the IRB-approved protocol and informed consent document and (b) the characteristics of the subject population being studied; AND
2. Is related or possibly related to participation in the research (*possibly related* means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); AND
3. Suggests that the research places subjects or others at greater risk of harm (including physical, psychological, economic, legal, or social harm) than was previously known or recognized.

Unanticipated Problems Involving Risks to Subjects or Others (UPIRSOs) may be medical or nonmedical in nature, and include – but are not limited to – *serious, unexpected, and related adverse events* and *unanticipated adverse device effects*. ***Please note*** that adverse events are reportable to the IRB as UPIRSOs **only** if they meet all 3 criteria listed above.

These UPIRSOs/SAEs will be reported to the IRB in accordance with IRB Policy 710, using the appropriate forms found on the website. All related events involving risk but not meeting the *prompt*

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

reporting requirements described in IRB Policy 710 should be reported to the IRB in summary form at the time of continuing review. If appropriate, such summary may be a simple brief statement that events have occurred at the expected frequency and level of severity as previously documented**.** In lieu of a summary of external events, a current DSMB report can be submitted for research studies that are subject to oversight by a DSMB (or other monitoring entity that is monitoring the study on behalf of an industry sponsor).
The DSMB is composed by:
1) Stuart A Weinzimer, MD. Professor of Pediatrics at Yale University. Dr. Weinzimer is an expert of type 1 diabetes and has participated to several clinical trials studying the efficacy of closed loop system in children with type 1 diabetes.
2) Eda Cengiz, MD. Associate Professor of Pediatrics at Yale University. Dr. Cengiz has a longstanding expertise in the pharmacokinetic properties of insulin analogues in type 1 diabetes. The PI will report to DSMB every 6 months by conference call and every year by in person meeting. Unscheduled meetings will be arranged in case any events should occur. The PI will provide the data obtained from the study and the potential adverse events reported by the patients. The DSMB will review the data and provide report the findings to the PI by email or in person meeting.
The DSMB will be responsible for reviewing comprehensive, cumulative, unblinded safety reports, and employing stopping rules if there is evidence of differential effects in either risk or benefit.

7. Plan for reporting adverse events to co-investigators on the study, as appropriate the protocol's research monitor(s), e.g., industrial sponsor, Yale Cancer Center Data and Safety Monitoring Committee (DSMC), Protocol Review Committee (PRC), DSMBs, study sponsors, funding and regulatory agencies, and regulatory and decision-making bodies.

For the current study, the following individuals, funding, and/or regulatory agencies will be notified:

☐ All Co-Investigators listed on the protocol.

☐ National Institutes of Health

☐ Data Safety Monitoring Board (DSMB)

The principal investigator (*Nicola Santoro*) will conduct a review of all adverse events upon completion of every study subject. The research participant will also be given a study safety questionnaire at the end of the study that asks about the degree to which they experienced any side effects. The principal investigator will evaluate the frequency and severity of the adverse events and determine if modifications to the protocol or consent form are required.

Please note: For any study that may be considered high risk, the IRB will be more focused on the safety requirements for the study and a DSMB will likely be required.

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022

For more guidance on Adverse Event reporting and DSMPs, see **IRB Policy 710 Reporting Unanticipated Problems Involving Risks to Subjects or Others, including Adverse Events**

APPROVED BY THE YALE UNIVERSITY IRB 12/1/2022