Study Title:

Oral Amino Acid Nutrition to Improve Glucose Excursions in PCOS (ORANGE Study)

Protocol Version Date: 06/09/2020

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COMIRB Protocol

COLORADO MULTIPLE INSTITUTIONAL REVIEW BOARD
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Project Title: Oral Amino Acid Nutrition to Improve Glucose Excursions in PCOS

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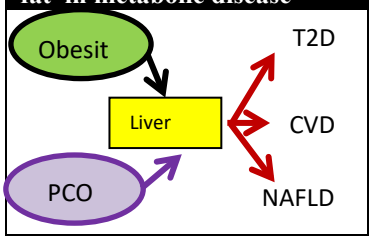
Principal Investigator: Melanie Cree Green, MD, PhD

I. Hypotheses and Specific Aims:

Polycystic Ovarian Syndrome (PCOS), characterized by excess testosterone and irregular menses, is a common endocrinopathy, affecting 10-15% of all women and up to 20% of obese women[1]. Obese adult women with PCOS
have a 4-fold increased risk of developing type 2 diabetes (T2D), higher rates of non-alcoholic fatty liver disease (NAFLD) and risk markers for cardiovascular disease (CVD). We have found that obese girls with PCOS have severe insulin resistance (IR) a precursor to T2D, high rates of hepatic steatosis (HS) (early NAFLD) and early risk markers for CVD[2,3]. The presence of HS is the strongest predictor for development of T2D and CVD[4]. It appears that the combination of obesity and PCOS are synergistic for increasing the risk of metabolic disease **(Figure 1).** Youth onset metabolic disease confers an early and more severe morbidity and earlier mortality than adult onset disease, emphasizing the urgency of treating this population[5]. Unfortunately, current therapeutic options for PCOS and HS are inadequate. *Obese girls with PCOS already have metabolic disease, are at high risk for severe disease as they age and therapeutic options are limited. Development of new interventions should be focused during this vulnerable yet reversible stage.*

Figure 1: Role of excess liver fat in metabolic disease



HS in PCOS appears to be due primarily to increased synthesis of new fat via de novo lipogenesis (DNL) and also relates to abnormalities in glucose metabolism including insulin resistance and hyperglycemia[2,3,6-10]. Beyond glucose, non-essential amino acids (NEAA) glutamine and alanine are substrates for increased gluconeogenesis and DNL[11,12]. NEAA and glucose are increased with excess caloric intake and muscle and hepatic IR[13,14]. Further, redundant cycling through the tricarboxylic acid cycle (TCA cycle) also leads to excess carbons available for DNL[9]. A potential target to reduce HS is to reduce the substrates available for DNL via modulating TCA cycle, glucose and NEAA excess.
Essential amino acids (EAA) are critical for multiple physiologic processes, most notably protein synthesis, and can only be obtained from the diet. A full complement of dietary EAA is required for adequate protein synthesis[15-17]. If protein breakdown is greater than protein synthesis, as seen in weight loss or increased metabolic stress such as sepsis, resulting excess NEAA are available as substrates for DNL and gluconeogenesis. Further, excess branched chain amino acids (BCAA), a subset of EAA, are increased when all of the EAA aren't available for protein synthesis. Excess BCAA relate to IR in T2D and obesity since by-products of their oxidation may cause muscle IR[18,19]. Women with PCOS have higher testosterone and thus an increased hormonal drive for protein synthesis, but do not have increased

Abbreviations used in this protocol: AHI=apnea hypopnea index; **BCAA**=branched chain amino acid; **BMI**=body mass index; **CAP**=controlled attenuation parameter; **CVD**=cardiovascular disease; **DHEAS=**dehydroepiandrosterone sulfate; **DNL**=de novo lipogenesis; **EAA**= essential amino acids; **EGP**= endogenous glucose production; **FFA**=free fatty acids; **GIR**=glucose infusion rate; **GLP-1**=glucagon-like peptide-1; **GLP-1 RA**=glucagon-like peptide-1 receptor agonist; **HOMA**=homeostasis model assessment; **HS**=hepatic steatosis; **IHTG**=intrahepatic triglyceride; **IR**=insulin resistance; **MRI**=magnetic resonance imaging; **MRS**=magnetic resonance spectroscopy; **NAFLD**=non-alcoholic fatty liver disease; **NEAA**=non-essential amino acids **NMR**=nuclear magnetic resonance; **OGTT**=oral glucose tolerance test; **OSA**=obstructive sleep apnea; **OSTT**=oral sugar tolerance test; **PCOS**=polycystic ovarian syndrome; **TCA**=tricarboxcylic acid cycle; **TG**=serum triglycerides; **T2D**= type 2 diabetes; **VAT**=visceral adipose tissue;

muscle mass, indicating that they may have increased protein turnover, resulting in excess NEAA and BCAA, placing them at unique risk[2,3]. Of importance, in the setting of weight loss, higher protein diets moderately improve IR and decrease HS in obesity[20,21], NAFLD[22], T2D[23,24] and PCOS[25]. We have found that balanced EAA supplements decrease HS in adults with IR or NAFLD[15,26,27] but the role of EAA supplementation to improve clinical outcomes via driving efficient protein synthesis and decrease substrates for DNL and gluconeogenesis has not been investigated in PCOS. *The goal of this project is to intervene with a well-tolerated and safe EAA dietary supplement to decrease HS and related IR in obese girls with PCOS at high risk for T2D, CVD and progressive liver disease via a blinded placebo controlled cross-over study.*

Specific Aim 1: In obese girls with PCOS and HS, determine if 4 weeks of EAA supplementation decreases HS via lower rates of hepatic DNL.
Strong preliminary data shape our working hypothesis that in obese girls with PCOS and HS, intrahepatic triglycerides will decrease due to decreased DNL following 4 weeks of EAA, as compared to placebo.

Specific Aim 2: In obese girls with PCOS and HS, determine if 4 weeks of EAA supplementation lowers T2D risk via decreased gluconeogenesis/gluconeogenic precursors and BCAA oxidation products/IR.

HYPOTHESIS:
We hypothesize, again based on preliminary data, that in obese girls with PCOS and HS, IR and post-prandial hyperglycemia will decrease after 4 weeks of EAA supplementation, as compared to placebo. EAA supplementation will improve postprandial metabolism by correcting EAA imbalances to allow for increased net protein synthesis. This will decrease NEAA substrates available for futile hepatic TCA cycling that creates gluconeogenesis precursors and decreases BCAA oxidation products that influence muscle IR.

II. Background and Significance:

PCOS affects 10-15% of U.S. women, and 15-20% of obese women, with an estimated economic burden of $4 billion and is increasing in prevalence in parallel with the increase in obesity[28,29]. PCOS develops in late puberty, and the phenotype of PCOS includes high androgens, irregular/infrequent menses and dermatologic manifestations including facial hair, balding and severe acne[1,30]. Metabolic disease is common in PCOS and in obese women with PCOS, IR is almost universal and is a major contributor to the earlier onset and rising incidence of inter-related NAFLD, T2D and CVD[30-35]. The primary therapy for PCOS with obesity is lifestyle modification which is unfortunately very challenging for youth due to peer, family and personal motivational issues. Current PCOS medical therapies include metformin, which only works in approximately half of patients and oral contraceptives which may actually increase CVD risk[1,30,36-41]. *Thus, PCOS is a common and challenging disease for youth, and an important gap exists in effective therapeutic options for PCOS*.

An estimated 50-70% of obese women with PCOS have elevated HS, compared to 20-30% of similarly obese women without PCOS [32,42]. Our previous data show that half of obese girls with PCOS have HS[2]. These findings indicate that hepatic pathology occurs early in the course of PCOS, and less severe abnormalities are even seen in normal weight girls with PCOS[3]. Furthermore, obese women with PCOS and HS have more IR than those without HS, indicating a link between HS and worsening IR [33]. IR in muscle, liver and adipose tissue is reported to be worse in PCOS adults than in adult women of similar BMI and thought to be related to increased androgens[43-46]. We also found worse adipose, liver and muscle IR in girls with PCOS,regardless of BMI,than BMI-similar non-PCOS girls, despite their young age [47]. The synergy between obesity and hyperandrogenism in PCOS likely has unique influence on fat metabolism, inflammation, NAFLD and IR [48-50]. Limited studies in adults with NAFLD indicate that upregulated rates of hepatic DNL and increased delivery of free fatty acids (FFA) are the primary contributors to the development of HS [6,51,52]. However, the unique mechanisms behind increased HS have not been well studied, as the majority of mechanistic NAFLD studies have focused on men.

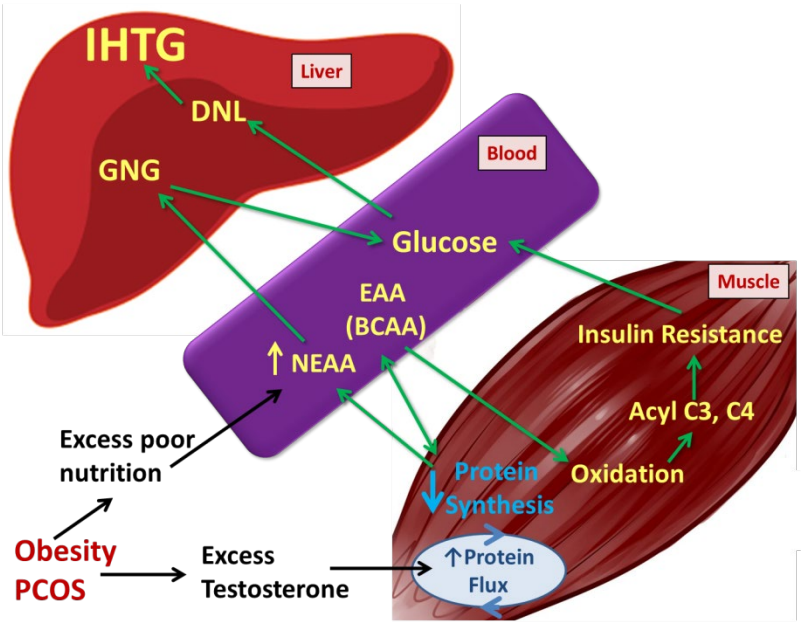
Understanding and treating the high rates of HS in girls with PCOS at the critical preclinical stages may be the best strategy to prevent future NAFLD, T2D and CVD. HS may contribute to worsening IR and

progression to T2D and can progress to cirrhosis and liver failure [4,34,35]. Elevated liver transaminases (a marker of NAFLD) predict hyperglycemia and T2D onset[53]. Conversely, treatment of NAFLD with an effector of bile acid metabolism improves glycemia[54], arguing for a tight connection between glycemia and liver health. IR in adipose, muscle and the liver increases as the degree of HS increases[55]. Unfortunately, youth onset NAFLD appears to be more severe at presentation and progress more rapidly than adult onset disease[5,56]. Thus treating NAFLD in youth has the potential of significant long term health benefits.

Both dietary and pharmacologic strategies have been employed to treat NAFLD[57,58]. Diets with decreased calories and simple carbohydrates have been shown to decrease rates of HS across the lifespan[22,23]. Fructose is preferentially used by the liver for DNL, and just 9 days of a low fructose diet reduced hepatic fat by 50% in youth with HS[15]. Unfortunately, this diet is nearly impossible to follow for most families. Diabetes medications that lower glucose and thus DNL precursors such as metformin have had variable success[59-61]. However, there are currently no standard of care medications to treat HS[57].

The interplay between protein, glucose and fat metabolism is dynamic and tightly regulated. However, the majority of research involving alterations in glucose and fat metabolism as they relate to T2D and NAFLD does not account for the role of protein. The balance of protein synthesis and breakdown is influenced by nutritional state, exercise, insulin and in particular testosterone. In men, testosterone concentrations of 400-700 ng/dL lead to increased muscle mass and absolute increases in protein synthesis[62,63]. In women with PCOS, testosterone concentrations are 40-125 ng/dL yet muscle mass is not increased, suggesting no increase in net protein synthesis[2,64,65]. States of decreased net protein synthesis secondary to increased protein turnover or lack of quality protein from starvation, lipodystrophy and surgical weight loss are known causes of HS[11,57,66,67]. The US dietary recommendation of protein is inadequate for obesity and weight loss[68]. In obese youth, excess calories are typically carbohydrates and fat and food may be deficient in quality protein and EAA[69-71]. This high calorie poor diet can lead to excess formation of NEAA and precursors for gluconeogenesis and DNL[8,9]. Additionally, BCAA not utilized for protein synthesis can be oxidized into acyl CoA products that induce IR and further provide glucose for DNL[72]. Indeed, alterations in BCAA metabolism relate to IR in obese adolescents[18], women with PCOS[73] and obese adults with T2D[74-76]. Thus, obese youth with PCOS have poor diet quality and likely have increased protein flux from androgens without increased synthesis, creating a unique pathology for development of HS (**Figure 2**).

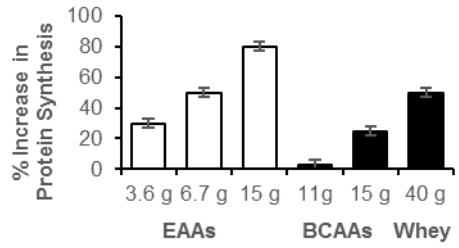
Figure 2: Inadequate EAA from poor nutrition and high protein flux from testosterone in PCOS causes hepatic steatosis and hyperglycemia



EAA: essential amino acids, NEAA: non essential amino acids, BCAA: branched chain amino acids, DNL: de novo lipogenesis, GNG: gluconeogensis, Acyl C3 and C4: acyl Co-A breakdown brokducts of BCAA oxidation, IHTG: intrahepatic tryglycerides

Multiple studies have shown that dietary intervention with a high protein hypocaloric diet induces weight loss, improvements in insulin sensitivity or lowering HS[77-84]. However, the majority of these studies are by default low in carbohydrates and it is unclear if metabolic improvement is from the protein affect, a decrease in carbohydrate or decrease in overall calories[85-88]. In women with PCOS, either a high protein diet or whey protein supplementation improved insulin sensitivity, but all participants also experienced weight loss[86,89-91]. Yet not all studies with a high protein diet or BCAA supplementation show improvements in metabolic disease[87,92-94]. These conflicting results are likely attributable to the failure to improve the pathology listed in **Figure 2**, i.e. not enough EAA to stimulate net protein synthesis or supplementation with a protein that is high in gluconeogenic precursors alanine and glutamine which can increase glycemia[95]. Indeed, we have shown that the ideal composition for increasing muscle protein synthesis appears to be a 15 gram dose of EAA, with more modest effects from isolated EAA's or intact proteins[16,96-99] **(Figure 3).**

Figure 3: 15 g of EAA stimulates the most net protein synthesis in adults



EAA supplementation is therefore a promising treatment for HS and IR, as opposed to complete protein source[94,100,101]. Just weeks of EAA supplementation decreases HS and IR in at-risk populations even in the absence of weight loss and metabolic studies in healthy men demonstrate a blunting of intrahepatic triglycerides (IHTG) synthesis with 3 days of EAA[15,26,27,102,103]. We previously found that ingestion of a mixture of EAAs stimulates more efficient reutilization of non-essential amino acids (NEAAs), and as a result reduces the plasma concentrations of NEAAs and substrate available for gluconeogenesis and DNL and that this is not disturbed in the presence of elevated free fatty acids[17,104]. Finally, the EAA leucine has metabolic signaling effects in addition to being crucial for protein synthesis and supplementation EAA higher in leucine have increased beneficial metabolic effects in those with pre-existing disease. EAA oral supplements are readily available, acceptable to patients and have minimal side effects[15,26,103]. *EAA supplementation treatment for metabolic disease in obese girls with PCOS is a simple intervention with a potential to reduce significant long-term morbidity and perhaps mortality.*

A. Preliminary Studies/Progress Report:

We have been increasing our knowledge of the pathophysiology of IR and HS in obese youth though 4 distinct studies (**Table 1,** * indicates Dr. Cree-Green as PI). All studies included stable isotope tracers, MRI measured IHTG and measurements targeted to better understand pathophysiologic mechanisms. The results of these studies which have informed our proposed study are shown below.

Table 1: Youth studies used for preliminary data

Study, N	Participant population	Relevant methods
Resistant N=80	Lean and obese youth With and without T2D	Tissue specific clamp assessed IR
Airs* N=73	Lean and obese girls With and without PCOS	Tissue specific clamp assessed IR Indirect measure of hepatic DNL Metabolomics
Apple* N=92	Obese girls With and without PCOS	Tissue specific OSTT assessed IR Direct measure of hepatic DNL
Plum* N=11	Obese girls With PCOS	Prolonged OSTT assessed IR Hepatic TCA cycle activity

- Obese girls with PCOS have severe IR and high rates of HS and hyperglycemia.

In our Resistant and APPLE studies, we examined the differential tissue-specific response to insulin in female youth with PCOS compared to my mentor's (Dr. Nadeau) studies of T2D, utilizing a 4 phase hyperinsulinemic euglycemic clamp with glucose and glycerol stable isotope tracers. By contrasting patterns of pathology between these disorders, we uncovered subtle differences that suggest unique mechanisms of metabolic change in PCOS. PCOS girls in AIRS study had mildly increased hepatically-derived serum triglycerides (TG) **(Table 2),** liver transaminase ALT as elevated as in T2D, and MRI-assessed rates of IHTG even higher than in T2D girls **(Figure 4)**. Most notably, we found that ≈50% of obese girls with PCOS had >5.5% liver fat (i.e. clinically significant HS), whereas 10% obese girls without PCOS had HS. These findings a have been duplicated in the APPLE cohort. Obese girls with PCOS had multi-tissue IR relative to similarly obese controls, but are not as IR as T2D girls[47]. The concentration of insulin required to suppress hepatic glucose release was double in girls with PCOS (p=0.04), indicating hepatic IR **(Figure 4, middle)**, and may be reflective of dysregulated gluconeogenesis. Muscle IR **(Figure 4, bottom)**, measured as insulin stimulated glucose uptake (GIR) was much lower (p<0.001) in PCOS. Simple clinical scores such as the fasting HOMA-IR as proposed as a secondary endpoint are also different between groups and correlate well with clamp IR in these girls (r=0.75, p<0.01; **Table 2)**. Additionally, obese girls with PCOS have an increased hemoglobin A1c, as an indicator of blood sugar over the previous 3 months and following a (75g glucose +25g fructose) oral sugar tolerance test (OSTT), nearly half of girls had impaired glucose tolerance, defined as a blood glucose > 140 mg/dl at 2 hours.

Figure 4: Obese PCOS girls have high IHTG, liver and muscle IR

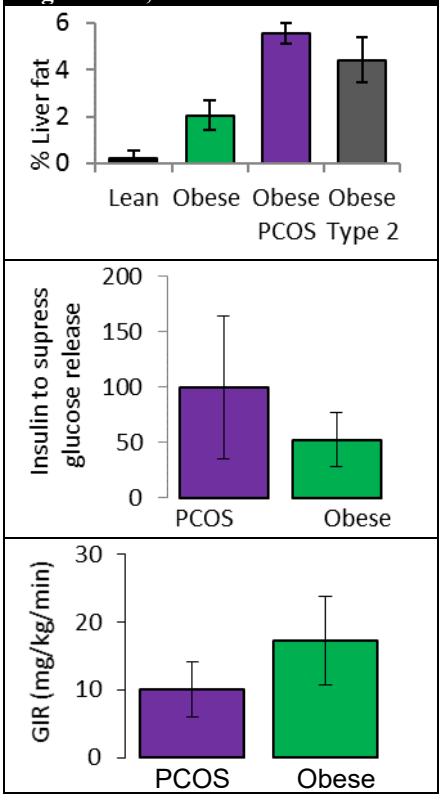
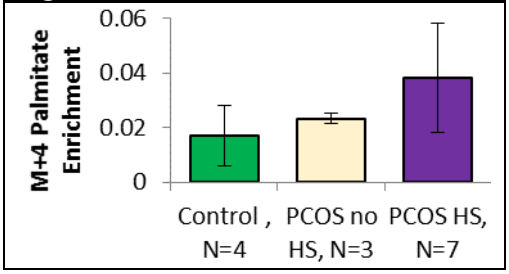


Table 2: Serum markers in obese girls

	Obese	PCOS	T2D
Number of Participants	19	38	40
ALT (IU/L)	29±3	38±3	40±2
Triglycerides (mg/dl)	78±8	129±7	207±7
HbA1C (%)	5.1±0.2	5.4±0.3	7.2±0.4
2h OSTT glucose >140 mg/dL	20%	50%	100%
HOMA-IR	3.6±1.3	5.9±0.4	-

- Excess hepatic triglyceride is from increased de novo lipogenesis in obese girls with PCOS. In our Resistant and APPLE studies, we examined the differential tissue-specific response to insulin in female youth with PCOS compared to my mentor's (Dr. Nadeau) studies of T2D, utilizing a 4 phase

Figure 5: Increased DNL in PCOS+HS

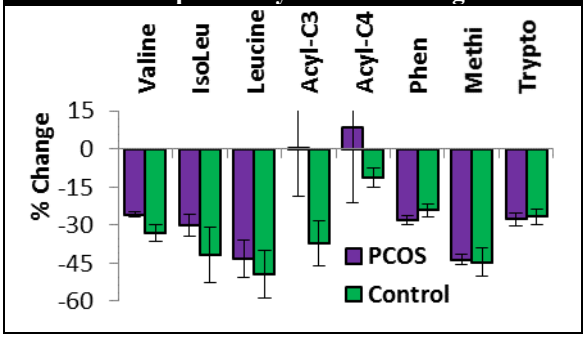


Obese girls with PCOS in the Airs cohort had higher serum concentrations of markers of DNL (i.e. long chain fatty acids with a double bond in the 7th carbon position; N7 FFA) (29±13 nmol/g obese vs. 45±15 PCOS, p<0.01)[64]. IHTG in PCOS girls weakly correlated with markers of overall lipolysis, such as FFA, but was closely related to N7 FFA (r=0.53, p=0.004). To follow-up on these findings, in the APPLE study, we are directly measuring the rate of hepatic DNL with an IV infusion of a 1,2C_{13} acetate tracer. We are performing studies during overnight fasting and then following a glucose ingestion, to better understand what happens during a normal physiologic state. The incorporation of this acetate tracer, expressed as tracer enrichment, is greatest in the newly secreted hepatic VLDL-TG in girls with HS and PCOS **(Figure 5).** Further, DNL is even increased in PCOS girls without HS compared to controls, demonstrating an independent role of PCOS. These changes are more evident following the glucose load, rather than fasting, again emphasizing the need to

study this metabolism in the fed state and the negative additive effect of PCOS and obesity.

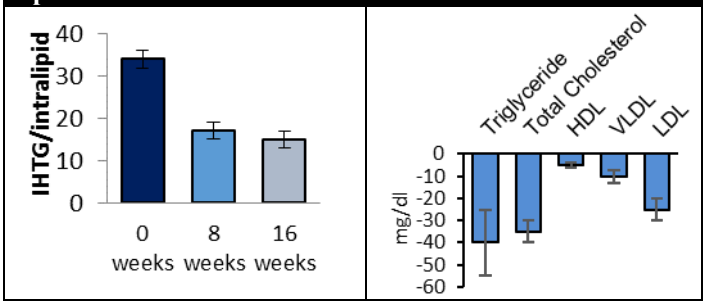
- EAA metabolism is altered in girls with PCOS. We performed untargeted metabolomics analysis in a pilot subset of girls enrolled in the AIRS cohort. We found that fasting BCAA are higher in girls with PCOS compared to controls, as were NEAA alanine (p=0.011) and glutamine (p=0.037) concentrations. Glutamine during the OSTT related to HbA1C (p=0.0091), demonstrating that an increased gluconeogenic precursor related to increased serum glucose concentrations. Further, higher BCAA valine related to worse IR as assessed with the clamp (p=0.045). During hyperinsulinemia, the concentrations of serum BCAA trended to not suppress nearly as much in the PCOS compared to the controls, and end products of BCAA oxidation, acyl-CoA C3 and C4, did not decrease **(Figure 6).** This suggests that protein synthesis as a depot for EAA was less, shunting the excess BCAA towards oxidation and muscle IR in PCOS. In contrast, EAA's (phenylalanine, methionine and tryptophan) did decrease similarly in PCOS as controls, suggesting that these may limit protein synthesis during hyperinsulinemia. These trends need to be confirmed in a physiologic setting, such as proposed.

Figure 6: Hyperinsulinemia induces BCAA oxidation not protein synthesis PCOS girls



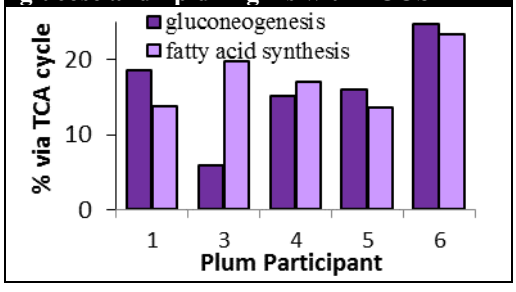
- EAA supplementation in older adults with IR decreased HS and improved insulin sensitivity. Older adults are similar to girls with PCOS in that they have worse IR and higher rates of pre-diabetes, distinct form changes in obesity. We have shown that when adults aged 65 years and older, without diabetes or hepatic steatosis, were given 11 grams of EAA twice a day for 16 weeks, serum lipids had improved by 4 weeks (**Figure 7**), and the concentration of IHTG decreased by 50%, as measured with ^{1}H-MR spectroscopy[26,103]. The supplements were well tolerated, with no adverse effects noted. Insulin sensitivity as assessed with fasting HOMA-IR was improved. Participants with the lowest insulin sensitivity and the highest IHTG experienced the greatest change with EAA supplementation.

Figure 7: EAA supplementation lowers serum and hepatic lipids in older adults with IR but not HS



- *Intrahepatic metabolism can be measured non-invasively with an U-C_{13} oral glycerol tracer, in obese adolescent girls.* Glycerol is nearly universally taken up by the liver, and when labeled with a carbon isotope can be used to trace hepatic metabolism. Our collaborator Dr. Malloy first described this methodology in adult men[105,106]. In our PLUM study, we have implemented this protocol in obese girls and made adaptations to account for their severe IR and prolonged post oral glucose response. We have determined that giving the glycerol tracer 3 hours after the sugar tolerance drink and sampling for an additional 3.5 hours provides the best data. We have also confirmed that the isotopomer outcomes of interest for Aim 2, including the percent of carbons which have come from hepatic anapleurotic cycling in the TCA cycle and then contribute to fatty acid synthesis and gluconeogenesis can be measured with this methodology in our patient population **(Figure 8).** The proportion of carbon TCA cycling in glucose trends to be related to worse HOMA-IR (R=-0.9, p=0.08 with 5 participants) and higher ALT,

Figure 8: Percent of carbons from excess TCA cycle activity in hepatically secreted glucose and lipid in girls with PCOS



suggesting that alternate sources of carbon such as from NEAA are producing glucose. *This data demonstrates that this methodology has been optimized for our patient population, and when combined with the clinical outcomes, rates of DNL and gluconeogenesis and the metabolomic amino acid data, can yield powerful insights into underlying mechanisms of pathology.*

III. Research Methods

A. Outcome Measure(s):

Primary Outcome Measure(s): Change in hepatic fat content per treatment type
Secondary Outcome Measure(s): Activity ratios of the hepatic pentose phosphate pathway, TCA cycle and TG synthesis, change in peripheral IR as assessed with an OSTT, measures of sympathetic nervous system activity, hepatic phosphate profile. Indirect measure of DNL (change in TG concentration with OSTT) Targeted glucose and fat metabolism metabolomics, and untargeted exploratory analysis, gut microbiota profile.
Likely contributors to above measures: Lipid/glucose markers:(fasting C-peptide and lipid panel, HbA1c); hepatic markers:(c-reactive protein, glucagon, GLP-1 and leptin at baseline, 5 min and 30 min post glucose load to assess changes with adiponectin, AST, ALT, GGT), sex-steroids:(DHEAS, free and total testosterone, sex hormone binding globulin, progesterone, estradiol); Body size and composition: (BMI, waist/hip ratio, DEXA, hepatic visceral fat via MRI[107-110]), Whole body fat oxidation at rest and following glucose ingestion as measured with a metabolic cart; Physical activity/ diet:(accelerometer, 3-day physical activity recall questionnaire (3DPAR); Food frequency questionnaire).Questionnaires for presence of obstructive sleep apnea. Questionnaires for perceived mental strengths and difficulty (note there is no assessment of suicidality on this tool). Obstructive Sleep Apnea, to be assessed with an overnight sleep study via WatchPAT.

B. Description of Population to be Enrolled:

Study staff aims to complete 20 obese girls with PCOS (untreated) and HS. This is the number of participants needed to be completed statistically, thus more participants may be enrolled, to allow for screen failures and dropouts. Total enrollment will be up to 50 participants as they will have a screening, then those without hepatic steatosis will be removed from the study.

Ethnic Categories	**Gender**		
	Females	Males	Total
Hispanic or Latino	20	0	**20**
Not Hispanic or Latino	30	0	**30**
Ethnic Categories: Total of All Participants	**50**	**0**	**50**
Racial Categories			
American Indian/Alaska Native	2	0	**2**
Asian	5	0	**5**
Native Hawaiian or Other Pacific Islander	5	0	**5**
Black or African American	15	0	**15**
White	23	0	**23**
Racial Categories: Total of All Participants *	**50**	**0**	**50**

Inclusion Criteria:

1) Female

2) Ages 12-21

3) Sedentary- less than 2 hours of moderate (jogging, swimming etc) exercise a week.

4) BMI equal or greater than the 90th percentile for age and gender

5) PCOS per the most stringent NIH criteria adapted for adolescents (irregular menses >24 months post-menarche and clinical or biochemical hypertestosteronemia)[1,30]

6) HS per FibroScan ultrasound, with CAP score of >225[111,112].

Exclusion Criteria:

1. Use of medications known to affect insulin sensitivity: metformin, oral glucocorticoids within 10 days, atypical antipsychotics, immunosuppressant agents, HIV medications, hormonal contraception. Dermal patch or vaginal ring contraception methods.
2. Currently pregnant or breastfeeding women. Development of pregnancy during the study period will necessitate withdrawal from the study.
3. Severe illness requiring hospitalization within 60 days
4. Diabetes, defined as Hemoglobin A1C > 6.4%
5. BMI percentile less than the 90th percentile for age and sex. Weight >325 lbs or <84 lbs.
6. Anemia, defined as Hemoglobin < 11 mg/dL
7. Diagnosed major psychiatric or developmental disorder limiting informed consent
8. Implanted metal devices that are not compatible with MRI
9. Use of blood pressure medications
10. Known liver disease other than NAFLD or AST or ALT >125 IU/L

C. Study Design and Research Methods

Overall Study Design:

Both aims with be accomplished from a cohort of 20 obese girls with PCOS and HS. Girls will complete a 12 week blinded placebo controlled cross-over study with 4 weeks each of EAA and placebo, with metabolic studies after each intervention and approximately 4 week wash out in-between **(Figure 9).** 4 weeks of EAA supplementation is adequate as it decreased IR and serum lipids in obese adults and IHTG can be changed within 3-9 days with more extreme dietary changes[15]. Participants will have a total of 5 visits, a Screening visit (Visit 1) will ensure eligibility, Study 1 (Visits 2 and 3), and Study 2 (Visits 4 and 5) (see Study Calendar). The study days will include an abdominal MRI, OSTT with isotope tracers and assessment of other contributors to HS and IR. The study days **(Figure 10)** will start in the afternoon on day 1 with MRI measures, with at least a 4 hour fast prior, followed by admission to the hospital. Participants will receive deuterated water overnight, and in the morning, they will be given U-C_{13} glycerol drink while participants
are fasting.

Figure 9: Overall Study Design

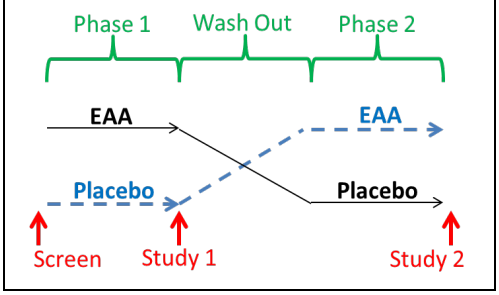
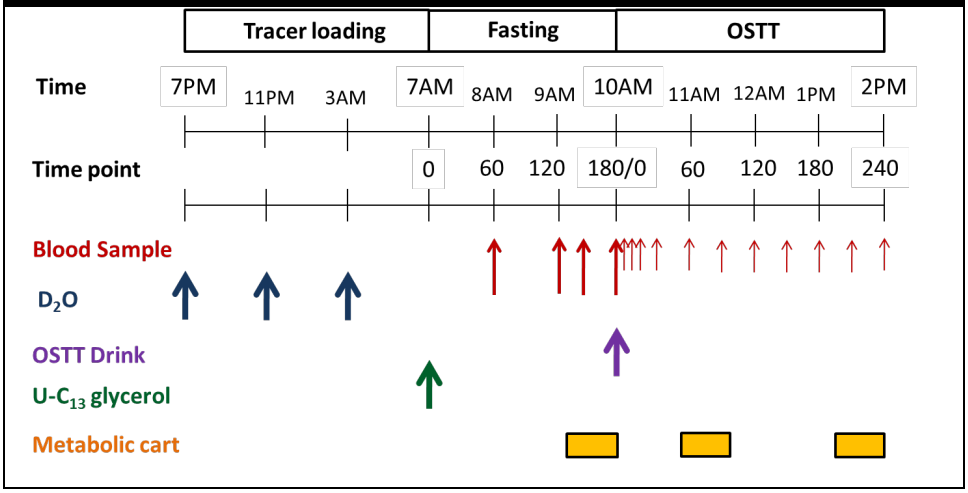


Figure 10: Study day procedure timeline



Blood will be drawn for DNL and gluoneogensis. The oral sugar tolerance test (75g glucose +25g fructose) drink will be given a few hours later. A metabolic cart will be used to measure the rate of fat oxidation fasting, and throughout the OSTT. This is similar to our current PLUM study and it is well tolerated in youth.

EAA Supplement: The formulation of EAA **(Table 3)** is similar to our previous studies with an increased proportion of leucine, although we will give 10 grams of EAA twice a day, as this stimulates maximal protein synthesis[15,26,103]. The increased leucine influences IR and also maximizes protein synthesis[94]. Supplements will be given twice a day, once during the morning and the second in the evening, as balancing EAA intake across the day has a greater effect on protein synthesis[113] and adolescents most frequently skip morning meals[100]. The supplement is a powder that is mixed in 8 ounces of water. Placebo will be matched by the pharmacy to match the EAA supplement in flavor and appearance. Despite the dogma that excess protein challenges the kidney, long-term high protein diets do not affect kidney function in healthy individuals[114] and we do not have safety concerns.

Table 3: Composition of 15 g EAA nutritional therapy

Histidine	6%
Isoleucine	11%
Leucine	23%
Lysine	16%
Methionine	5%
Phenylalanine	14%
Threonine	8%
Tryptophan	3%
Valine	14%

Participants: Recruitment: Girls will be recruited from several Children's Hospital Colorado clinics and from the community. Based on a 50% prevalence of HS in this patient population, up to 50 girls will be screened. Based on an approximately 5% drop out rate in my cross-sectional trials and my mentors clinical trials, we will aim to complete 20 girls with PCOS with HS. Inclusion criteria: PCOS per the most stringent NIH criteria adapted for adolescents (irregular menses >24 months post-menarche and clinical or biochemical hypertestosteronemia)[1,30]; age 12-21 years, sedentary status as exercise training affects IR (<2 hours of physical activity/week), BMI>90%ile for age and sex, HS per FibroScan ultrasound, with CAP score of >225[111,112]. Exclusion criteria: diabetes, liver disease other than NAFLD, liver transaminases >125 IU/L, medications affecting IR including metformin or hormonal contraception, hemoglobin <11 mg/dl, and MRI-incompatible metal.

Aim 1: Hepatic steatosis and DNL: IHTG percent will be assessed with MRI utilizing the Dixon method as in our previous studies[2,47,64]. The Dixon method has excellent correlation with liver biopsy and is preferable to spectroscopy in obese participants, where spectroscopy coil depth of penetration is limiting. Visceral and subcutaneous fat will also be calculated from MRI as in our previous studies. Rates of hepatic DNL will be measured from the tracer incorporation of deuterated water into VLDL secreted palmitate with samples analyzed with GCMS by collaborator Dr. Bergman, as previously described in youth[51,115,116]. We will utilize deuterated water rather than U-C_{13} acetate as in the APPLE study to reduce participant burden and blood volume draws. Further, both tracers have a carbon label and cannot be combined in a single protocol.

Aim 2: Insulin resistance, hyperglycemia and hepatic glucose metabolism. After 3 doses of overnight deuterated water (approximate times shown in **Figure 10**), participants will be given a U-C_{13} glycerol isotope drink per **Figure 10**[105]. The glycerol drink is given in the morning while participant is fasting and

samples are collected at frequent intervals per **Figure 10** for positional NMR isotopomer analysis by collaborator Eunsook Jin at UT Southwestern[105]. Analyses will include modeling metabolic flux utilizing custom designed software and endpoints will include rates of excess TCA cycling contributing to triglyceride synthesis and gluconeogenesis as shown in **Figure 8**. This will be followed by a OSTT (75-gram glucose and 25-gram fructose) with frequent sampling of glucose, insulin, C-peptide. Fasting insulin sensitivity will be calculated with HOMA-IR (fasting glucose* fasting insulin/22.4). The Matsuda[117] index will be used to estimate whole body post-prandial insulin sensitivity and indices of hepatic [Glucose0–30(AUC)/insulin0–30(AUC)] and muscle insulin sensitivity [dGlucose/dtime ÷ insulin] with calculations starting at the downslope of glucose concentration[118]. Blood glucose will be measured at the 2 hour time point and >140 mg/dL is defined as impaired glucose tolerance. Rates of fasting gluconeogenesis will be measured from the tracer incorporation of deuterated water into glucose, with samples analyzed with GCMS by collaborator Dr. Bergman, as previously described in youth[51,115,116]. Metabolomics: Targeted amino acid[119] and amino acid breakdown product metabolomics profiles will be measured by the University of Colorado metabolomics core on fasting and 120 minute post glucose drink samples (https://www.dalessandrolab.com/home).

Study Calendar	Screen	Phase I		Phase 2	
	Visit 1	Visit 2 –MRI	Visit 3 – Overnight	Visit 4 –MRI	Visit 5 – Overnight
Consenting and Eligibility Assessment	X				
History & Physical	X				
Intravenous Blood Draw	X		X		X
Fibroscan -Liver ultrasound	X		X		X
Randomization- Placebo vs EAA	X				
Crossover- Placebo vs EAA			X		
Accelerometer teaching	X				
Gut Bacteria Collection			X		X
Urine Pregnancy Test			X		X
Questionnaires- SEARCH Food frequency, Activity 3DPAR, Strengths and Difficulties and Sleep assessments			X		X
DEXA Scan (located in Leprino building)			X		X
Oral Glycerol Tracer with D_20			X		X
Oral Sugar Tolerance Test (OSTT)			X		X
WatchPAT sleep study			X		X
Metabolic Cart			X		X
EndoPat and Dynapulse		X		X	
MRI of abdomen and liver		X		X	
P MRS of Liver		X		X	
Total Time of visit (approximately)	2 Hours	2 Hours	22 Hours	2 Hours	22 Hours
Location of Visit	CHCO CTRC Outpatient	UCD Outpatient, Brain Imaging Center	CHCO or University Hospital Inpatient	UCD Outpatient, Brain Imaging Center	CHCO or University Hospital Inpatient

Timeline: The time between the initial screening visit and completion of the study will be less than 6 months. After confirmation of eligibility and randomization, participants will be on 4 weeks of EAA or placebo and then Phase I study visits will be completed (Visits 2 and 3). There will be approximately 4-

week washout period and then participants will crossover treatment, and complete another 4 weeks of EAA or placebo. Phase II study visits (Visits 4 and 5) will be completed.

Overall enrollment procedure plan per group
VISIT 1 (SCREEN VISIT)
Pediatric CTRC Outpatient unit: Participants will begin with a medical screening and physical exam for inclusion/exclusion criteria evaluation. During this visit, patients will review and complete consent documents, have demographics and medical history confirmed, assess allergies and further evaluation of inclusion/exclusion criteria, have blood samples drawn, and have anthropometrics completed. HbA1c, ALT, AST, CBC panel and testosterone panel will be drawn at the beginning of the visit after consent in all participants. PCOS labs may be performed for confirmation of PCOS status, if not performed previously. Whole blood for DNA samples for future genetic analysis associated with hepatic steatosis will also be drawn and stored at this time. Participants will also have a Fibroscan (liver ultrasound) to check for HS (CAP score of >225).

After eligibility assessment, participants who qualify for the study will be randomized to placebo or EAA by the pharmacy. Both study staff and participant will be blinded. The participant will be scheduled for Visits 2 and 3 after being on placebo or EAA supplements for 4 weeks.

Screening lab test	**Purpose for test**
HbA1C	Rule out type 2 diabetes, if > 6.4% participant to be excluded
ALT, AST	Ensure no severe liver disease, if >125 IU/L participant to be excluded normal participant is excluded
Hemoglobin & Hematocrit (part of CBC)	If participant is Anemic, they will be excluded
Testosterone	Test for hyperandrogenism – required to meet NIH criteria for PCOS
<u>Optional PCOS labs</u> PCOS status must be confirmed prior to enrollment. Referring physicians often do not perform the entire recommend work-up for oligomenorrhea (per 2013 Endocrine Society Clinical Guidelines for PCOS). The values are typically expected in PCOS publications	
TSH, total T4	Ensure no hypo or hyperthyroidism causing amenorrhea
LH, FSH	Rule out primary ovarian failure
17-hydroxyprogesterone	Rule out late onset congenital adrenal hyperplasia
DHEAS	Rule out adrenal tumor
Prolactin	Rule out prolactin secreting brain tumor

VISIT 2 (MRI VISIT)
<u>Overall Plan:</u> UCD Research MRI (Brain Imaging Center): Participants will be asked to fast for 4-6 hours prior to this visit. The imaging will be of the liver. Two different studies may be conducted. One will be standard MRI of the mid abdomen to assess the amount of subcutaneous fat, visceral fat and percent liver fat. The second type of scan is optional pending scheduling. It will be ^{31}P spectroscopy to measure the concentrations of phosphate molecules including PDE, PME and PCr. Vascular Endothelial function

and Heart Rate Variability will be assessed with Endopat and Dynapulse, which take approximately 30 minutes.

^{31}P MRS of the Liver and Abdominal Imaging (pending scheduling)

MRS Data acquisition: Imaging and MRS will be performed on a Seimens 3 Tesla MRI magnet. A custom ^{1}H/^{31}P abdominal coil will be used for imaging and MRS (Clinical MR Solutions, Brookfield, WI) as in our previous ^{31}P work[120]. The coil will be a concentric probe with an inner coil 16 cm in diameter (for ^{31}P) and a 20 cm outer coil (for ^{1}H scout imaging and shimming). A 2 cm x 2 cm x 2 cm area of focus is found in the liver in homogenous tissue for MRS, similar to our previous studies[109]. A ^{31}P MRS scan will then be performed for baseline measurements. We will continue to work with our current collaborators Mark Brown, PhD, Assistant Professor, Department of Radiology, UC Denver Anschutz and Bradley Newcomer, PhD, Professor, Department of Radiography, University of Alabama at Birmingham to optimize the MR signal collection. Visceral adiposity will be measured using the gold standard of an MRI slice at L4-L5. Hepatic fat fraction will be performed using modification of the Dixon method as in our previous studies[121]. Hepatic fibrosis will be measured with a fibroscan sequence.

MRS Data Analysis: For the ^{31}P data, peak positions and areas of interest [phosphocreatine (PCr), inorganic free phosphate (Pi), β-ATP(3 peaks), α-ATP(2 peaks), γ-ATP(2 peaks), and PME] will be determined by time domain fitting with jMRUi [122,123], utilizing AMARES (A Method of Accurate, Robust and Efficient Spectral fitting), a nonlinear least-square-fitting algorithm using our previously built prior knowledge files **[124]**. We have utilized this method for muscle **^{31}**P analysis for the previous 6 years, and have extensive experience with this analysis. The adipose data will be analyzed by radiology MRI research imaging, supervised by Ann Scherzinger, PhD, as in previous protocols (COMIRB #'s 10-1288 and 14-0542).

Gut Bacteria Collection: The gut microbiome consists of the microorganisms, predominantly bacteria, that inhabit the gastrointestinal tract and are estimated to outnumber mammalian cells by up to a factor of 10, and their genes outnumber human genes by a factor of over 100 [69]. It is possible that gut microbiota contribute to the development of NAFLD, and this has not been explored in PCOS. We will collect a sample to define the microbiota in girls who are already undergoing extensive metabolic profiling. The gut microbiota will be collected with BBL culture swabs (Becton, Dickinson and Company, Sparks, Maryland) one week prior to visit 3. Fecal samples will be collected from the first bowel movement of any day the week before visit 3 and stored in the freezer. Fecal samples are being collected for present and future research markers in the mircrobiome. Fecal samples are routinely collected in research and pose little risk to participants. For all samples, bacterial DNA will be extracted from the swab using established methods and the V4 region of 16S bacterial rRNA will be amplified using previously published primers and PCR conditions. [66, 67, 68] To provide a full picture of microbial diversity in the gut, we have combined phylogenetic and Operation Taxonomic Units (OTC)-based methods for comparing communities. Grouping bacterial rRNA sequences by similarity is important for asking questions about which particular species, genera, phyla, etc, contribute to differences between samples. To choose OTUs, groups of similar 16S bacterial rRNA sequences are identified, and candidate OTUs are identified as sets of sequences connected to each other. Candidate OTUs are considered valid if the average density of connection is above 70% (i.e., if 70% of the possible pairwise connections between sequences in the set exist.

Accelerometer: One week prior to the overnight visit, the participant will be provided two accelerometers (GT3X BT by Actigraph and ActiWatch by Philips Respironics) to be worn for seven days to measure level of habitual physical activity, which affects insulin sensitivity, and sleep patterns. Accelerometers are effective tools for the objective measurement of physical activity [125] because they have the ability to continuously record physical activity data and such data can be used to estimate METs of activity. They provide more detailed information than pedometers, which only measure walking steps, and help get around the recall bias of questionnaires. We are currently using the GT3X BT Actigraph in adolescents in our other diabetes studies; therefore, we are familiar with their use in this population and have the necessary computer software and interpretation skills. The Actiwatch is being used as a tool for objective measurement of sleep patterns. The Actiwatch is fitted with a LED monitor that detects multiple spectrums of light to better assess sleep patterns in this population.

***One week prior to overnight visit, participant will receive via mail the accelerometers, sleep diary and stool sample collection kit (Gut bacteria collection) ***

VISIT 3 (ORAL GLYCEROL TRACER WITH D20 AND ORAL SUGAR TOLERANCE TEST)

Children's Hospital Colorado or University Hopsital CTRC Inpatient unit Only under extenuating circumstances, if no rooms available in either hospital, we may have patients go home after WatchPAT is placed, and drink deuterated water at home, and come back the following morning at 6am: Participants will be asked not to have caffeine or exercise for 3 days prior to this visit. During admission, participant will be provided with a study diet dinner and snack. Patients will be admitted to the Pediatric CTRC for a monitored overnight fast. Participants will be questioned regarding changes in concomitant medications and medical history, height, weight, BP. Participants will also have a Fibroscan (liver ultrasound) on the day of admission or the morning of the OSTT (pending staff availability).

A DEXA scan will be performed to assess body composition. A urine pregnancy test will be done on all female participants prior to the DEXA scan. If a female participant is confirmed to be pregnant, she will be withdrawn from the study and referred to her primary care physician for follow-up.

The following morning, a blood sample for baseline metabolic labs will be drawn the modified OSTT will be completed **(Figure 10)**. Additional blood will be drawn and stored for future inflammatory markers as well as for targeted and untargeted metabolomics analysis.

WatchPAT: During the hospital overnight stay, trained study staff will place the WatchPAT sleep monitor. The primary measures will be for oxygen saturation and apnea hypopnea index (AHI). Each participant will wear the watch with a one-time use finger cuff as recommended by the FDA. The watch will be placed by 8 PM, and will be removed the following morning. The watch pat data collection will not be ideal, due to waking the participants to drink the deuterated water. However, the primary endpoint is the apnea hypopnea index, and we can still get a rough estimate of this information, even with less than ideal sleep.

Details of Stable Glycerol Isotope Tracer studies with an OSTT: Participants will fast overnight, and drink 3 doses of D_2O three times during the night with doses at 6-8PM, 10-11 PM and 2-3 AM as described previously in obese youth. The total D_2O to be given (3 mL per kg of body water) is designed to raise body deuterium levels to 0.3% and was administrated in three doses to maintain the steady state until the end of the study[126]. Serum samples will be collected in the morning before the glycerol, and then throughout the OSTT, and VLDL TG separated and then enrichment in palmitate measured. By evaluating the incorporation of the D_2O into palmitate, we will able to assess the DNL expressed as a percentage of DNL. We will also measure the rate of gluconeogenesis by measuring the enrichment in serial plasma glucose samples.

The following morning at approximately 7:00AM, a 50 mg/kg of an oral $^{U}C_{13}$-glycerol tracer will be consumed mixed with water. Blood samples will be collected and at approximately 10AM, participants will consume a 75 gram glucose load with an additional 25 grams of fructose to stimulate hepatic de novo lipogenesis. Analysis of serum glucose isotopomers will be performed by our collaborator Eunsook Jin at UT Southwestern, using NMR isopomomer analysis and pathway modeling software. Lipolysis will be modeled using FFA concentrations and whole body IR by a time modified version of the Matsuda model.

Using a metabolic cart and hood, resting VO2 (ml/kg/min) and VCO2 (ml/kg/min) measurements (REE) will be collected the morning of the OSTT prior to the start of the OSTT, and two more times during the OSTT. This is required to determine what portion of ingested carbohydrates are subject to oxidative and non-oxidative glucose disposal[127].

Study diet: Variations in diet, activity and circadian rhythms affect metabolism [121]. Therefore, OSTT studies will be performed in the AM, in the follicular phase where possible, preceded by 3 days of no strenuous physical activity. Participants will be advised to eat a typical diet for the 3 days preceding the study. On the day of study, they will have a snack and dinner selected from limited choices on the Children's hospital carbohydrate counting diet plan, with at least 20 grams of carbs in the snack and 40 grams of carbs for dinner. Participants will ingest their study drink (amino acid or placebo) in the morning of the day of admission, and then after the MRI with their snack.

Note: Participants will stop taking placebo or EAA supplements after completion of overnight visit for approximately 4 weeks (washout period). Then participants will crossover to EAA or placebo. The pharmacy will dispense the proper supplement, since study staff and participant will be blinded. The participant will be scheduled for Visits 4 and 5 after being on placebo or EAA supplements for 4 weeks.

VISIT 4 (MRI VISIT)
See Visit 2 Description.

VISIT 5 (ORAL GLYCEROL TRACER WITH D20 AND ORAL SUGAR TOLERANCE TEST)
See Visit 3 Description.

Purpose for lab test to be drawn:

OSTT Labs	Purpose
Lipids and glycerol samples for tracer analysis	Determination of hepatic metabolism flux
Glucose	Determination of IR
Insulin	Determination IR
FFA	Measure of lipolysis
Glycerol	Measure of lipolysis
Glucagon	Gut hormone known to influence hepatic IR
GLP-1	Gut hormone known to influence hepatic IR
CRP	Marker of inflammation, know to effect IR
Leptin	Gut hormone known to influence hepatic IR
Adiponectin	Adipokine thought to influence adipose IR
Estradiol	Known to effect IR and HDNL
Progesterone	Demonstrate the participant is in the follicular phase of cycle, required for publication
Metabolomics	Correlation with hepatic metabolism flux
1 sample for genetic analysis	Measure for common polymorphisms associated with hepatic steatosis[126,128]
Stored blood	Future markers of glucose and fat metabolism, CVD or hormones related to PCOS

Purpose for questionnaires being done:

Questionnaire	Description	Study Validity
Adolescent Sleep Hygiene Scale	Measurement of sleep patterns/habits	Cronbach's alpha ranges from .46-.74; total scale alpha = .80 LeBourgeois et al. 2005. *The Relationship Between Reported Sleep Quality and Sleep Hygiene in Italian and American Adolescents*

Center for Epidemiological Studies Depression (CES-D)	Measuring for depression	See Table 3 below, adapted from: Stockings et al. 2015. *Symptom screening scales for detecting major depressive disorder in children and adolescents: A systematic review and meta-analysis of reliability validity and diagnostic utility*
Cleveland Adolescent Sleepiness Questionnaire	Measurement of sleepiness during a typical week	alpha = 0.89 Spilsbury et al. 2007. *The Cleveland Adolescent Sleepiness Questionnaire: A New Measure to Assess Excessive Daytime Sleepiness in Adolescents*
SDQ: Strengths and Difficulties Questionnaire	Behavioral screening questionnaire	The internal reliability of the various self report scales was assessed using Cronbach's alpha coefficient. This was 0.82 for the total difficulties, 0.75 for emotional symptoms, 0.72 for conduct problems, 0.69 for hyperactivity, 0.65 for prosocial behaviour, and 0.61 for peer problems. Goodman et al. 2003. *The Strengths and Difficulties Questionnaire: a pilot study on the validity of the self-report version*
Sleep Disturbances Scale for Children	Gain understanding of sleep-wake rhythm and any problems in sleep behavior	Internal consistency ranged from .71-.79l test-retest reliability r = .71 Bruni et al. 1996. *The Sleep Disturbance Scale for Children (SDSC)Construct ion and validation of an instrument to evaluate sleep disturbances in childhood and adolescence*
3DPAR: Activities Scale	Measuring activity in the 3 days previous as a typical activity score	Interrater and test-retest reliability was 0.99 and 0.98, respectively (P < 0.01). The correlation between relative energy expenditure from the PDPAR (kcal.kg-1.l.d-1) and pedometer and Caltrac counts was 0.88 (P < 0.01) and 0.77 (P < 0.01), respectively. The correlation between percentage heart rate range (HRmax-HR-rest) and mean energy expenditure from the PDPAR was 0.53 (P < 0.01). The correlation between 1-min heart rates > 50% HRR sustained for 20 min and the number of 30-min blocks with a relative energy expenditure of at least four metabolic equivalent tasks (MET) was 0.63 (P < 0.01). The PDPAR provides valid and reliable estimates of physical activity and also accurately identifies bouts of moderate to vigorous activity. Weston et al. 1997 *Validation of an instrument for measurement of physical activity in youth*
Food Frequency Questionnaire	Measuring typical food intake over	The mean correlations, adjusted for measurement error, of food groups and

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

Table 3
Validation evidence for the Center for Epidemiologic Studies Depression Scale (CES-D) in child and adolescent samples.

Source	N	Age and Gender (% m, % f)	Sample (location)	Scale name (no. of items)	Reliability	Criterion	Cutoff	Sensitivity	Specificity	PPV	NPV	AUC
Clinical												
Logsdon and Myers (2010)	59	13–18 (0, 100)	Adolescent mothers at 4–6 weeks postpartum (USA)	CES-D (20)	α=0.84	K-SADS-PL	16	0.7	0.52	0.25	0.12	0.62
Aebi et al. (2009)	140	Mean: 15.5 (33, 67)	Adolescents diagnosed with major depressive disorders (Switzerland)	CES-D (20)	α=0.83	Clinical interview	21	0.86	0.86	–	–	0.94
Non-clinical												
Betancourt et al. (2012)	367	10–17 (33, 67)	Children and adolescents (Rwanda)	CES-DC (20)	α=0.86	MINI-KID	≥ 30	0.82	0.72	–	–	0.83
Cuijpers et al. (2008)	1392	14–16 (52, 48)	Adolescents (Netherlands)	CES-D (20)	α=0.93	MINI	22	0.9	0.74	–	–	0.90
Thrane et al. (2004)	213	9–16 (54, 46)	Adolescents from three American Indian reservations (USA)	CES-D (20)	α=0.80	–	–	–	–	–	–	–
Yang et al. (2004)	2440	12–16 (52, 48)	Adolescents (Taiwan)	CES-D (20)	α=0.9	K-SADS-E	90th % tile	0.41	0.9	–	–	0.9
Prescott et al. (1998)	556	Mean: 16.8	Adolescent students from grades 9–12 (USA)	CES-D (20)	–	DISC	16	0.79	0.74	0.24	0.96	0.74
Garrison et al. (1991)	1231	12–14 (100, 0)	Child and adolescent boys from school sample (USA)	CES-D (20)	α=0.81	K-SADS-P	12	0.85	0.49	0.16	0.98	0.61
Garrison et al. (1991)	1234	12–14 (0, 100)	Child and adolescent girls from a school sample (USA)	CES-D (20)	α=0.86	K-SADS-P	22	0.83	0.77	0.32	0.98	0.77
Roberts et al. (1991)	1710	14–18 (47, 53)	Adolescents of nine senior high schools (USA)	CES-D (20)	α=0.89	K-SADS	24	0.84	0.75	0.08	0.99	–
Roberts et al. (1991)	804	14–18 (100,0)	Adolescent male sample of nine senior high schools (Roberts et al., 1991; USA)	CES-D (20)	–	K-SADS	22	–	–	–	–	0.87
Roberts et al. (1991)	906	14–18 (0,100)	Adolescent female enrolment of nine senior high schools (Roberts et al., 1991; USA)	CES-D (20)	–	K-SADS	24	–	–	–	–	0.83
Fendrich et al. (1990)	220	12–18	Children and adolescents at risk for depression according to their parents' diagnosis (USA)	CES-DC (20)	α=0.89	K-SADS-E	≥ 16	0.71	0.62	0.15	0.96	–

Note: *N*=Number of participants in the study sample. PPV=Positive predictive value. NPV=Negative predictive value. AUC=Area under the curve analysis. CES-D=Center for Epidemiologic Studies Depression Scale. CES-DC=Center for Epidemiologic Studies Depression Scale Child Version. α=Cronbach's alpha reliability co-efficient. K-SADS-PL: The Schedule for Affective Disorders and Schizophrenia for School-Age Children – Present and Lifetime version. MINI-KID: The Mini-International Neuropsychiatric Interview. MINI: The Mini-International Neuropsychiatric Interview for children. K-SADS: The Schedule for Affective Disorders and Schizophrenia for School-Age Children. K-SADS-E: The Schedule for Affective Disorders and Schizophrenia for School-Age Children – Epidemiological version. DISC: The National Institute of Mental Health Diagnostic Interview Schedule for Children. K-SADS-P: The Schedule for Affective Disorders and Schizophrenia for School-Age Children Present Episode version.

LIFESTYLE PRESCRIPTION

1) At the time of screening, all participants will receive standard lifestyle recommendations including the reduction of liquid carbohydrate and portion control. We will not perform extensive counseling at this time as major changes in diet could affect the results. However, variability in the consumption of sugar sweetened beverage may affect results, and thus we will discourage these in all participants. Further this is the first step of standard lifestyle counseling for this population.

2) The final visit will conclude with education regarding the importance of physical activity, diet and lifestyle modification to mediate the risks associated with sedentary lifestyle and an exercise prescription designed to increase physical activity. The information at this visit will build on the previous education, and be personalized to include the basal metabolic rate calculations and specific dietary recommendations based on the food frequency survey. The exercise information and prescription are the standard of care used in our Children's Hospital Colorado Pediatric Metabolic Syndrome Clinic, designed by the Children's Hospital Colorado Pediatric Exercise Physiologist. Families will be provided with standard information about follow up care with their primary care provider and contact information for Children's Hospital Colorado Diabetes and Child Health Clinics if needed regarding any abnormal study findings. In addition, the participant/family will be provided with copies of their study lab results, DEXA scan and physical activity monitoring. A results letter will be provided to the family within 6 months of completion of the study about clinically relevant results obtained during the study. Participants will be encouraged to contact study staff with questions regarding the results letter.

Follow-up from Sleep study results:

As discussed above, we anticipate that approximately 30-40% of our participants will have an abnormal apnea hyponea index (AHI), requiring some type of follow-up. We have worked with Drs. Ann Halbower, Stephen Hawkins and Ben Hughes, our primary pediatric sleep pulmonologists to develop a post-study follow-up algorithm. Of note, Dr. Hallbower is currently working with Kaiser to verify the accuracy of the results from the WatchPAT device as compared to inpatient polysomnograms in children younger than 12, and is very familiar with this device, it's output and limitations. Our youth fall into a grey zone in terms of what is an abnormal sleep study, as pediatric criteria are defined for less than 12, and adult for 18 or older. The international accepted clinical criteria are listed below, as well as the American Academy of Sleep Medicine's recommendations of how to handle age 12-17:

Pediatrics:

Mild OSA	Moderate OSA	Severe OSA
1 to 4.9	5 to 9.9	>10

Adults

Mild OSA	Moderate OSA	Severe OSA
5 to 14.9 + symptoms	15-30	>30

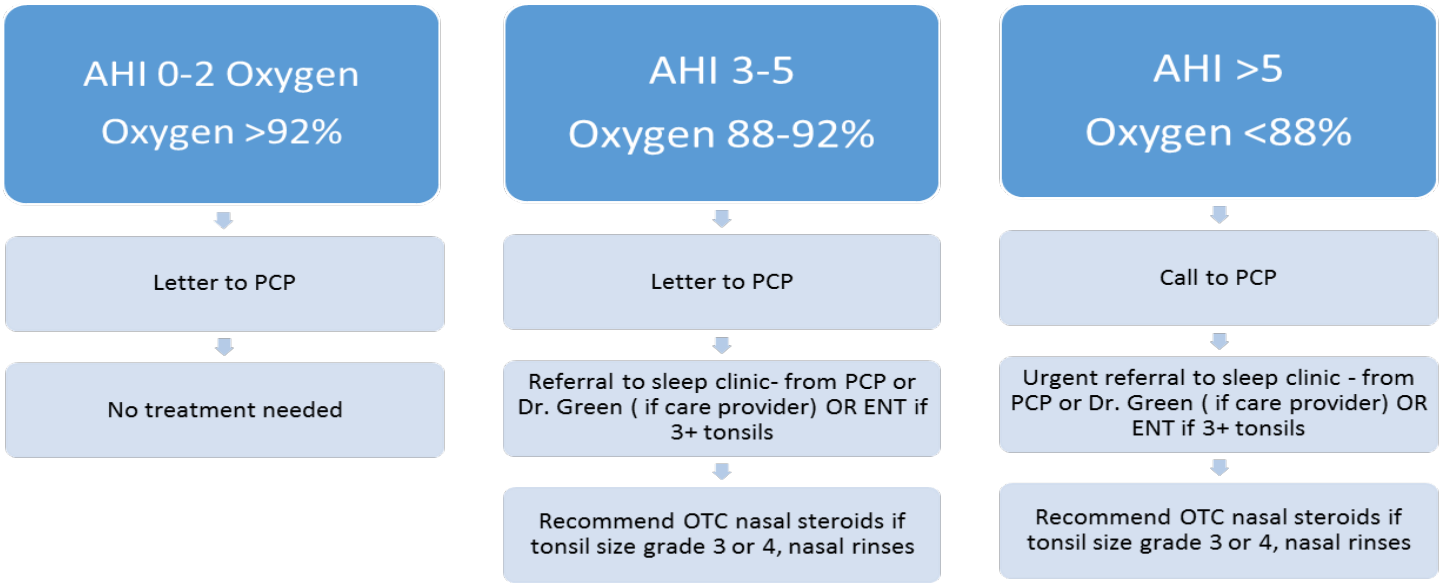
A. Ages for Which Pediatric Respiratory Scoring Rules Apply

1. Criteria for respiratory events during sleep for infants and children can be used for children <18 years, but an individual sleep specialist can choose to score children ≥13 years using adult criteria.[N1] RECOMMENDED

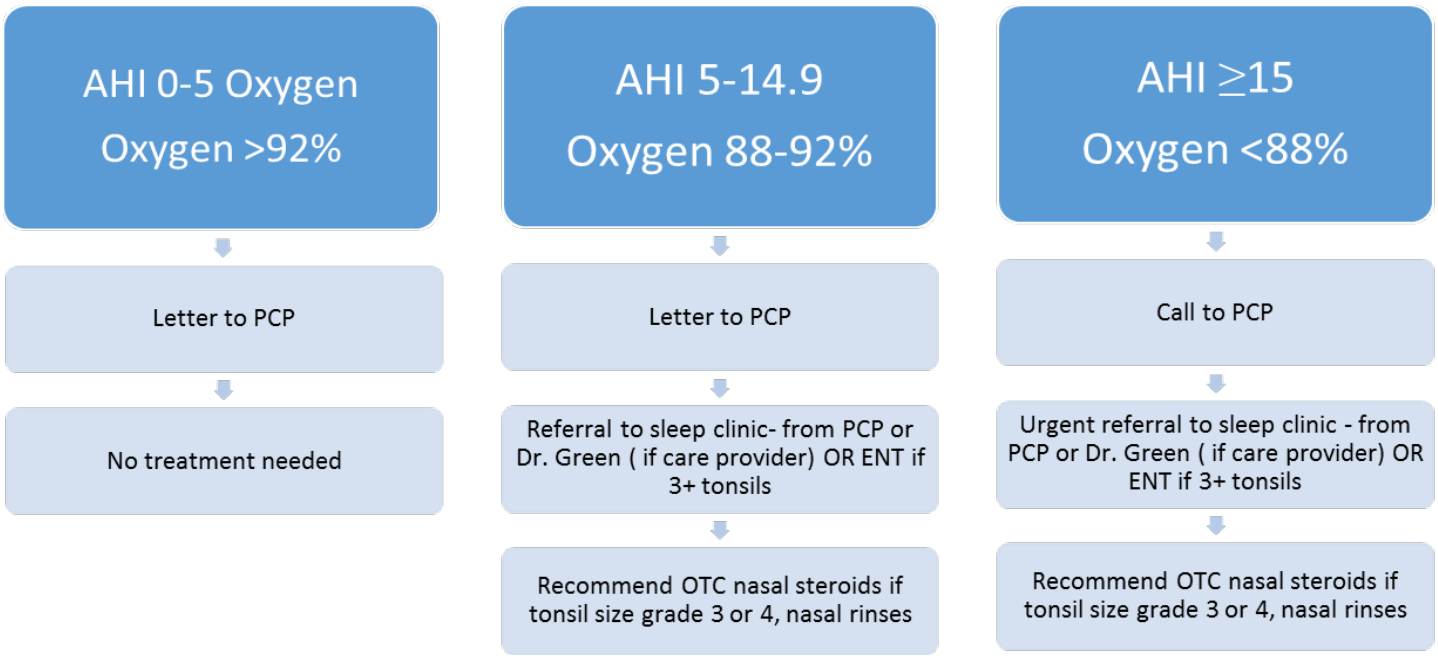
Note 1. Several studies suggest that the apnea hypopnea index (AHI) will be higher in adolescent patients when using pediatric compared to the adult rules presented in the 2007 version of the AASM scoring manual. As adult hypopnea rule 1A and pediatric hypopnea rules are similar, there may now be less difference in the AHI when using adult versus pediatric rules.

Since the recommendations are not concrete for the 12-17 year old range, we sought to follow what is being done in clinical practice at Children's Hospital Colorado sleep clinics, were a patient to have an inpatient polysomnogram. Sleep studies are read within 5 business days, and we will adhere to this same turnaround timeline. In terms of interpretation, currently, the pediatric guidelines are being applied for the 12-17 year old age group but 2 is considered normal in this age, and thus our post-study for the 12-17 year olds will follow this, and are shown below. Approximately half of the participants have a patient relationship with the PI Dr. Green, and thus she can order F/U evaluation if needed, and if not, Dr. Green will request the follow-up be arranged by the primary care provider. We have 2 algorithms by age.

For participants 12-17 years of age:



For participants ≥18 years of age:



PARTICIPANT RECRUITMENT/CONSENT/PAYMENT

1. **Participant Recruitment Plan**

Participants will be recruited from pediatric endocrine, PCOS, Lifestyle, adolescent and gynecology clinics, and from the community. We receive 4-8 new PCOS referrals a month, showing the feasibility of recruiting the required participants. Further, we enrolled >100 obese girls in studies in the last 60 months. The PI and Co-I's have a treatment relationship with girls from clinic or participants can call from study

advertisements. Protected health information will only be accessible by study investigators. The initial patient contact will be made by personnel who have a treatment relationship with the participant.

2. **Informed Consent Plan**

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

3. **Special Consent/Assent Plan**

Consent will be obtained from all participants in the study. Following explanation, all participants below 18 years old will co-sign the consent form in addition to the parents signing the consent form. All participants age 18 or older will sign the standard consent form.

4. **Participant Compensation, Incentives and Rewards**

Participants will be compensated with Target gift cards for completion of each study visit. The initial visit consisting of informed consent, lab draw, and abdominal ultrasound fibroscan will result in a $50 gift card. Each of the 2 MRI's (Visits 2 and 4) will be $50 each. Each of the two overnight visits (Visits 3 and 5) will be $150 each. In an effort to maintain participant retention and completion of the study, the participants will be rewarded an additional $50 if they complete all study visits 1-5. Compensation for all completed visits will total $500.

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

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

1. Blood Sampling

Description: Blood will be drawn for Complete Blood Count (CBC), HbA1c, total and free testosterone, and sex hormone binding globulin. If participants have not had a full evaluation for oligomenorrhea, Prolactin, DHEAS, LH, FSH, TSH, total T4 or 17-OH progesterone may be drawn.
Risk: Minimal. Risk of pain, bruising at site of blood draw, excessive amount of blood.
Minimizing Risk: Certain studies at our institution draw over 7ml/kg in 6 weeks, or up to 7 ml/kg in a single draw, but include iron supplementation. Otherwise, the routine guidelines in our Pediatric CTRC are 2.5ml/kg for a single draw and no more than 5 ml/kg over a 4 week period. Our baseline visit will include 11.5 ml of blood (HbA1c, Hb, Cr, AST, ALT) and 25.5 ml of blood for PCOS patients (additional draw for T4, TSH, prolactin, LH, FSH, 17OH progesterone and DHEAS). The OSTT visit includes up to 300 ml of blood which will occur within 4 weeks of the initial visit. Thus, our OSTT visit is within the NIH Clinical Center guidelines of 9 ml/kg in 6-8 weeks and within Children's Hospital Colorado's institutional guidelines of 5 ml/kg. In addition, by study design, participants are screened by our baseline CBC and excluded if anemic, further increasing the safety of the study regarding blood draws. We will use a minimum weight cutoff of 38 kg to remain below the most conservative pediatric CTRC blood drawing guidelines. This screening also helps to increase the safety of the blood draw. In addition, the blood planned to be frozen and held could also be omitted if needed to reduce blood volume for a particular participant. Finally, our CTRC has a system to track other studies participants might enroll in, and we ask

during our consent process if the participant has been involved in any other studies in the past 6 weeks to avoid excessive blood drawing.
Justification: Screening laboratory measurements are necessary to assure that patients meet inclusion/exclusion criteria before any further study is completed. A CBC is necessary as a screening lab, to rule out anemia. A hemoglobin A1c can be used to rule out diabetes. Hormone levels of free and total testosterone, and sex hormone binding globulin are needed to categorize patients as having PCOS, and prolactin, DHEAS, LH, FSH, TSH, total T4 or 17-OH progesterone to rule out other causes of oligomenorrhea, if not done previously.

2. IV Risks
Description: One peripheral IV will be placed during the OSTT for drawing blood samples.
Risk: There is temporary discomfort when the needle goes in and 10% of the time there is a small amount of bleeding under the skin that may produce a bruise. Rarely, there is a risk of a blood clot forming or infection. We will use a low dose of a medication called heparin to try to prevent blood clotting.
Justification/Minimization: These studies involve sampling blood at multiple time points. Thus, an IV is needed, so as to avoid multiple needle sticks. These studies are focused on measured rates of change which necessitates the sampling of the same test over time. Proper sterile technique will be used with blood draws and IV placement to decrease the infection risk. EMLA cream will be used if participant desires to minimize pain of IV.

3. Oral Sugar Tolerance Test (OSTT):
Description: An OSTT will be performed with multiple blood draws. The purpose of the OSTT is to provide a controlled oral stimulus to effect changes in lipolysis and hepatic glucose release.
Risk: The participants rarely experience nausea within 15 min of consuming the drink, however, the amount of carbohydrate is very similar to a large soda, which is regularly consumed by this patient population.
Justification/Minimization: A standard oral challenge is needed to study lipogenesis, lipolysis and gluconeognesis in the fed state. We have chosen to start with a standard glucose and fructose load, to simplify the mathematical modeling. Dynamic carbohydrate metabolism in youth is made more relevant by the recently reported TODAY study, showing a decline in beta cell function in youth with newly diagnosed type 2 diabetes that was much more rapid than what has been reported in adults, and not prevented by metformin in the majority of the youth[129]. Our team of investigators, CTRC research nursing staff and physicians are well experienced with the OSTT blood draw procedure. A floor nurse located on the will be available during our inpatient visits and patients will be distracted by TV or other similar means during the OSTT, to minimize queasiness.

4. Stable Isotope Studies Glycerol:
Description: Oral stable isotope tracer of glycerol will be utilized to determine rates of intrahepatic substrate flux. These are substances normally present or produced in the body, and thus pose no more risk than typical glucose infusions. Measurements of these metabolic processes are only able to be made with the utilization of stable isotope tracers.
Risk: We are utilizing an isotope which already exists in all humans, but are simply increasing the percentage. We are only giving this medication orally. These are NOT radioactive substances.
Justification/Minimization: Pyrogen-free $^{2}C_{13}$ glycerol will be obtained from the manufacturer and delivered to CHCO IDS. The IDS pharmacist will deliver the tracer to the 9th floor inpatient CTRC once ordered by the physician or outpatient if patient is admitted to University Hospital.

4. Stable Isotope Studies Deuterated water:
Description: Oral stable isotope tracer of deuterated water will be utilized to determine rates of de novo lipogenesis and gluconeogenesis. These are substances normally present or produced in the body, and thus pose no more risk than typical water unless consumed in large amounts, to increase total body enrichment in excess of 20%[130]. Measurements of these metabolic processes are only able to be made with the utilization of stable isotope tracers.
Risk: We are utilizing an isotope which already exists in all humans, but are simply increasing the percentage. We are only giving this medication orally. These are NOT radioactive substances. The

planned dose is 3 ml/kg as has been used previously in obese youth[126], with a goal enrichment of 0.3%, well below the 20% known to cause side effects. At the proposed dose, few subject experienced nausea, but it was extremely well tolerated (personal communication with PI's Dr. Santoro and Parks).
Justification/Minimization: Pyrogen-free deuterated water will be obtained from the manufacturer and delivered to CHCO IDS. The IDS pharmacist will deliver the tracer to the 9th floor inpatient CTRC once ordered by the physician or outpatient if patient is admitted to University Hospital.

6. Standard Diet
Description: A dinner and snack will be provided during the overnight visit. selected from limited choices on the Children's Hospital cafeteria carbohydrate counting diet plan, with at least 20 grams of carbs in the snack and 40 grams of carbs for dinner.
Risk: None
Justification/Minimization: Variations in diet, activity and circadian rhythms affect metabolism [121].

7. Magnetic Resonance Imaging (MRI)
Description: The MRI will usually be obtained the day of admission to CHC), at the UCD Brain Imaging Center on the Fitzsimmons campus. A trained research radiographer who is supervised by Dr. Mark Brown, of UCD radiology, will perform an abdominal MRI to obtain hepatic, visceral and subcutaneous fat on a 3.0 T whole–body MRI scanner (Siemens MAGNETROM, Malverne, PA). Participants will lie supine while these measurements are obtained, need to hold reasonably still during the scan and cannot weigh >325 lbs. A second sequence to measure the amount of fibrosis (if any present) in the liver will be performed. A specialized phosphorus coil will be utilized to measure the concentration of ^{31}P via MRS.
Risks: Minimal. Participants may develop claustrophobia in the magnet.
Minimizing Risk: The participant is provided with audio protection and optional television to help increase comfort. Some participants might feel claustrophobic while having an MRI and the scan will be stopped if it cannot be tolerated. In addition, any participants with implanted metal that is not cleared by the MRI technician may not be able to have the MRI due to the type of magnet involved.
Justification/Minimization: MRI is a non-invasive and non-radiation method to assess body fat, and mitochondrial function. The risks are minimized by assuring patient comfort prior to starting the scan, placing eye goggle that plays movies on the participants. Further, per standard protocol, no patient will be placed into the scanner if they do not meet the rigorous safety standards for the MRI, including the absence of non-compatible implanted metal.

8. Body Composition
Description: Body composition will be measured using the DEXA technique and will be used to derive fat-free mass and % body fat. This technique relies on the absorption of dual electron wavelengths for the assessment of body fat, lean tissue, and bone mineral density. During the procedure, the participant will be supine on the measurement table, and the arm of the machine will slowly pass over their body.
Risk: Minimal. Radiation exposure
Justification/Minimization: Body composition is best assed via DEXA, and the amount of muscle mass is needed to standardize the OSTT results, since body weight can vary greatly. This procedure will deliver the radiation exposure that is 2 times the level of background radiation in Colorado. Participants will be tested for pregnancy immediately prior to DEXA, to ensure that they are not pregnant.

9. Endopat and Dynapulse
Description: The Dynapulse Pathway and the EndoPat system are noninvasive portable systems that measure brachial artery distensibility and endothelial function, utilizing a standard sphygmomanometer cuff inflated in the same fashion as a sphygmomanometer to obtain blood pressure. The instrument derives brachial artery distensibility using the technique of pulse waveform analysis of arterial pressure signals obtained from the sphygmomanometer. Measurement of heart rate variability for autonomic tone will be performed using the Endopat
Risk: This procedure may lead to mild discomfort due to the blood pressure cuff being inflated.
Justification/Minimization: Endothelial function is a novel measure in PCOS and will aid in the determination of cardiovascular dysfunction with this population.

10. Body fat distribution

Description: Height, weight, waist circumference, and hip circumference will be measured. Body fat distribution will be determined using the waist-to-hip ratio where the waist circumference is measured 1/2 the distance from the xiphoid process to the navel and the hip circumference is measured at the level of the greater trochanter.

Risk: None

Justification/Minimization: IR has been associated with central obesity, as has hyperandrogenism. Whereas we are measuring central obesity with MRI, it is important to see if this simple non-invasive measure matches the MRI results, as it is a much simpler measure to follow clinically.

11: Accelerometer:

Description: Participant will be provided two accelerometers (GT3X BT by Actigraph and ActiWatch by Philips Respironics) to be worn for seven days to measure level of habitual physical activity, which affects insulin sensitivity, and sleep patterns. The accelerometers will be worn on the participant's wrist. The wrist position has been validated to hip position actigraphy in this population.

Risk: There is no risk involved with the accelerometer.

Justification/Minimization: Accelerometers are effective tools for the objective measurement of physical activity [125] because they have the ability to continuously record physical activity data and such data can be used to estimate METs of activity. They provide more detailed information than pedometers, which only measure walking steps, and help get around the recall bias of questionnaires. We are currently using the GT3X BT Actigraph in adolescents in our other diabetes studies; therefore, we are familiar with their use in this population and have the necessary computer software and interpretation skills. The Actiwatch is being used as a tool for objective measurement of sleep patterns. The Actiwatch is fitted with a LED monitor that records multiple spectrums of light to better assess sleep patterns in this population.

12: Metabolic Cart:

Description: The metabolic cart measures the amount of air that the participant breathes in and out. The machine attaches to the participant's mouth through a tube, or a plastic bubble that is placed over the participant's head. There is the potential for experiencing claustrophobia from having the plastic bubble over the participant's head. A metabolic cart will be utilized multiple times during the OSTT study day to measure rates of oxygen consumption and carbon dioxide release. These rates can be utilized to calculate rates of carbohydrate and fat oxidation and resting energy expenditure.

Risk: Minimal risk of claustrophobia.

Justification/Minimization: These studies are well tolerated by youth, and involve placing a clear plastic hood over the participant's head for approximately 20 minutes. The data collected from the baseline study is also very useful for assisting obese participants in determining their true caloric needs, and useful in setting dietary goals for weight loss. This piece of information is thus utilized in post-study nutritional counseling.

13. Food Frequency Questionnaire (SEARCH FFQ)

Description: Customary macronutrient pattern will be ascertained by diet interview at the time of admission using a SEARCH FFQ, modified to incorporate common food choices among ethnically and regionally diverse youth aged 10-19 participating in another large childhood diabetes study, SEARCH (48). The instrument is self-administered with staff support to provide instructions, answer questions, and to review the form after completion, and captures the last week of dietary intake.

Risk: None

Justification/Minimization: Several of the measurements being assed are affected by prior nutritional intake. Furthermore, participants will receive dietary counseling at the end of the study, and by knowing what their previous dietary pattern is, suggestions for improvement can be tailored to their specific dietary habits.

14. 3DPar Questionnaire

Description: A questionnaire (3DPAR) recalling the physical activity levels of the three previous days will be completed at visit 3 and visit 5.

Risk: None

Justification/Minimization: Physical activity can directly affect insulin sensitivity, our primary outcome measure. The 3DPar is a well validated measure to asses 3 days of physical activity in youth, and includes a variety of youth centric activities.

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

16. WatchPAT and Questionnaires to assess for Obstructive Sleep Apnea
Description: The WatchPAT is a noninvasive portable system that measures the oxygen saturation and apnea hypopnea index. Three surveys querying signs and symptoms of obstructive sleep apnea.
Risk: No risk associated with the questionnaires and the WathchPAT,other than a mild discomfort from having to wear the watch and cuff around finger during sleep. It is possible that we will discover that the participant has obstructive sleep apnea, and will need to be referred for further clinical care.
Justification: Obstructive sleep apnea is associated with obesity, and can worsen both fatty liver and insulin resistance. Thus the presence of OSA must be accounted for when measuring either of these outcomes. These surveys selected are currently being utilized by the NIH multiple center study in obese youth at risk for diabetes, and are well validated in youth from multi-ethnic populations. If OSA is suspected during the course of the screen, the participants will be referred for further evaluation and treatment. The WatchPAT is an FDA approved device that can be used specifically for oxygen saturation and apnea hypopnea index and is approved in children as young as 12 years of age, within the age range of our study population.

17: Gut Bacteria Collection:
Description: A week prior to visit three, participants will be provided with stool collection swabs to collect a small sample of stool from the toilet paper they use after having a bowel movement.
Risk: Although the risk is minimal, participants may feel uncomfortable taking a sample of stool from the toilet paper following a bowel movement. All participants will be instructed to follow proper bathroom etiquette as fecal matter can transmit diseases.
Justification: Studies in obese individuals with type 2 diabetes have alterations in the gut microbiota that may be related to NAFLD. These studies have not been performed in PCOS.

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

E. Benefits of the study:

Benefits to Society:

PCOS affects 6-15% of the female population in the US, has an estimated $4 billion economic burden and the associated irregular periods, obesity, fatty liver disease and excessive facial hair are especially socially difficult for teens. Current treatment options for PCOS are limited. Women in their 20's and 30's with PCOS already have evidence of cardiovascular disease and diabetes, making adolescent studies, when the disease starts, crucial to understanding disease development. Current therapies options are limited and minimally efficacious. This understanding will lead to the development of more effective early treatments, before diabetes and cardiovascular disease develop.

Knowledge to be gained:

A better understanding of how NAFLD develops in girls with PCOS could lead to more effective treatment strategies. This understanding could ameliorate development of diabetes and heart disease for these girls as they become adults, and may also help with many of the health and social difficulties teens with PCOS experience. Since PCOS is one of the most common endocrine diseases in the US female population, improving PCOS care could have major health implications. The data to be generated from this project will be utilized to inform R01 grant applications of treatment trials with new medications. Baseline and placebo phase data will add to our knowledge of NALFD in girls with PCOS. This study will also test the efficacy of a food based supplement to decrease hepatic steatosis and lead to a new treatment option.

Individual: Participant will benefit from in depth testing for pre-diabetes, fatty liver disease and sleep disorders that are not clinically offered. They will receive extensive counseling for both dietary and exercise lifestyle changes. Similar participants who completed related protocols (10-1288 and 14-0542) and received this counseling have higher rates of weight loss upon clinical follow-up than those children being seen in obesity clinics alone. Further, participants may benefit from the amino acid supplement and all participants will receive this therapy.

Benefits to participant:

1) All participants will be undergoing measures that can identify insulin resistance, hyperlipidemia, NAFLD or early cardiovascular disease. These measures are not typically done within the scope of daily pediatric practice, and participants would likely not otherwise know this information. If they have one of these conditions, they will be referred for appropriate follow-up and treatment. If the participants have signs of obstructive sleep apnea, they will be referred to the sleep clinic for appropriate evaluation and treatment.

2) All of the participants enrolled must be sedentary, with less than 2 hours a week of physical activity. This is less than the time recommended by the US Preventive Task Force and the American College of Sports Medicine for this age group. This lifestyle puts them at risk for several diseases including diabetes and cardiac disease later in life, even if they don't have evidence of disease at this time. At the end of the study, all participants will receive counseling on how to increase their activity levels by trained study staff. Increased activity has been shown to reverse the risk for diseases later in life.

3) All participants will complete a 7 day food questionnaire. This will be reviewed with them, and healthier food choices and meal planning will be discussed with both the participant and their parent by the PI or PRA, all of who are trained in providing diet prescriptions. Additionally, obese participants will be counseled on a weight loss diet. Over 80% of obese adolescents are obese as adults, if they do not change their eating habits and lose weight when they are still a teen.

4) The participants will benefit from getting a sleep study during their overnight stay at the hospital and discovering if they have obstructive sleep apnea. Depending on the results they may be referred to sleep clinic for further evaluation of sleep apnea.

5) All participants will receive the amino acid supplement, which may decrease hepatic steatosis and improved glucose metabolism.

Evidence of Direct Benefit

We believe that this protocol is in the 405 risk category for pediatric research. Participant s can benefit from the above study measurements. Further, at the conclusion of the study, all participants in the protocol will also be given counseling on the benefits of exercise as discussed above, and given an exercise prescription. The participants may also be re-contacted once by phone call to follow-up on their study results and exercise recommendations. They may also be followed up in a clinical setting if PI notes further benefit of clinical follow up. Sedentary participants will thus gain direct benefit from the study through the benefits of specialized counseling and recommendations for increased physical activity that would not otherwise be available to them.

Sedentary and obese adolescents are at increased risk for diabetes, reduced bone mineral density, reduced exercise capacity, cardiovascular dysfunction, and increased visceral and liver fat. Therefore, obese non-PCOS participants may benefit from the results of glucose testing, insulin resistance assessment, DEXA, cardiovascular measures, and MRI testing. The DEXA scan can detect evidence of osteopenia, which is becoming more prevalent in adolescents, especially those who are sedentary females. The MRI of the liver can detect early evidence of fatty liver disease or fibrosis. The blood tests

done for screening can detect alterations in blood glucose, pubertal sex hormones, as well as abnormalities in fasting lipids. The risk of all of these endpoints is increased in sedentary participants, especially those who are also obese, and if left untreated can increase long term health risk, thus the benefit of detecting any of these would directly impact both the health and the longevity of the individual participant.

Inclusion criteria is less than 2 hours of exercise per week, a level of activity well below that recommended by the U.S. Surgeon General for youth (Children and teenagers should exercise for 1 hour of vigorous physical activity daily and weight-bearing activities that strengthen their bones). Numerous studies have linked low activity with development of diabetes, heart disease and insulin resistance; all of the end-points which we are studying. Recent studies have shown that sedentary lifestyle increases risk of cardiovascular disease and all-cause mortality. Youth in the U.S. now suffer from obesity in epidemic proportions, with about 32% of US adolescents currently being overweight and 17% being obese. A sedentary state is an increasingly common problem in the U.S., especially for adolescents, as a recent study showed that the most sedentary groups in the United States were adolescents and adults over 60 years. Adolescents in this study spent about 60% of their waking time in sedentary pursuits, making sedentary adolescents a critical group to study. The amount of time spent in sedentary behaviors has been independently associated with increased risk of weight gain and increased risk of metabolic syndrome, diabetes, and heart disease. In light of these links to adverse health outcomes and the continued increase in the prevalence of overweight and obesity in the United States, sedentary behaviors have emerged as an important target of health promotion and obesity and disease prevention efforts, complementing efforts to increase levels physical activity. For this reason, sedentary lifestyle can be considered a pre-disease state.

The primary benefit from the OSTT would be the discovery of pre-diabetes via a non-invasive measurement of glucose tolerance. As mentioned before, surrogate measures are unable to detect insulin resistance adequately, and insulin resistance is a strong predictor of NAFLD. Discovery of insulin resistance would enable us to recommend education and an exercise and diet plan to treat or prevent further development of insulin resistance, and diabetes. The direct benefit of the isotopic tracers would be the differentiation of whether the liver, and/or the muscle and/or the adipose tissue is involved in the insulin resistance. Again, if this were true the individual could benefit from referral to the metabolic syndrome clinic or the GI clinic to be followed for evidence of hepatic disease that may need to be treated if there is any disease progression. Fifty percent of obese girls with PCOS, a hormonal disorder affecting 15-20% of obese females, have hepatic steatosis (HS), which causes premature mortality due to an elevated risk for type 2 diabetes (T2D), cardiovascular disease (CVD) and progressive liver disease in early adulthood. The proposed project will combine a tolerable and relatively inexpensive therapeutic option with both clinical outcomes and innovative methods to quantify intrahepatic metabolism. Treatment options for PCOS are limited and implementation of effective lifestyle changes are very difficult for obese adolescents. This work would contribute to the development of a dietary intervention that could treat NAFLD in PCOS thus decreasing current and long term risks for T2D, CVD and advanced NAFLD and prolonging life for these high risk girls.

WatchPAT results: The patient population is at high risk for OSA. Untreated OSA is thought to contribute to worse fatty liver disease and glucose metabolism. By identifying that these youth have a problem, they are then in the position to work with their physicians to address this, and potentially improve their metabolic health long-term. Additionally, treatment of sleep disordered breathing is associated with weight loss, and this is a goal for all of these participants.

F. Alternative Treatment

The alternative is for participants to not participate in the study

G. Consideration of Specific Participant Categories

1. Inclusion of Women

All participants will be women, as PCOS only occurs in females.

2. Inclusion of Minorities

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

3. Inclusion of Children

All participants will be between ages 12 and 21. Insulin sensitivity needs to be studied in the adolescent age group as available is scarce in this age group and it is critical to understand the pathophysiology of PCOS in its developing stages.

IV. Potential Scientific Problems:

Limitation of Method Development:

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

1) Measurement of Hepatic Substrate flux in youth during fasting.

a. Purpose: Asses hepatic substrate flux in youth looking at shifts from pentose phosphate pathway (PEP), TCA cycle and fatty acid synthesis

i. Rational: utilizing and oral glycerol model with NMR isotopmer analysis is a newer technique. We have validated the timing with an OSTT in youth. However, this relies on consistent OSTT responses. When we anticipate that the response to the OSTT may change, the timing and thus results would vary, and we are thus better off using a fasting model. This has been done in adults, but not in our youth.

ii. Measurement: NMR isotopomer analysis with pathway flux calculations and modeling in the fasting state

iii. Revision plan if model not correct: 1) increase the amount of tracer given 2) increase the frequency of blood draws in the later sampling period.

Overall Project Limitations:

We expect to complete 20 participants in 2.5 years. As with any clinical trial, recruitment and retention can be challenging. We have successfully recruited over 120 obese girls with untreated PCOS, and thus our recruitment goals are in line with previous work. We have never needed to recruit in the community or other endocrinology practices, but can if needed. Within our department we have been successful with 3 month, 21 month and 3 year interventional protocols and we have designed the study with techniques found to be successful in similarly obese adolescent populations. Adherence to treatment is also a challenge, and we will use texting and emails as preferred contacts in youth to send supplement reminders. Major changes to diet and exercise could alter results, and we will request participants not to initiate major lifestyle changes during the study. An alternative would be to recommend lifestyle changes for all girls, although from our clinical experience these are poorly followed.

Aim 1: We anticipate that after EAA, absolute IHTG will decrease by 3% compared to placebo, secondary to decreased DNL. Based the mean of 11±3.5% IHTG in PCOS girls with HS, this is less than the relative 50% decrease we found in most of older adult participants. Further, 9 days of low fructose dietary modification reduced IHTG by 50% via decreased DNL in obese youth with HS, demonstrating that IHTG can be rapidly changed with dietary modification and relates to decreased DNL[132]. However, this patient population does have a very poor diet, and it is possible that those with excessive soda intake will not benefit as anticipated if EAA cannot overcome the excess glucose. An alternative would be to provide the diet for participants for 3 days prior to studies. We will be using a FibroScan ultrasound based method to determine the presence of HS at screening. MRI is the most sensitive and repeatable measure for IHTG. Ideally we would also perform an MRI for IHTG at screening to determine eligibility, however the cost and time of this for the 50% of girls to be excluded is excessive and the processing time for the results is not conducive for a screening test. Whereas our lab has not used deuterated water to measure DNL[51], this method has been used successfully in other youth populations by our collaborator Dr. Parks, and we thus are confident that we will be able to perform this analysis.

Aim 2: We expect that insulin sensitivity will improve following EAA and that the glucose concentration at 2 hours of the OSTT will decrease. The reproducibility of glucose measurement from an OSTT in any individual is only 75%, however, this is when the effects of exercise, diet, sleep and menstrual cycle are not controlled. An alternate approach for assessing insulin sensitivity would be a hyperinsulnemic euglycemic clamp, for which we have extensive experience. We have not elected to use this as it is not physiologic and does not allow for isotopomer assessments. We expect to measure hepatic metabolism with metabolomics and isotopomer analysis and to integrate these results with clinical outcomes. We hypothesize that those with worse HS will have increased futile TCA cycling and increase BCAA which will improve with improved insulin sensitivity and decreases in liver fat after EAA. If our hypotheses are incorrect, the data may suggest additional mechanistic pathways unique to PCOS. Interpretation of the resulting data requires extensive knowledge of biochemistry and I have the unique skills, time, institutional support, collaborators, and mentoring to be successful.

V. Data Management and Security Plan

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

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

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

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

VI. Data and Safety Monitoring Plan

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

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

Multiple Institutional Review Board, regulatory officials from the institution where the research is being conducted to monitor safety and compliance with policies.

A. Adverse Events (AE)

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

1. Adverse Event Definition

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

a) Adverse Event Reporting

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

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

B. Serious Adverse Events (SAE)

1. Serious Adverse Event Definition

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

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

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

- Infection related to blood draw or IV placement

a) Serious Adverse Event Reporting

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

C. Participant Discontinuation Criteria

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

1. Inability to complete study procedures
2. Abnormal screening labs (LFT's >125 IU, HbA1C>6.4%, Hg <11 mg/dL)
3. Participant becomes pregnant during study

D. Protocol Stopping Criteria

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

VII. Data Analysis Plan:

Overall Project Statistical Plan:

Sample size analysis: Sample size is based on the power to detect a change in IHTG with treatment with EAA as compared to placebo (primary endpoint). Assuming a 2-sided α=0.05, 20 participants provides 86% power to detect a change of 2.8% in IHTG, assuming a 3.9% standard deviation of change, which corresponds to a correlation of 0.8 between repeated measures. For Aim secondary measures, a 2 sample size of 20 gives 99% power to detect a change of 14.4 mg/dL in 2 hour OSTT glucose and 81% power to detect a change of 1.8 in HOMA-IR, assuming a standard deviation of 14.4 mg/dL and 2.7, respectively. Estimates of effect size and variability were conservatively estimated from the PI's extensive pilot data.

Analysis plan: No interim analyses are planned. Prior to the start of any formal analyses, unusual values that need to be queried, patterns of missing values, and whether their distributions are non-Gaussian will be identified. Demographic and clinical characteristics will be summarized with descriptive statistics. Analyses will follow the "intent-to-treat" principle so that study hypotheses are tested under realistic conditions in which not all participants adhere to the intervention. For the primary outcome, we will compare IHTG with and without EAA treatment using a paired t-test or a Wilcoxon signed rank test. Similar analyses will be used for secondary outcomes (2 hour OSTT glucose, HOMA-IR, metabolomics markers, proportion of direct/indirect glucose, EAA concentrations). Linear models will be used to identify the variables that are associated with change in IHTG, such 2 hour OSTT glucose, HOMA-IR, metabolomics markers, etc.

All measures will be adjusted for OSA status (yes/no) and/or severity of AHI.

Secondary outcomes include a Pearson correlation test of potentially related variables followed by targeted multiple regression to assess the relationship between hepatic fat and adipose IR, PEP, TCA, TG synthesis pathways.

Likely contributors to endpoints: Variables known to affect IHTG and IR will be measured: lipid/glucose markers (lipid panel, HbA1c); inflammatory markers (C-reactive protein, AST, ALT, GGT); glucose-related hormones (glucagon, GLP-1 and leptin), sex-steroids (DHEA-S, free and total testosterone, sex hormone binding globulin, progesterone, estradiol); body composition (BMI, waist:hip ratio, lean and fat mass by DEXA); Variations in diet, activity, menstrual phase and circadian rhythms affect metabolism and will be assessed: dietary intake (7 day food diary), habitual activity (3 day questionnaire and accelerometer for 7 days prior to study) and sleep timing and quality (sleep questionnaires and sleep and light watch). OSTT studies will be performed in the morning fasting, in the follicular phase of the menstrual cycle where possible, preceded by 3 days of no strenuous physical activity and a typical diet for the participant.

Non-Isotope OSTT calculations: The Matsuda model of IR will be used but adapted for the 6.5 hour OSTT, and B-cell function [insulinogenic index (ΔI30/ΔG30) and (ΔC30/ΔG30) and disposition index (1/IFasting × ΔI30/ΔG30) and (1/CFasting ×ΔC30/ΔG30) will be calculated[109,129,133,134]. Differences between groups will be evaluated with and without controlling for activity and sleep status.

Fitting glucose and glycerol concentrations curves: Using computed data points, glycerol and glucose concentrations will be fitted with an appropriate model. In control participants, concentration typically shows 3 phases in OSTT [135]. Cubic polynomial, piecewise-linear, spline, and dynamic models have been used for fitting Ra in previous work[130,136]. We will assume a cubic polynomial model; we will validate estimates obtained with the cubic polynomial model by computing comparable estimates using at least one other model form. In previous work comparing different models (piecewise-linear, cubic polynomial, dynamic), results have been relatively unaffected by choice of model. If we find that this is not the case for our data, then we would certainly consider the advantages of adopting a more complex approach, such as the state-space, for describing concentration changes.

Metabolomics data: Differences in targeted metabolic concentrations will be compared between groups, after appropriate cleaning of the data, as performed by the processing site. Changes in metabolites over time will be compared between groups, to determine the times that demonstrate the largest differences. Results will also be compared to isotopomer results, to see if a metabolomics endpoint can yield similar information to the isotopomer analysis.

Gut Microbiota: The microbiota profile is entirely exploratory, and will be related to status of hepatic steatosis and 2 hour glucose status of impaired glucose tolerance or not.

VIII. Summarize Knowledge to be Gained:

Overall Expected Results*:* We anticipate that we will determine which specific areas of hepatic metabolism are upregulated in girls with hepatic steatosis and can these be changed with EAA supplementation. This data would then provide the background evidence for future larger therapeutic trials. We will also continue to refine the isotopomer protocol with extension into fasting.

Significance: Recent evidence in adults with PCOS indicates that 60-70% have NAFLD[32,42]. Our data shows that > 45% of obese girls with PCOS have NAFLD, likely an underestimate due to exclusion criteria of T2D and weight >250 lbs in the previous protocols. The majority of NAFLD studies have focused on males, and none have been performed in multi-race cohorts of girls with PCOS, despite this high prevalence. NAFLD has been described as the primary driver of worsening metabolic syndrome and CVD in obesity across populations, and can progress to cirrhosis and liver failure[34,35]. Thus, understanding the hepatic metabolism associated with NAFLD is critical to designing strategies to improve the long-term health of these girls and reduce their risk of T2D, CVD and liver failure. Further, we will be testing a well-tolerated oral nutritional supplement.

IX. References:

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