Study Title:

Assessment of hepatic glucose and fat regulation in overweight adolescent girls (APPLE Study)

Protocol Version Date: 11/26/2018

NCT02157974

COMIRB Protocol

COLORADO MULTIPLE INSTITUTIONAL REVIEW BOARD
CAMPUS BOX F-490 TELEPHONE: 303-724-1055 Fax: 303-724-0990

Project Title: Assessment of hepatic glucose and fat regulation in overweight adolescent girls

Version Date: November 26th 2018

Principal Investigator: Melanie Cree Green, MD, PhD
Mentor: Kristen Nadeau, MD, MS

I. Hypotheses and Specific Aims:

HYPOTHESIS:
Obese girls with PCOS, when compared to obese controls, will have hepatic and adipose insulin resistance (IR) that is not overcome with endogenous insulin secretion following an oral glucose (OG) load. IR will be worse in girls taking oral contraceptives, and improved in girls taking metformin. Hepatic IR and fat will relate to a failure to suppress hepatic gluconeogenesis and utilize carbohydrates during feeding.

Specific Aims:
Aim 1: Optimize novel minimally-invasive physiologic methods to study liver and adipose IR.
Rationale: Our preliminary pediatric PCOS data show high rates of NAFLD, altered interactions between hepatic and adipose IR during IV studies, and abnormalities during an OG load. A single less invasive protocol to synthesize these findings is needed, and this is a novel approach.
Methods: 1) Optimize a combined oral glucose and stable isotope tracer model to assess hepatic and adipose IR, while simultaneously accounting for post-prandial hormone changes. This aim will be accomplished with completion of the first 15 subjects, who may or may not have PCOS.
Aim 1A: Develop a mathematical model to describe dynamics of glucose and glycerol during an OG and quantify key aspects of liver & adipose metabolism
Rationale: Tissue specific IR is detectable following an OG that includes stable isotope tracers, but mathematical modeling is required to correctly interpret data collected before and after an OG load.
Methods: In the initial 6 subjects we will 1) Develop a differential equations-based mathematical model of glucose and glycerol dynamics and fit to data computed from stable isotope tracer data. 2) Analyze model to characterize tissue specific IR in PCOS subjects. 3) Develop a model of glucose passage through the gastrointestinal tract to assess glucose absorption in PCOS subjects.
Aim 1B: Compare the OG glucose and glycerol rates of appearance to hyperinsulinemic euglycemic rates of appearance
Rationale: We need to assure that the new method produces rates of appearance (Ra) of hepatic glucose release and adipocyte free fatty acid release that are within range of those calculated following the hyperinsulinemic euglycemic clamp which is currently accepted as the gold standard method to assess IR using only an IV approach.
Methods: An additional 9 subjects will be enrolled and studied with the OG model. Data from the raw suppressed glucose and glycerol Ra, insulin concentration required for 50 percent of peak suppression and percent suppression of basal Ra will be compared between OG subjects and a matched cohort of girls whom have already undergone hyperinsulinemic euglycemic clamps with identical glucose and glycerol tracer infusion rates.
Aim 1C: Optimize timing of data collection with ^{31}P-MRS in the liver related to a glucose load.
Rationale: Studies in adults have shown that glycogen synthesis increases by 1 hour post glucose load. A preliminary step to glycogen synthesis or hepatic glucose utilization is passage of glucose

through glucose-6-phosphate (G-6-P). Increased concentrations of G-6-P should be detectable 45 min after a glucose load.
Methods: We will collect ^{31}Phos data from the liver when subjects are fasted, and 45 min following an 75 gram oral glucose load.

Specific Aim 2: Test the impact of oral glucose on hepatic and adipocyte insulin sensitivity in PCOS, and the effect of common medical therapies metformin and oral contraceptives on hepatic and adipose insulin sensitivity
Rationale: Insulin sensitivity is traditionally assessed with IV only methods. However these to not allow for the contributions of gut hormones, and are non-physiologic. We have previously found significant decreases in hepatic and adipose insulin sensitivity in girls with PCOS and need to determine if this is also seen following a glucose load, as this would be reflective of daily life. Further, the effect of current therapies has not been assessed with this method.
Methods: Perform OGTT's with tracers in obese girls with and without PCOS, and girls with PCOS who have been taking oral contraceptives or metformin.
Specific Aim 3: Determine the role of hepatic and adipose IR in the rate of hepatic de novo lipogenesis (HDNL) and the effect of common medical therapies metformin, oral contraceptives and GLP-1 receptor agonists on HDNL
Rationale: Studies indicate that increased HDNL is the primary cause of increased liver fat. Our preliminary data demonstrates that hepatic fat is 3 times elevated in girls with PCOS compared to weight similar controls.GLP-1 concentrations are lower in girls with PCOS and hepatic steatosis.
Methods: HDNL will be assessed in the fasted and fed state utilizing an acetate tracer in a subset of subjects. Rates of HDNL will be compared to measures of hepatic and adipose insulin sensitivity.

II. Background and Significance:

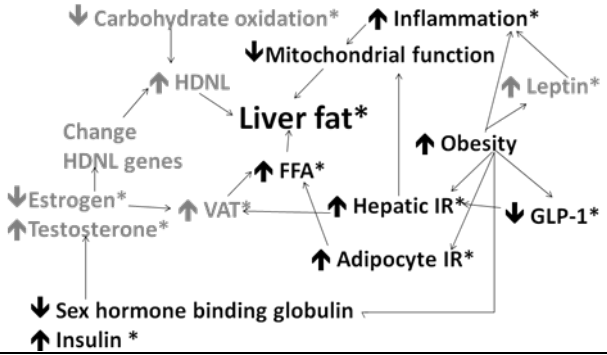
a) Overall background:

Polycystic Ovarian Syndrome (PCOS) affects 6-10% of U.S. women, with an estimated economic burden of $4 billion, and is increasing in prevalence in parallel with the obesity epidemic[1,2]. PCOS includes elevated androgens, and higher rates of insulin resistance (IR), type 2 diabetes (T2D), nonalcoholic fatty liver disease (NAFLD) and cardiovascular disease (CVD) [3-6]. The increasing rates of obesity-related PCOS are a major contributor to the earlier onset and rising incidence of T2D, NAFLD and CVD[3-7]. Current PCOS therapies are marginally efficacious, and one of them, oral contraceptives, may adversely affect CVD risk [8-14]. *Despite the high prevalence and serious morbidity associated with PCOS, a gap exists in the current therapeutic options.*

b) Mechanisms of insulin resistance in PCOS

Alterations in hepatic metabolism may be central to IR and cardiometabolic disease in PCOS. An estimated 50-70% of obese women with PCOS have NAFLD, compared to 20-30% of obese women without PCOS [7,15]. Furthermore, obese women with PCOS and NAFLD are more IR than those without NAFLD, indicating a link between NAFLD and worsening IR in PCOS [4]. Liver enzyme concentrations, as a marker of NAFLD, independently predict dysglycemia and T2D onset[16], and a new NAFLD medication decreased dysglycemia[17], arguing for a tight connection between glycemia and liver health. In obese non-PCOS youth, NAFLD correlates with adipose, hepatic and muscle IR, is worsened by fructose consumption, and reversed by weight

Figure 1: Proposed Mechanism of NAFLD in PCOS



Mechanisms influenced by hyperandrogenism and unique in PCOS vs. T2D are in grey. Items with an * will be studied in this grant. HDNL = hepatic de novo lipogenesis, VAT=visceral adipose tissue, FFA=free fatty acids, GLP-1=Glucagon Like Peptide

loss[18-21]. Animal models of primary hepatic IR demonstrate the causal role of hepatic dysfunction in the development of NAFLD, T2D and CVD [22,23]. IR in muscle, liver and adipose tissue are reported in PCOS adults[24-27], and we found the same in girls with PCOS, despite their young age. The synergy between obesity and hyperandrogenism relate to alterations in fat metabolism, inflammation, NAFLD and IR in PCOS adults[28-30]. Our proposed mechanism for development of excess liver fat from adipose and hepatic IR is shown in Figure 1. *An understanding of the complex physiology between tissue-specific IR, hepatic fat and androgens in PCOS is lacking. Addressing this gap is my intermediate goal and is an aim this protocol.*

c) Current research methods to assess IR

Research methods to assess early tissue-specific IR require complex protocols and few models exist which incorporate oral feedings[31-33]. Current data for evaluating both hepatic and adipose IR have employed intravenous (IV) approaches, which fail to account for contributions of hormones such as glucagon, glucagon like peptide (GLP-1) or leptin, which may play a crucial role in hepatic and adipose signaling[20]. Further, the maximum exogenous insulin doses that can be administered safely IV often produce lower serum insulin concentrations than generated endogenously following an oral glucose load, and the prolonged fasting required for IV studies is at the limit of tolerability in youth. *Thus, a gap exists in the methodology to assess hepatic glucose metabolism and adipose IR in a comprehensive, minimally-invasive, yet physiologic setting. Addressing this gap is the first step towards addressing my long terms goals and is a focus of this protocol.*

A. Preliminary Studies/Progress Report:

SA1: Optimize novel minimally-invasive physiologic methods to study liver metabolism

SA 1 Pilot Data on Liver fat and Insulin Resistance in youth with T2D: In our recent adolescent studies, we examined the differential tissue-specific expression of IR in youth with T2D and PCOS utilizing a sophisticated 3 stage hyperinsulinemic euglycemic clamp with multiple stable isotope tracers. By contrasting patterns of pathology between PCOS, obese controls and T2D we are uncovering subtle differences suggesting unique mechanisms of hepatic IR in PCOS. T2D youth appear similar to T2D adults in having adipose, hepatic and muscle IR. However, the IR is more severe than in adults, with unexpectedly progressed markers of CVD and NAFLD at diagnosis despite their young age [34]. Adipose IR in our T2D youth is exhibited by a persistent glycerol rate of appearance (Ra) despite very high induced insulin concentrations (200 mU/L) and hepatic IR is demonstrated by a doubling in the insulin concentration required for 50% suppression of glucose Ra (IC50 Ra)(Figure 2A). This IR translates to persistent elevations in serum free fatty acids (FFA) and glucose in these individuals. Elevated hepatic fat content (Figure 2B) correlates with both elevated serum FFA concentrations and glycerol Ra in T2D, indicating that hepatic steatosis is related to adipocyte IR. Muscle IR, assessed by glucose clearance rate, is very tightly correlated with both glycerol Ra and serum FFA concentrations during hyperinsulinemia, indicating that adipocyte IR may impact muscle IR as well. Finally, our T2D youth have muscle mitochondrial dysfunction as assessed with ^{31}P magnetic resonance spectroscopy (MRS), which relates to muscle IR.

Figure 2: Hepatic Measures in Girls

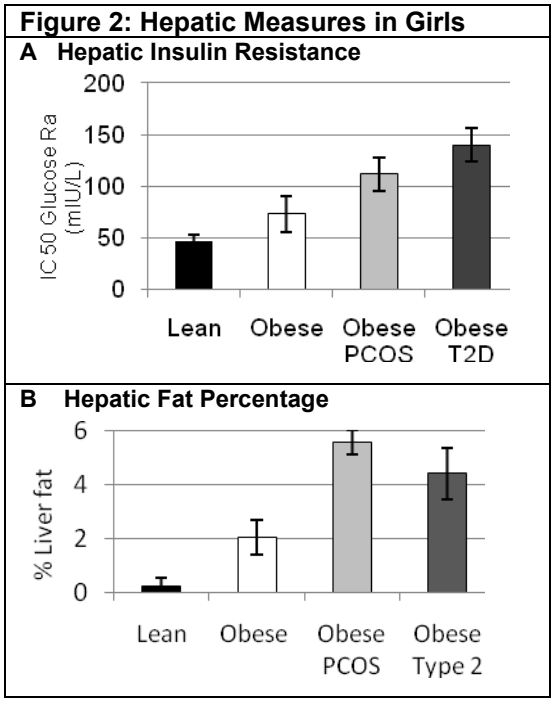


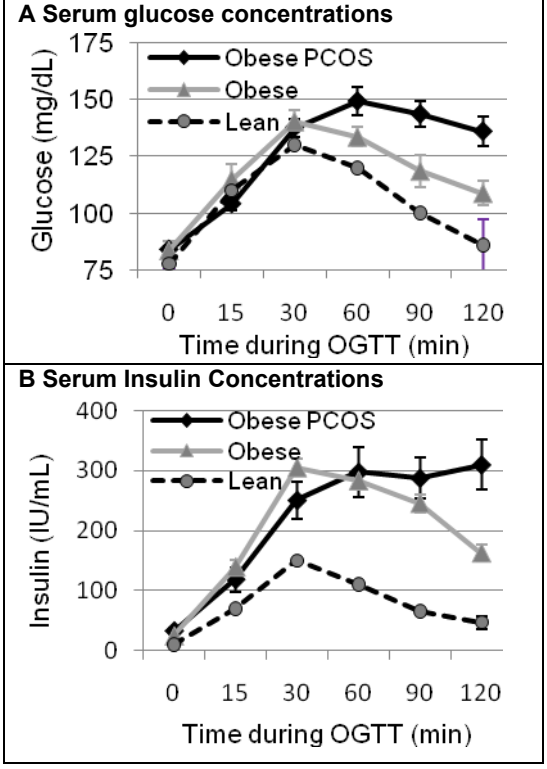
Table 1: Serum Markers in Obese Girls			
	Control	PCOS	T2D
Number	19	38	40
AST (IU/L)	19±2	36±2	32±2
ALT (IU/L)	29±3	38±3	40±2
Triglycerides (mg/dl)	78±8	129±7	207±7
Adiponectin (ng/dL)	8.4±0.9	5.8±0.4	5.7±0.1
Leptin (ng/dL)	37±6	43±3	36±1
CRP (ng/dL)	1.7±1.4	4.3±0.6	4.1±0.1
HbA1C (%)	5.1±0.2	5.4±0.3	7.2±0.4
Oral Disposition Index (oDI)	5.2±2.2	3.2±0.5	N/A
Fasting FFA (mmol/l)	523±37	629±22	N/A
FFA n7 (nmol/g)	29±13	45±15	N/A

Pilot Data on Liver fat and Insulin Resistance in youth with PCOS:
Obese girls with PCOS have moderate adipose, hepatic (Figure 2A) and muscle IR relative to obese controls, but are not as IR as T2D girls. Adipokines such as adiponectin are similarly low in PCOS and T2D girls, whereas leptin is higher in PCOS than even T2D (Table 1). Unlike T2D, muscle mitochondrial function is not impaired in PCOS compared to obese controls. PCOS girls have increased hepatically-derived serum triglycerides, liver enzymes as elevated as in T2D, and MRI-assessed rates of hepatic steatosis even higher than in T2D girls (Figure 2B). Hepatic fat in PCOS girls is weakly associated to markers of overall lipolysis, but is more closely related to visceral adipose tissue (VAT) and plasma markers of hepatic *de novo* lipogenesis (FFA n7, Table 1). Further, during IV-induced hyperinsulinemia during the clamp, girls with PCOS have lower rates of carbohydrate oxidation than obese controls (0.013±0.0001 vs. 0.017±0.002 mmol/min/kg), indicating preferential storing of glucose as evidence of metabolic inflexibility. Finally, markers of CVD, including carotid plaque development and exercise tolerance are similar between PCOS and T2D, indicating that PCOS status may be a significant risk factor for CVD even in youth. In summary, in youth with PCOS, excess liver fat may relate to adipose IR, hepatic IR and metabolic inflexibility with increased de novo lipogenesis from carbohydrates. Targeting improved understanding of these pathologic processes in a physiologic model is a logical step for reducing progression of T2D, CVD and NAFLD in PCOS youth.

Preliminary data from oral glucose tolerance tests in girls with PCOS:

Obese girls with PCOS have high 2 hour oral glucose tolerance test (OGTT) glucose and insulin concentrations (Figure 3 A and B). Of note, the PCOS OGTT insulin concentrations are higher than those achieved during the clamp 245±40IU/ml). Despite the relative hyperinsulinemia, they have poor insulin secretion relative to IR, i.e. a lower OGTT-derived oral disposition index (oDI) vs. obese controls (Table 1) indicating the presence of both β-cell dysfunction and IR. Girls with PCOS and an oDI of <1, an established risk for T2D[35], have half the insulin sensitivity vs. those with an oDI of >1 (5.3±2.1 vs. 10.8±0.8 mg/kg lean/min). Thus it may be that inadequate insulin secretion during an OGTT allows for persistent lipolysis and gluconeogenesis, which increases hepatic fat content and worsens muscle IR. GLP-1 and leptin, both hormones affected by oral ingestion, may be involved in this physiologic interplay, which is completely ignored with hyperinsulinemic clamp IV glucose delivery. The combination of isotope tracers and oral nutrient delivery is required to answer this critical question. This data is one of the primary reasons that we need to move to an oral model.

Figure 3: OGTT Plasma Results



Data and methods to study hepatic glucose use:
Hepatic glucose utilization may relate to hepatic de novo lipogenesis (HDNL). Liver glucose uptake occurs via the Glut-2 glucose receptor and is concentration dependant. Thus girls with increased glucose following an oral glucose load may preferentially have increased HDNL from increased glucose supply. It is unknown if there is a decrease in gluconeogenesis with a shift towards lipogenesis as another possible contributor to increased hepatic fat in PCOS. Hepatic glucose uptake can be demonstrated with either increased glycogen synthesis, as assessed with carbon MRS, or via increased glucose-6-phosphate. Increased glycogen synthesis has been demonstrated in subjects with insulin resistance. Hepatic phosphomonoesters **(**PME) largely represent G-6-P,and are detectable with 31Phosphorus spectroscopy. PME differentiate between patients with and without liver disease, change with nutritional manipulation and can be measured with ^{31}P MRS [36,37]. Specifically, increased G-6-P activity has been shown fasted in adults with NAFLD. Increased G-6-P change following a nutrient load was demonstrated in healthy patients relative to no change in subjects with cancer related liver disease, but has not been examined n NALFD or obesity[37]. We thus propose to study gluconeogenesis non-invasively by combining an oral glucose (OG) load with ^{31}P MRS, an entirely novel approach.

Preliminary data from polysomnograms in girls with PCOS:
Results from the inpatient polysomnograms that were performed in this protocol indicate the there is a strong relationship between obstructive sleep apnea (OSA) defined as an apnea hypopnea index (AHI) of 5 events per hour or greater and fatty liver disease. Girls with OSA also had higher serum triglycerides and glucoses 2 hours after the glucola drink, despite similar BMI's (Table 1).

	Mean AHI (apnea/hour)	**BMI %ile**	**Hepatic fat fraction (>5.5%= fatty liver)**	**Serum TG, mg/dL (>150 abnormal)**	**2 hour glucose, mg/dL (>140 pre-diabetes)**
AHI >5, N=13	14.6±3.5	98±1	10.5±2.6	162±19	153±6
AHI< 5, N=13	1.3±0.4	97±1	5.3±1	110±12	131±5

Further, at least 50% of the girls studied had mild to moderate obstructive sleep apnea, and a few had severe sleep apnea, although there was a bias to performing sleep studies in girls with symptoms suggestive of OSA, so this is not a true prevalence estimate from this obese female population. Clinically, from the PI's PCOS clinic, approximate 1/3 of obese girls with PCOS are requiring continuous positive airway pressure therapy for OSA. Thus, it may be that the presence and severity of sleep apnea is a confounder for our primary outcome of altered hepatic metabolism. Accordingly, we need to quantify the presence and severity of OSA in all of our subjects to be enrolled in 16-2399.

Traditionally, inpatient overnight polysomnograms are performed in youth, to determine OSA. However, through our experience in performing these in this protocol, these can only be performed at Children's Hospital Colorado on Thursday nights, which greatly limits scheduling and enrollment. Additionally, whereas the price for these was $500 a piece, this has now been changed, and is currently nearly $2,000 per patient, as a research price. Due to these limitations, we have sought alternative means of quantifying the degree and severity of OSA.

A wearable sleep device, WatchPAT, designed for home use has been utilized for the last 5 years clinically in adults. In July of 2016, the FDA approved the use of this device down to the age of 12 specifically for the oxygen saturation and apnea hypopnea index, but not the respiratory disturbance index (>17 years only). We have included the device company provided general description of the device, as well as the descriptions of limitation and use with individuals 12-17 years. As many of the limitations of pediatric use pertain to placement and utilization of the device,

we will perform the sleep study while the patients are having their already planned and approved overnight stay in the hospital, and will have the device placed by our study personnel, who will be trained in correct placement and interpretation. Our study team also has extensive experience with similar devices from this company, and they are a supportive and easy to work with company.

Known obstructive sleep apnea or treatment with CPAP will not be an exclusion criteria, however, those individuals already utilizing CPAP at home will be requested to bring it for admission and use it during the WatchPAT study, so that we capture their typical home sleep patterns.

III. Research Methods

A. Outcome Measure(s):

Primary Outcome Measure(s): Insulin concentration suppressing 50% glucose Ra (IC_{50} glucose Ra) during OGTT.

Secondary Outcome Measure(s): Hepatic PME/ATP ratio change after OG load, Glycerol Ra during OGTT, Hepatic fat fraction from MRI, Rate of hepatic de novo lipogenesis.

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 asses change with OG, 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[38-41]), Whole body fat oxidation at rest and following glucose ingestion as measured with a metabolic cart; metformin, OCP, Physical activity/ diet:(accelerometer, activity survey (3DPAR); Food frequency survey).Questionnaires for presence of obstructive sleep apnea. Questionnaires for perceived mental strengths and difficulty (note there is no assessment of suicidality on this tool). Obstructive Sleep Apnea, to be assessed with an overnight sleep study via WatchPAT

B. Description of Population to be Enrolled:

60 obese adolescent females with PCOS will be studied compared to 20 obese non-PCOS controls. This is number of studies needed to be completed statistically, thus more subjects may be enrolled, to allow for screen failures and dropouts. Total enrollment will be up to 105 subjects.

Ethnic Categories	**Gender**		
	Females	Males	Total
Hispanic or Latino	27	0	**27**
Not Hispanic or Latino	78	0	**78**
Ethnic Categories: Total of All Subjects	**105**	**0**	**105**
Racial Categories			
American Indian/Alaska Native	3	0	**3**
Asian	4	0	**4**
Native Hawaiian or Other Pacific Islander	3	0	**3**
Black or African American	20	0	**20**
White	75	0	**75**
Racial Categories: Total of All Subjects*	**105**	**0**	**105**

Inclusion Criteria:

1) Female

2) Ages 12-21

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

4) For PCOS groups: (NIH definition) irregular menstrual cycles at least 1.5 years after menarche and either clinical evidence of hyperandogenism or elevated Testosterone (above the norms for age/tanner stage) at time of screening or documented prior to initiation of therapy for OCP and metformin groups.

5) For PCOS groups: patients un-treated or currently treated with either Metformin or OCP's for at least 6 months.

6) For non-PCOS groups: regular menstrual cycles and no clinical evidence of hyperandrogenism

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

Exclusion Criteria:

1. For non-treated PCOS and non PCOS group: Use of medications known to affect insulin sensitivity: oral glucocorticoids within 10 days, atypical antipsychotics, immunosuppressant agents, HIV medications, oral contraceptives- Subjects may withdraw from oral contraceptive (OCP's) to meet the inclusion criteria only if a good pregnancy prevention plan can be created. If they are CHC patients, and have documented abnormal testosterone concentrations in our computer system prior to starting OCP's, they may screen while on OCP's. They must have been off the medication for at least 6 months prior to doing the MRI.

2. For Treated PCOS groups: Use of medications known to affect insulin sensitivity other than Metformin or OCP's: oral glucocorticoids within 10 days, atypical antipsychotics, immunosuppressant agents, HIV medications

3. Currently pregnant or breastfeeding women. Development of pregnancy during the study period will necessitate withdrawal from the study.

4. Severe illness requiring hospitalization within 60 days

5. Diabetes, defined as Hemoglobin A1C > 6.4%

6. BMI percentile less than the 90th percentile for age, sex and weight > 300 lbs

7. Anemia, defined as Hemoglobin < 9 mg/dL

8. Diagnosed major psychiatric or developmental disorder limiting informed consent

9. Implanted metal devices that are not compatible with MRI

10. Use of blood pressure medications

11. Liver disease other than NAFLD or AST or ALT >150 mg/mL

12. History of renal disease

13. History of clotting disorders or Warfarin use

Rationale for Inclusion of Non-PCOS Subjects

Obese adolescents are at risk of T2D and its complications, reduced exercise capacity, increased liver and visceral fat, muscle dysfunction and cardiovascular dysfunction therefore the tests performed screening for each of these problems provide useful information to these subjects. Likewise, sedentary subjects of any weight are at increased risk of reduced bone mineral density (BMD), T2D, reduced exercise capacity, increased liver and visceral fat, muscle dysfunction and cardiovascular dysfunction. Therefore, all of the subjects may benefit from the results of DEXA, glucose testing and exercise prescription and dietary counseling.

C. Study Design and Research Methods

Study Calendar	Visit 1 – Screen	Visit 2 –MRI	Visit 3 – Overnight
Consenting and Eligibility Assessment	X		
History & Physical	X		
Intravenous Blood Draw	X		X
Finger Stick Blood Draw		X	
Urine Pregnancy Test			X
Accelerometer Teaching	X		
Gut Bacteria Collection			X
Questionnaires- SEARCH Food frequency, Activity 3DPAR, Strengths and Difficulties, a sleep diary, and Sleep assessments			X
Sleep Study with salivary melatonin collection (pending scheduling)			X
WatchPAT sleep study			X
DEXA Scan			X
Glucose, Glycerol and Acetate Tracers			X
Study Drug (Byetta) GLP-1 Injection			X
EndoPat and Dynapulse		X	
MRI of abdomen and liver, P MRS of Liver		X	
Metabolic Cart			X
Total Time of visit (approximately)	3 Hours	2 Hours	24 Hours
Location of Visit	CHCO CTRC Outpatient	UCD Outpatient Brain Imaging Center	CHCO Inpatient Hospital

The study consists of 3 visits - 2 outpatient visits and one overnight inpatient visit. Inpatient visit will last approximately 24 hours, outpatient screening visit approximately 3 hours, outpatient MRI visit approximately 2 hours. The 3 visits would be completed within 4 months time.

Overall enrollment procedure plan per group

Test Group	Screening	MRI/MRS	Overnight stay	Glucose and Glycerol tracers	DEXA	Overnight acetate tracer	GLP-1 Injection (Byetta)
PCOS	40-50	50	50	50	50	30	10
PCOS w/ Metformin	10-13	10	10	10	10	5	0
PCOS w/ OCP's	10-12	10	10	10	10	5	0
Obese	20-30	20	20	20	20	10	0
Total	Up to 105	90	90	90	90	50	10

VISIT 1 (SCREEN VISIT)
Pediatric CTRC Outpatient unit: Participants will begin with a medical screening and physical exam. During this visit, patients will review and complete consent documents, have demographics

and medical history confirmed, assess allergies and inclusion/exclusion criteria, have blood samples drawn, and have anthropometrics completed. HbA1c, ALT, AST, hemoglobin and testosterone samples will be drawn at the beginning of the visit after consent in all subjects. PCOS subjects will have more labs performed for confirmation of PCOS status, if not performed previously.

Screening lab test	Purpose for test
HbA1C	Rule out type 2 diabetes, if > 6.5% subject to be excluded
ALT, AST	Ensure no severe liver disease, if >4x subject to be excluded normal subject is excluded
Hemoglobin & Hematocrit (part of CBC)	If subject is Anemic, they will be excluded
Testosterone	Test for hyperandrogenism – required to meet NIH citeria for PCOS
Optional PCOS labs PCOS status must be confirmed prior to enrollment in PCOS group. 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
17hydroprogesterone	Rule out late onset congenital adrenal hyperplasia
DHEAS	Rule out adrenal tumor
Prolactin	Rule out prolactin secreting brain tumor

Accelerometer: At the completion of the screening visit, the subject will be given an accelerometer to be worn for the following seven days to measure level of habitual physical activity (MTI Actigraph by Actigraph), which affects insulin sensitivity. Accelerometers are effective tools for the objective measurement of physical activity [42] 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 MTI 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.

VISIT 2 (MRI/CV VISIT)

Overall Plan: UCD Research MRI (Brain Imaging Center): Subjects 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 will be ^{31}P spectroscopy to measure the concentrations of phosphate molecules including PDE, PME and PCr. This will be done twice, before and 30-45 min following a standard 75 gram glucose load. The MRI time is approximately an hour in a 2 hour visit. Vascular Endothelial function will be assessed with Endopat and Dynapulse, which take approximately 30 minutes.

^{31}P MRS of the Liver and Abdominal Imaging

MRS Data acquisition: Imaging and MRS will be performed on a General Electric (GE) 3 Tesla MRI magnet (GE, Milwaukee, WI), upgraded with GE MRS research software. 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[43]. 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[40]. 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. Subjects will then consume 75 grams of glucose and the scan will be repeated every 10 min for an hour. 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[34].

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 [44,45], utilizing AMARES (A Method of Accurate, Robust and Efficient Spectral fitting), a nonlinear least-square-fitting algorithm using our previously built prior knowledge files [46]. We have utilized this method for muscle ^{31}P analysis for the previous 6 years, and have extensive experience with this analysis. Percent PME relative to all other phosphate peaks will be calculated before and every 10 min following a glucose load, and percent suppression calculated. The adipose data will be analyzed by Collaborate Ann Scherzinger, PhD, as in previous protocols (COMIRB 10-1288).

Sleep Study: (Based on sleep study technician availability, participant may or may not have the sleep study). If subjects have had a previous clinical sleep study, we will collect that appointment's data for analysis. Pediatric CTRC Inpatient unit: Polysomnographic (PSG) Methods and Analysis will be performed overnight into visit 3 using pediatric criteria as suggested by the American Thoracic Society and standard sleep scoring techniques for evaluating sleep structure and respiratory patterns. A polysomnogram will be performed overnight on all subjects, by a trained pediatric polysomnographic technician. A sleep study is a routine, noninvasive clinical test. A parent will remain with the child throughout testing. During the sleep study, surface electrodes and monitoring devices (acquired digitally by Somnoligica, Broomfield, CO) will measure signals from: the central EEG, right and left electro-oculogram, surface EMG, ECG, chest and abdominal wall motion, pulse oximetry (Masimo, Irvine, California), and end-tidal PCO_2 (Novametrix, Wallingford, CT). Airflow will be measured by oro-nasal thermistor and by nasal pressure in order to obtain a quantitative signal (Protech, Mukilteo, Washington). All studies are monitored with real-time video for motion analysis and snoring recording. Prior to falling asleep and from 5 AN to noon, salivary melatonin samples will be collected once an hour to determine how melatonin offset relates to PSG abnormalities.

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]. 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 routinely collected in research and pose little risk to subjects. 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.

VISIT 3 (ORAL GLUCOSE TOLERANCE TEST WITH ISOTOPE TRACERS)

Pediatric CTRC Inpatient unit: Subjects will be asked not to have caffeine or exercise for 3 days prior to this visit. During admission, subjects will be provided with a study diet dinner and snack. Patients will be admitted to the Pediatric CTRC for a monitored overnight fast and in select subjects an overnight stable isotope tracer infusion. Subjects will be questioned regarding compliance, adverse events, changes in concomitant medications and medical history, height, weight, BP, and blood sugars.

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

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 following morning, a blood sample for baseline metabolic labs will be drawn and infusion studies with the modified OGTT will be completed.

Study Drug Injections Exenatide (Byetta:

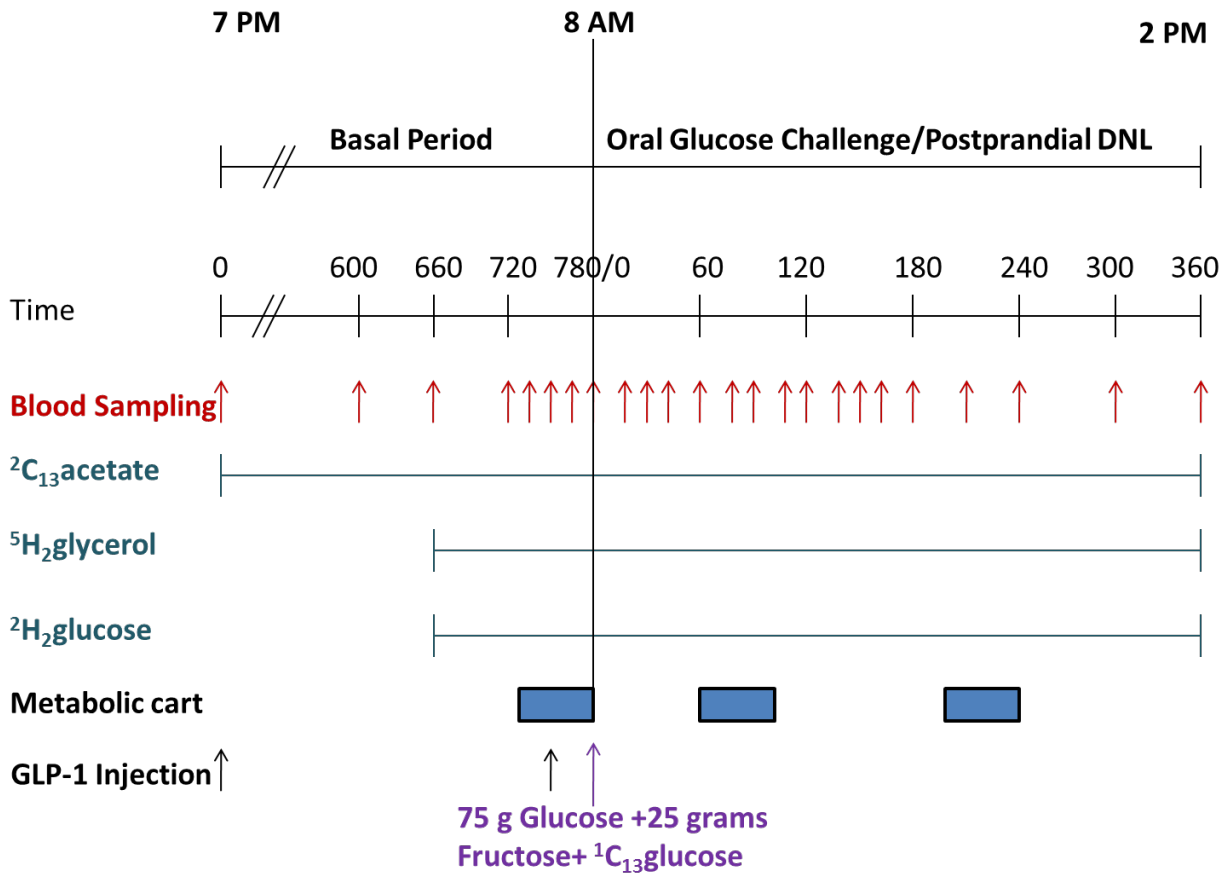
A subset of 10 untreated girls with PCOS who are receiving the overnight Acetate infusion will be administered two injections of Exenatide (Byetta) (5 mcg) during the in-patient admission for the OGTT. The first injection will be administered the night of admission at 6:30-7 PM. 30 minutes prior to the study diet containing a fixed macronutrient distribution listed below. The subject will be monitored throughout the night by nursing staff. The second injection will be administered at approximately 8 AM, 30 minutes prior to the OGTT load of 75 grams glucose. Following the second injection, study staff and CTRC nursing staff will be bedside for the reminder of the patient's inpatient admission at CHCO. Blood glucose measurements will be taken regularly for 6 hours post OGTT.

Details of Stable Isotope Tracer studies with an OGTT: After the overnight fast, subjects will consume a 75 grams glucose load with an additional 25 grams of fructose to stimulate hepatic de novo lipogenesis, and a $^{1}C_{13}$ glucose tracer (40 mg/kg) to calculate the rate of appearance of exogenous glucose[21].

The stable isotope method to be utilized is an OGTT with IV glucose and glycerol stable isotope tracers in all subjects, and acetate tracers in select subjects, based on power analysis and cost. For the glucose and glycerol tracers, we will utilize the tracer doses as in our previous hyperinsulinemic clamp studies, as these rates successfully detected basal and stimulated glucose and glycerol Ra. Whole-body lipolysis will be measured under basal conditions and during the OGTT (75 grams glucose + 25 grams fructose + $^{1}C_{13}$ tracer), using a primed (1.6 µmol/kg), constant (0.11 µmol/kg/min) infusion of $^{2}H_{5}$glycerol[47]. Hepatic IR will be measured under basal conditions and during the OGTT, using a primed (4.5 mg/kg), constant (0.03 mg/kg/min) infusion of 6,6-$^{2}H_{2}$glucose[48]. Blood sampling will continue for 6 hours post-OG to allow return to baseline, based on our pilot data shown in Figure 2 that 2 hours is inadequate. Blood for tracer analysis and glucose and insulin concentrations will be drawn at baseline, every 10 min during the last 40 min of the basal period, and every 15 min post OG load.

The rate of the acetate tracer will be 2 umol/kg/min with no prime, based on previous studies [49]. The oral challenge study design is shown in Figure 4. Hepatic De Novo lipogenesis will be measured in the fasting state, and in the fed state following the glucose load, by analyzing the incorporation of the C_{13} tracer into hepatically secreted lipids such as 16 and 18 carbon long chain

Figure 4: Proposed Oral Glucose Challenge



The study will have 2 physiologic states. 1. A basal fasted state, and 2. A post-prandial state following a glucose load. Hepatic IR is assessed glucose tracers, adipose IR with the glycerol tracer, and Hepatic De Novo Lipogenesis with the acetate tracer.

fatty acids in VLDL[49]. Blood will be drawn at baseline and for the last 4 hours of the basal state and the postprandial state.

A total of 40 girls will receive the overnight acetate infusion. This total will be broken down into 20 un-treated PCOS patients (10 without Byetta and 10 with Byetta), 10 obese control patients and 10 from PCOS patients treated with Metformin or OCP (5 from each group). Analysis of $^{2}H_{5}$glycerol, 6,6-$^{2}H_{2}$ and long chain fatty acid incorporation of $^{2}C_{13}$ Acetate, will be performed by our collaborator Bryan Bergman, PhD, using a modification of the negative ion chemical ionization gas chromatography mass spectrometry as in our previous studies [49,50].

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 OGTT prior to the start of the OGTT, as well as, 30 min after the start of the OGTT and 3 hours and 30 min after the start of the OGTT. An additional test may be administered if subject's glucose levels have not returned to baseline by the third metabolic cart test. This is required to determine what portion of ingested carbohydrates are being oxidized as opposed to stored, or utilized for HDNL as well as help distinguish between rates of oxidative and non-oxidative glucose disposal[51].

Study diet: Variations in diet, activity and circadian rhythms affect metabolism [34]. Therefore, OGTT studies will be performed in the AM fasting, in the follicular phase where possible, preceded by 3

days of no strenuous physical activity and a fixed macronutrient, high carbohydrate (65% carbohydrate, 20% fat, 15% protein), fixed grams of fructose, Calculated as Females: ([8.365 (weight in kg) + 465 (height in m) + 200] X Activity Factor x 1.25), dinner and snack provided by the Colorado CCTSI metabolic kitchen (similar to our previous studies[34]). 1.25 x weight maintenance was chosen as this as most similar to our subjects food consumption based on pilot subject's food frequency questionnaires and optimal for detection of hepatic glucose Ra [52].

Purpose for lab test to be drawn:

OGTT Labs	Purpose
Glucose Tracer	Determination of hepatic IR
Glycerol Tracer	Determination of adipose IR
Glucose Concentrations	Determination of hepatic IR
Insulin	Determination of hepatic, adipose IR
FFA	Measure of lipolysis
Glucagon	Gut hormone known to influence hepatic IR
GLP-1	Gut hormone known to influence hepatic IR
Acetate Tracer	Measurement of HDNL
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 subject is in the follicular phase of cycle, required for publication
Stored samples for future metabolic studies (not DNA)	Derminiation of potential other contributors to altered glucose and fat metabolism

LIFESTYLE PRESCRIPTION

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 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. This Exercise Physiologist will also be available to the study for consultation as needed. 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 subject/family will be provided with copies of their study lab results, DEXA scan, physical activity monitoring. Study staff will also call the family within 6 months of after completion of the study to check-in on the recommended follow up care and answer any questions about test results.

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

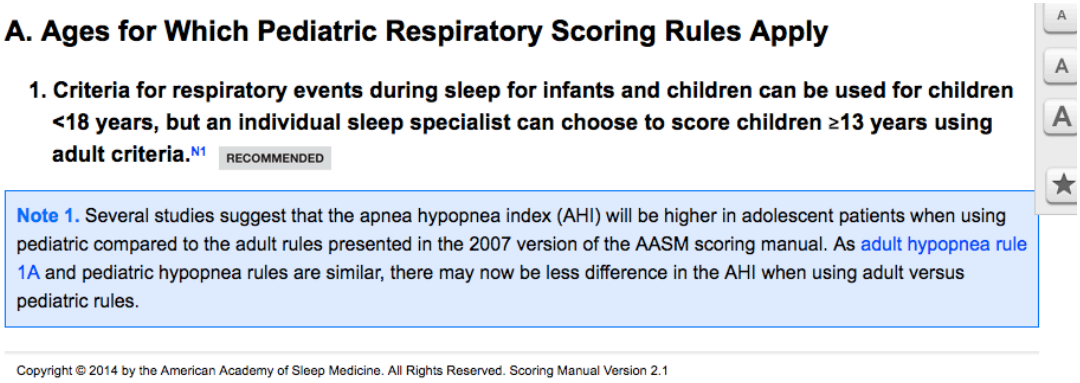
to verify the accuracy of the results from the WatchPAT device as compared to inpatient polysomnograms in children younger than 12, and is very familiar with this device, it's output and limitations. Our youth fall into a grey zone in terms of what is an abnormal sleep study, as pediatric criteria are 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



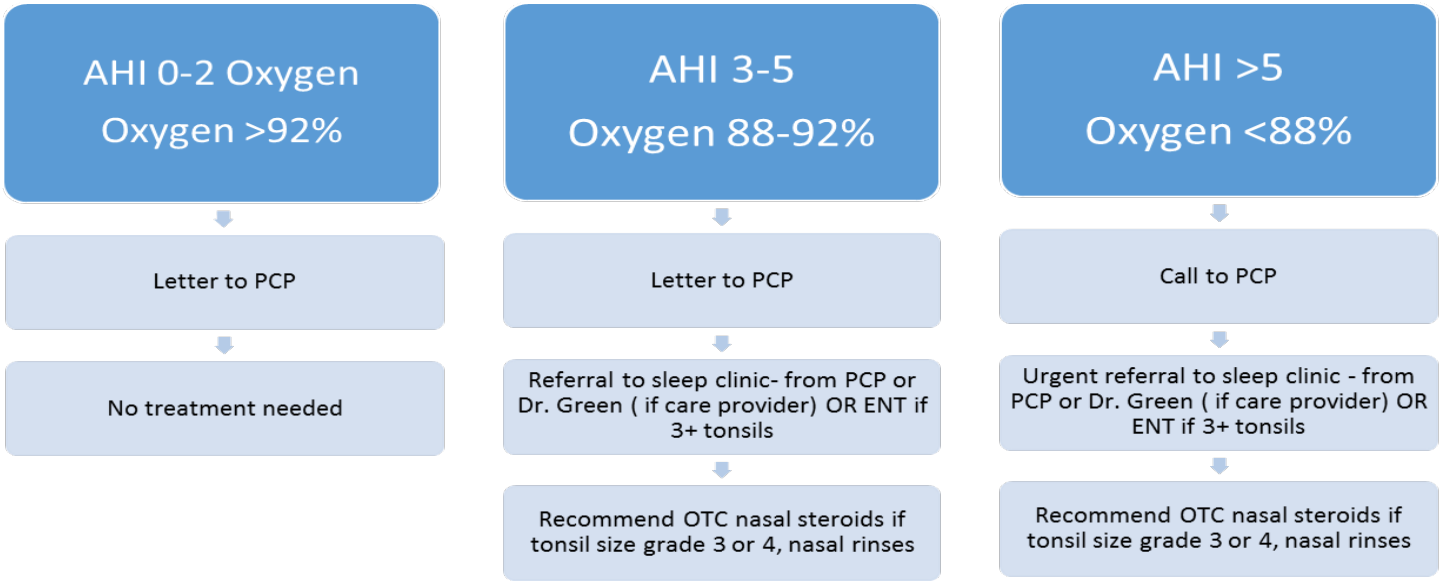
A. Ages for Which Pediatric Respiratory Scoring Rules Apply

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

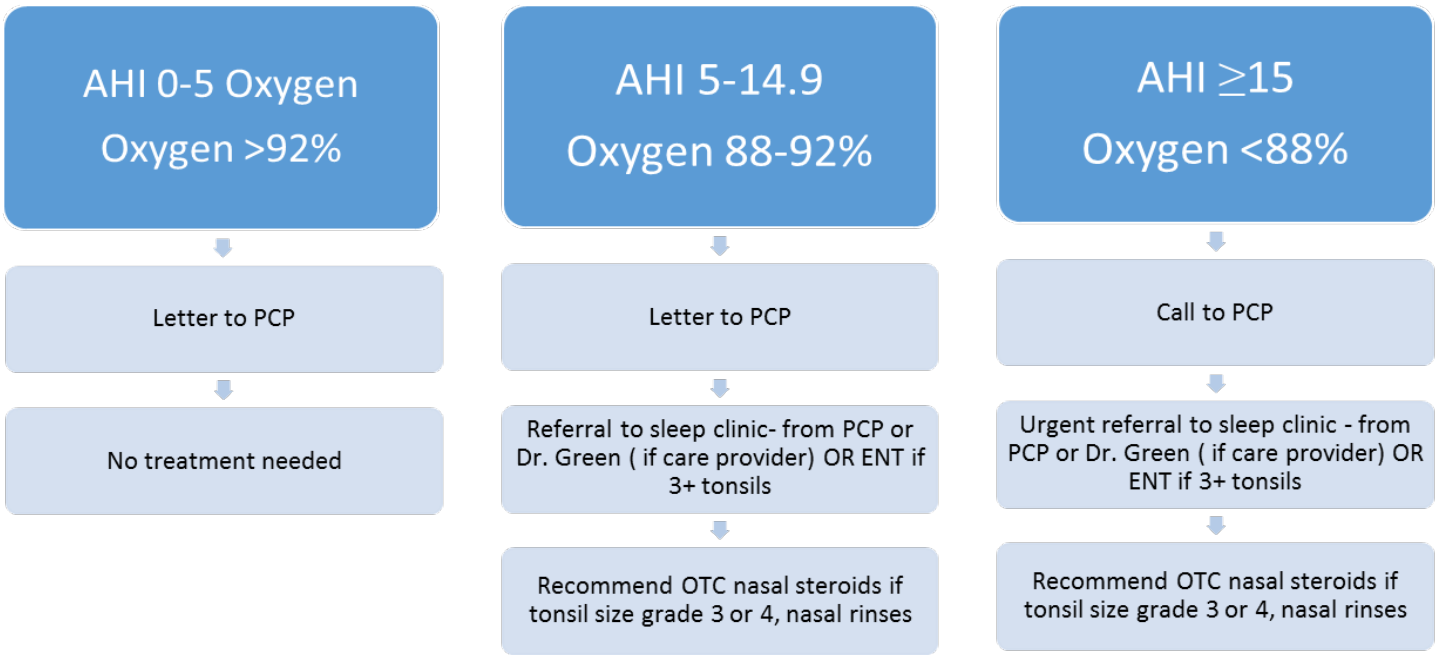
Note 1. Several studies suggest that the apnea hypopnea index (AHI) will be higher in adolescent patients when using pediatric compared to the adult rules presented in the 2007 version of the AASM scoring manual. As adult hypopnea rule 1A and pediatric hypopnea rules are similar, there may now be less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



SUBJECT RECRUITMENT/CONSENT/PAYMENT

1. **Subject Recruitment Plan**

Subjects 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 subjects. Further, we enrolled >100 obese girls in studies in the last 30 months. The PI and Co-I's have a treatment relationship with girls from clinic or subjects

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 subject.

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 subject and guardian verbally in the language they understand. The explanation will be conducted in a quiet environment with adequate time given for the subject and guardian to review the study procedure before the commencement of the study. Asking the subject to explain the study in their own words will assess the subject's understanding. If non-English speaking subjects are enrolled in the study, the investigators will adhere to Section 10C of the COMIRB Instructions for Clinical Investigators regarding the consent of these subjects. The qualified personnel mentioned above will then obtain written consent from the guardian and assent from the subject, co-signed on the consent form, or in subjects who are 18 years or older, direct consent. The PI will make a good faith effort to obtain both parent signatures. The subject 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 subjects below 18 years old will co-sign the consent form in addition to the parents signing the consent form. All subjects age 18 or older will sign the standard consent form.

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

Subjects will be compensated with Target gift cards during each scheduled visit. The initial visit consisting of informed consent, lab draw and questionnaires and the 2nd for the MRI will result in a $50 gift card. The 3rd and final visit consisting of the overnight portion will reward a $100 gift card. Compensation for all completed visits will total $200.

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

1. Blood Sampling

Description: Blood will be drawn for Complete Blood Count, HbA1c, total and free testosterone, and sex hormone binding globulin. If subjects 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 5ml/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 OGTT visit includes 207.5 ml of blood which will occur within 4 weeks of the initial visit. Thus, our OGTT visit is within the NIH Clinical Center guidelines of 9ml/kg in 6-8 weeks and within Children's Hospital Colorado's institutional guidelines of 5 ml/kg. In addition, by study design, subjects 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. In addition, we pre-screen subjects with a hemoglobin and hematocrit at their baseline visit, and exclude subjects with anemia. 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 subject. Finally, our CTRC has a system to track other studies subjects might enroll in, and we ask during our consent process if the subject 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: Two peripheral IV's will be placed during the OGTT. One will be to infuse the isotopes, and the other will be 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.
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 subject desires to minimize pain of IV.

3. Oral Glucose Tolerance Test (OGTT):
Description: An OGTT will be performed with multiple blood draws over 6 hours. The purpose of the OGTT is to provide a controlled oral stimulus to effect changes in lipolysis and hepatic glucose release. Subjects will also drink a glucose drink as part of the liver spectroscopy.
Risk: The subjects 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[53]. Our team of investigators, CTRC pediatric research nursing staff and physicians are well experienced with the OGTT blood draw procedure. A floor nurse located on the 9th floor of CHC will be available during our inpatient visits and patients will be distracted by TV or other similar means during the OGTT, to minimize queasiness.

4. Stable Isotope Studies:
Description: Stable isotope traces of glycerol, glucose and acetate will be utilized to determine rates of lipolysis, glucose release and hepatic de novo lipogenesis. 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 isotopes which already exist in all humans, but are simply increasing the percentage. The risk would be if these infusions were to become contaminated during preparation. These are NOT radioactive substances.
Justification/Minimization: These isotopes are specially produced to be sterile and pyrogen-free by the manufacturer, and in addition, after being reconstituted by the Children's Hospital Colorado Investigational Drug Pharmacy, they are retested for pyrogens and sterility prior to use at the University of Colorado CTRC laboratory and the University of Colorado Clinical Laboratory, respectively, and discarded if not used in the standard expiration period. This is the standard procedure used by numerous studies at the University of Colorado. The stable glucose and glycerol isotopes have previously been determined to be usable for research at our institution (currently being used in our protocols 06-0665, 07-0988, 10-1288) and others, and an IND is not required. To date, we have had no complications from the stable isotope infusions. However, as with any infusion, there is the possibility of infection.

Sterile and pyrogen-free $^{2}H_{5}$glycerol, 6,6-$^{2}H_{2}$ glucose and 1,2 -$^{2}C_{13}$ Acetate will be obtained in powder form the manufacturer. The CTRC pharmacist will reconstitute the glucose with sterile technique, filter (0.22 micron) for additional sterilization, aliquot, and freeze at –20 C in the CTRC investigational pharmacy freezer. One aliquot is then sent to the CTRC laboratory for confirmatory quantitative pyrogenicity testing and a second aliquot to the Children's Hospital laboratory for anaerobic and aerobic culture to confirm sterility.

5: Finger stick for glucose measurement during MRI:
Description: A glucometer will be utilized to measure fasting and 1 hour blood sugars following the glucose drink in the MRI.
Risk: There is a small amount of pain with the finger poke and risk of infection.
Justification/Minimization: The change in blood sugar needs to be assessed to correlate with changes in the glucose-6-phosphate concentrations. The finger will be well cleaned and dried with an alcohol pad. 1 time use Lancet's will be utilized to avoid the potential for blood exposure to other patients that has occurred with multi-use Lancet devices.

6. Standard Diet
Description: A dinner and snack will be provided from the CTRC the night prior to the OGTT with tracers. The diet will be composed of 65% carbohydrates, 25% fat, and 10% protein at 1.25 daily needs and will be will be provided by the CTRC. *Risk:* None
Justification/Minimization: 1.25 x weight maintenance was chosen as this as most similar to our subjects food consumption based on pilot subject's food frequency questionnaires and optimal for detection of hepatic Ra [52].

7. Magnetic Resonance Imaging (MRI)
Description: The MRI will usually be obtained the day of admission to CHC, at the UCHSC brain imaging center on the Fitzsimmons campus. Debra Singel, a trained research radiographer who is supervised by Dr. Mark Brown, of UCHSC radiology, will perform an abdominal MRI to obtain hepatic, visceral and subcutaneous fat on a 3.0 T whole–body MRI scanner (GE Medical Systems, Waukesha, WI). When this machine is being replaced, the imaging only will be performed on a clinical magnet on the Anshutz Campus. Subjects will lie supine while these measurements are obtained, need to hold reasonably still during the scan and cannot weigh >275 lbs. In some subjects, a specialized phosphorus coil will be utilized to measure the concentration of ^{31}P via MRS to calculate glucose-6-Phosphate concentrations before and after the glucose drink.
Risks: Minimal. Subjects may develop claustrophobia in the magnet.
Minimizing Risk: The subject is provided with audio protection and optional television to help increase comfort. Some subjects might feel claustrophobic while having an MRI and the scan will be stopped if it cannot be tolerated. In addition, any subjects with implanted metal cannot have an MRI due to the 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 place movies on the subjects. 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 on the pediatric CTRC 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 subject 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 OGTT 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.

Subjects 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.
Risk: This procedure may lead to mild discomfort due to the blood pressure cuff being inflated.
Justification/Minimization: Endothelial function is an novel measure in PCOS and will aid in the determination of cardiovascular dysfunction with this population. A trained cardiovascular technician will be on hand to witness the procedure.

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: Each subject will wear an accelerometer once (MTI Actigraph by Actigraph) to measure habitual level of physical activity, which affects insulin sensitivity. The accelerometer will be worn in a pouch that rests on the subject's hip and is positioned upright against the body to measure movement, similar to a pedometer.
Risk: There is no risk involved with the accelerometer.
Justification/Minimization: Accelerometers are effective tools for the objective measurement of physical activity [42] 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 MTI 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.

12: Metabolic Cart:
Description: The metabolic cart measures the amount of air that the subject breathes in and out. The machine attaches to the subject's mouth through a tube, or a plastic bubble that is placed over the subject's head. There is the potential for experiencing claustrophobia from having the plastic bubble over the subject's head. A metabolic cart will be utilized multiple times during the OGTT 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 hood over the subjects head for approximately 20 minutes. The data collected from the baseline study is also very useful for assisting obese subjects 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. Further, subjects 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 screening.
Risk: None
Justification/Minimization: Physical activity can directly effect insulin sensitivity, our primary outcome measure. The 3DPar is a well validated measure to asses 3 days of physical activity in youth, an 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 you at risk for depression or anxiety, but identify poor coping skills. It does not directly assess depression or suicidality.

16. WatchPAT and Questionnaire to assess for Obstructive Sleep Apnea
Description: The WatchPAT is a noninvasive portable system that measures the oxygen saturation and apnea hypopnea index. This is a 1 page survey 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. The survey selected is currently being utilized by the NIH multiple center study in obese youth at risk for diabetes, and is well validate in youth from multi-ethnic populations. If OSA is suspected during the course of the screen, the subjects 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. Overnight sleep study with salivary melatonin sampling (Pending scheduling availability)
Description: Stickers will be attached to the head and chest for approximately 11 hours to measure brain activity, oxygen status and breathing overnight. Saliva will be collected hourly while awake.
Risks: Discomforts include unsettling to sleep away from home in a strange place, and the risk of mild irritation from the sticker adhesive. The swab to collect spit can make your mouth feel dry when it is first placed.
Justification: Obstructive sleep apnea is associated with obesity, and can worsen both fatty liver and insulin resistance. The presence of OSA is often missed by questionnaires, and can only be assessed with a sleep study. The melatonin will provide data on the chemical signaling involved in falling asleep and waking up, as they relate to changes seen with the sleep study. Saliva is good alternative to blood sampling in pediatric populations.

18: 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, subjects 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 sickness.

19: Risk of stopping oral contraceptives
Description: OCP's will alter study variables, and cannot be used for 6 months prior to participating in the study unless they are enrolled as a PCOS patient treated with OCP's. If a non-treated subject is taking OCP's and wishes to participate, they will need to stop therapy.
Risk: 1) Subjects are at risk of having an increase in acne and hair growth, and a potential for some irregular menstrual bleeding if these symptoms were present prior to starting oral contraceptives. 2) The second risk is to those subjects who are also using the oral contraceptive for pregnancy prevention.
Justification/Minimization: For acne and hair growth, the severity of these changes in the short time off the OCP's is likely to be low, and have a minimal impact on the subjects. Furthermore, these symptoms will again subside once the subjects restart their OCP's. OCP prescribers will be notified of subjects' intent to stop OCP's for the study. The risk of pregnancy is likely low, as many women with PCOS have infertility issues due to anovulation. To decrease the risk further, all subjects who are sexually active will be provided with barrier protection in the form of condoms, and will be counseled about safe sexual practices. Subjects can additionally have a IUD placed by their gynecologist, which is not an exclusion to the study.

20. Study Medication Injection (Byetta)
Description: When Byetta is administered as a monotherapy for a prolonged duration, the most significant adverse events were nausea, vomiting and Dyspepsia. Hypoglycemia was rarely encountered. Adverse events were most common at the maximal dose of 10 mcg, and rare at the introduction dose of 5 mcg. Every effort will be made to monitor for these adverse events and study staff will also monitor for signs of hypoglycemia via glucometer readings.
Justification/Minimization: GLP-1 receptor agonists such as exenatide (Byetta) aid in secretion of insulin and suppress the action of glucagon. Our preliminary data show increased glucose concentrations secondary to relative inadequate insulin secretion following an oral glucose load in adolescent girls with PCOS compared to their obese control counterparts. Participants in this overnight study will only receive 2 doses of this short acting medication, and the well tolerated lower risk 5 mcg dose. They will be monitored at CHCO inpatient following the first injection of 5 mcg of Byetta. Patient will be monitored for excessive vomiting and dehydration overnight. Vital signs will be taken overnight every 4 hours by clinical inpatient nurses per CHCO overnight nursing policy. The patient will be instructed to alert the night nursing staff if any nausea or dehydration occurs overnight and study staff will be notified. During the study day (visit 3), study staff and CTRC nursing staff will be bedside monitoring patient from 6AM to 2PM periodically taken vital signs and assessing signs of excessive vomiting and dehydration. Glucometer readings will be taken at regular intervals for 6 hours after the OGTT to measure blood glucose concentrations. Study staff and research nursing will continuously monitor patient until discharge from hospital. At the time of discharge, the half-life of Byetta will have expired.

21. 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 subjects 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 effects 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. We will also begin to learn the effect of the three existing medical therapies.

Individual: Subjects 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 subjects who completed a related protocol (10-1288) and received this counseling have higher rates of weight loss upon clinical follow-up than those children being seen in obesity clinics alone.

Benefits to participant:

1) All subjects 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 subjects 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 subjects have obstructive sleep apnea, they will be referred to the sleep clinic for appropriate treatment.

2) All of the subjects enrolled must be sedentary, with less than 3 hours a week of physical activity. This is less than the time recommend 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 subjects will receive counseling on how to increase their activity levels by either Amy Baumgartner, MS or Greg Coe, MS, both of whom have degrees in exercise physiology, and are experts in providing exercise prescriptions. Increased activity has been shown to reverse the risk for diseases later in life.

3) All subjects will complete a 3 day food questionnaire. This will be reviewed with them, and healthier food choices and meal planning will be discussed with both the subject and their parent by the PI or PRA, all of who are trained in providing diet prescriptions. Additionally, obese subjects 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.

Evidence of Direct Benefit

We believe that this protocol is in the 405 risk category for pediatric research. Subjects can benefit from the above study measurements. Further, at the conclusion of the study, all subjects in the

protocol will also be given counseling on the benefits of exercise as discussed above, and given an exercise prescription. The subjects will also be re-contacted once by phone call to follow-up on their study results and exercise recommendations. Sedentary subjects 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 subjects 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. 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 subjects, 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 subject.

Inclusion criteria is less than 3 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.

Because of their sedentary nature, there are several primary direct benefits to the non-PCOS subjects from the OGTT, which justify the risk. The primary benefit from the OGTT 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. Thus, it is very possible that we may find evidence of NAFLD in the obese non-PCOS population. 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, diabetes and NAFLD. 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.

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 subjects to not participate in the study

G. Consideration of Specific Subject Categories

1. Inclusion of Women

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

2. Inclusion of Minorities

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

3. Inclusion of Children

All subjects will be between ages 12 and 21. Insulin sensitivity needs to be studied in the adolescent age group as no data is currently available 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 the entire subject set, which allow for comparisons between obese girls with and without PCOS. OGTT with tracers will be conducted in all subjects, and overnight assessment of HDNL in 20 subjects (10 with PCOS and 10 without). However, data from the first 6-12 subjects (both PCOS and non-PCOS) will be immediately evaluated to see if any changes are needed in terms of protocol details. The specific points that are to be evaluated are listed below:

1) OGTT with glucose and glycerol tracers

a. Purpose: Develop an oral stimulus model to assess hepatic and adipose insulin sensitivity.

i. Question 1. Is 4 hours post oral load long enough to model hepatic and adipose insulin sensitivity?

1. Rationale: Our preliminary data from OGTT's in obese girls indicates that subjects' insulin and glucose concentrations have not returned to baseline by 2 hours. Data from adults showed that all non-diabetic adults were back to baseline by 3.5 hours.
2. Measurement. Serum glucose and insulin will be measured post-glucose load for 4 hours in the first 6 subjects. If 4 hours post glucose values are within 10 mg/dL for glucose and 10 IU/mL for insulin relative to baseline values, 4 hours will be considered sufficient.

ii. We are revising the protocol to 6 hours based on our results from our first several subjects.Question 2: Are glucose and glycerol Ra during the OGTT similar to those obtained with a hyper-insulinemic euglycemic clamp.

1. Rational: Peak insulin concentrations are similar between an OGTT and a clamp, thus Ra rates should also be similar. Comparisons in adults of the glucose Ra from clamps and an oral meal challenge were similar.
2. Measurement: The peak suppressed glucose and glycerol Ra for the first 12 subjects will be compared to peak suppressed Ra values calculated from data from 70 girls during hyper-insulinemic euglycemic clamps.
3. Revision plan if model not correct: None. Findings of either similar or different glucose and glycerol Ra are valuable information.

2) Measurement of Hepatic De Novo Lipogenesis (HDNL)

a. Purpose: Optimize a model to asses HDNL with minimal blood draws, minimal time of tracer intervention and no interference with measures of glucose and glycerol Ra

i. Rational: HDNL can be difficult to measure. Previous studies have used weeks of deuterated water loading or large volume deuterated water overnight. However Deuterated water cannot be used at that same time as other tracers methods. The use of $^{1}C_{13}$ acetate requires the use of patented MIDA calculations, to which we do not have access. Rate's of HDNL are low, and glucose loading may be required for detection. We will be using $^{2}C_{13}$ acetate which will allow us to avoid use of the MIDA calculations. We will infuse the tracer for 19 hours, and also precede the tracer study with a dinner and snack of 1.25 REE meal, which can increase fasting lipogenesis rates.

ii. Measurement: Enrichment of long chain fatty acid moieties after 19 hours of infusion rate will be measured.

iii. Revision plan if model not correct: 1) increase the time for overnight acetate infusion or 2) increase rate of acetate tracer infusion either by giving a prime at that start of infusion, or increasing the hourly rate of acetate infusion.

b. Purpose: Measure HDNL in the fed state concomitantly during the OGTT with tracers

i. Rational: HDNL needs to be measured in the fasted and the fed state. We will be using the OGTT to also measure glucose and glycerol kinetics. There is a chance that the oral $^{1}C_{13}$ glucose needed for the glucose model may be incorporated into long chain fatty acids via HDNL, and falsely elevated the rate of HDNL calculated.

ii. Measurement: Measure the enrichment of long chain fatty acids during the OGTT with tracers, without the acetate tracer

iii. Revision plan if needed: Calculate the mean rate of incorporation of oral $^{1}C_{13}$ into long change fatty acids and develop a correction factor to apply to overall rates of HDNL.

3) Measurement of Glucose-6-Phosphate(G6P) in liver before and after glucose load

a. Purpose: Determine if glucose flux through the liver can be measured with MRS Phos

i. Rational: Increased rates of HDNL may be related to preferential uptake and utilization of oral glucose to the liver. Phosphorylation is the first step following hepatic uptake of glucose. G6P can be measured with MRS Phos technologies

ii. Measurements: The area under the curve of G-6-P will be measured before and 45 min after a glucose load. Serum glucose peaks between 30 and 60 min in the majority of subjects, which is why this time point was selected. Further, liver studies using carbon MRS have demonstrated increased glycogen production 60 min following a oral glucose load, and G-6-P is a precursor step for this process.

Overall Project Limitations:

1) Subject recruitment is always a potential concern, but is minimized by a streamlined recruitment system and experience with this population; the previous, more intensive study is well tolerated in youth and enrolled faster than projected. In addition, we had >100 new PCOS referrals to our pediatric endocrinology department in 2013 alone, and the PI has a clinic specifically for girls with PCOS.

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

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 (Redcaps).

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 the mentor Kristen Nadeau, MD will monitor the protocol and the safety of the research subjects. The PI will review all laboratory data and report any abnormal values to the patient and guardian and instruct the subject to follow-up with their PCP. 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 (age, 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 subject 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 hyperinsulinemic euglycemic clamp 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 untoward medical event associated with the use of a drug in humans, whether or not considered drug-related. AEs also include 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.

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 randomization) are not considered or recorded as AEs or SAEs unless the condition worsens in intensity or frequency after randomization. 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 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 Safety Officer and COMIRB and any other required institutional monitoring committee.

C. Subject Discontinuation Criteria

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

1. Inability to complete study procedures
2. Abnormal screening labs (LFT's >4 times elevate, HbA1C>6.4%, Hg <9 mg/dL)
3. Subject becomes pregnant during study

D. Protocol Stopping Criteria

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

VII. Data Analysis Plan:

Statistical Plan for Method Development: The previously described tracer protocol and mathematical modeling with any refinements will be performed in an additional 9 subjects. Outcome measures (raw suppressed glucose and glycerol Ra, insulin concentration required for 50 percent of peak suppression and percent suppression of basal Ra) will be compared between the OG subjects and a matched cohort of girls who have undergone hyperinsulinemic euglycemic clamps with identical glucose and glycerol tracer infusion rates. Subjects in the two groups will be matched on PCOS diagnosis, race, percent body fat, percent liver fat, and time post-menarche. An equivalence design will be used to compare the outcomes in the two groups and to demonstrate whether there are clinically significant differences in outcomes from the OG model and the hyperinsulinemic clamp. For purposes of sample size calculation, we used estimates from the investigator's pilot study. Using an equivalence limit of one standard deviation for each outcome measure (SD=50 units for glycerol IC50 and SD=39 units for glucose IC50) and a correlation of 0.2 between outcomes on matched subjects, 15 matched pairs gives 80% power to demonstrate equivalence. 70 girls have completed the hyperinsulinemic euglycemic clamps, so adequate matching will be possible.

Overall Project Statistical Plan:

Power calculation: The primary outcome is hepatic IR measured as IC_{50} glucose Ra. Using our preliminary data, a two-tailed t-test and an alpha of 0.05, completing 13 controls and 32 PCOS provides 86% power to detect a difference of 35 IU/L in the insulin concentration.

Differences between the two primary groups (PCOS, controls) will be assessed with a t test or for non-normally distributed data a non-parametric test such as Mann-Whitney U. Secondary outcomes include a Pearson correlation test of potentially related variables followed by targeted multiple regression to assess the relationship between hepatic IR or hepatic fat and adipose IR, hepatic gluconeogenesis, VAT, carbohydrate oxidation, androgens, metformin or OPC use and presence of OSA. As an exploratory analysis, the four groups will also be compared using an ANOVA. We recognized that we may not be powered for these analyses, but the data will be used as hypothesis generating for future studies.

The rate of hepatic De Novo lipogenesis is a secondary outcome. Based on our preliminary data that plasma markers of hepatic De Novo lipogenesis are 40% greater in those with fatty liver disease and the use of a high carbohydrate and fructose drink to increase the differences between groups, 10 subjects in each group will be needed to detect a difference in hepatic De Novo lipogenesis[21,49,52]. The rate of hepatic De Novo lipogenesis will be correlated with measures of hepatic and adipose IR and MRI measures of NAFLD.

Non-Isotope OGTT calculations: The Matsuda model of IR will be used, and B-cell function [insulinogenic index ($\Delta I30/\Delta G30$) and ($\Delta C30/\Delta G30$) and disposition index ($1/IFasting \times \Delta I30/\Delta G30$) and ($1/CFasting \times \Delta C30/\Delta G30$) will be calculated[40,53-55].

Proposed Mathematical Model of Glucose and Glycerol Dynamics: The differential equations-based isotope labeled oral glucose minimal model (OMM*) [56] will be adapted to describe glucose and glycerol dynamics present in the collected data. In OMM*, the oral glucose minimal model (OMM) is modified to account for labeled glucose oral data obtained with the inclusion of a stable isotope tracer. This refinement allows an estimate of insulin sensitivity which is unique in that it accounts for both stimulation of adipose and muscle glucose uptake and inhibition of hepatic glucose release. In OMM*, glucose concentration G and insulin action on glucose disposal X are governed by the following equations:

$$\frac{dG}{dt} = -[S_G^* + X^*] \cdot G + \frac{R_a(\alpha, t)}{V*}$$
$$\frac{dX*}{dt} = -p_2^* \cdot X^* + p_3^* \cdot [I - I_b]$$

where S_G* is the fractional glucose effectiveness; Ra(α, t) is the glucose rate of appearance; V* is the distribution volume; p_2*and p_3* are rate constants; I is plasma insulin concentration; and I_b is basal plasma insulin concentration. The Ra in this equation represents a best fit (see Fitting Ra below) to Ra values computed from data (see Proposed Isotope Tracer Calculations below). This model will allow differentiation between exogenous and endogenous contributions to changes in glucose concentration. A similar model, modified to reflect the absence of an exogenous glycerol contribution from OGTT, will be used to describe glycerol dynamics. The concomitant study of glycerol has been shown to not interfere or contribute to glucose Ra.[57]

Proposed Isotope Tracer Calculations: The glycerol and glucose Ra during fasting and the entire post-glucose load period will be calculated using the non-steady-state equation of Steele[58,59]. The glucose rate of disappearance (Rd), and metabolic clearance rate (MCR) will be calculated for the same time periods [58,59]:

$$R_a\ (\mathrm{mg \cdot kg^{-1} \cdot min^{-1}}) = (F - V((C_1 + C_2)/2)((IE_2 - IE_1)/(t_2 - t_1)))/((IE_2 + IE_1)/2)$$
$$R_d\ (\mathrm{mg \cdot kg^{-1} \cdot min^{-1}}) = R_a - V((C_2 - C_1)/(t_2 - t_1))$$
$$MCR\ (\mathrm{ml \cdot kg^{-1} \cdot min^{-1}}) = R_d/((C_1 + C_2)/2)$$

where F represents isotope infusion rate; IE_1 and IE_2 are isotopic enrichments at sampling time points 1 (t_1) and 2 (t_2), respectively; C_1 and C_2 are metabolite concentrations at t_1 and t_2; and V is the estimated volume distribution (glucose is 180 ml/kg and glycerol 27 ml/kg). Ra glucose from the glucose drink (Ra $_{EXO}$) and endogenous glucose production (Ra $_{ENDO}$) will be calculated:

$$\%R_a \text{ from EXO} = (E_P/E_D)100$$
$$R_{a,EXO} = R_a(\%R_a \text{ from EXO})/100$$
$$R_{a,ENDO} = R_a - R_{a,EXO}$$

where E_D and E_P are the enrichments of the 1-^{13}C-glucose in the drink and plasma, respectively. Ra, Rd and MCR will be presented with and without correction for serum insulin concentrations.

Fitting Ra: Using computed data points, glycerol and glucose Ra will be fitted with an appropriate model. In control subjects, Ra typically shows 3 phases in OGTT [60]. Cubic polynomial, piecewise-linear, spline, and dynamic models have been used for fitting Ra in previous work[61,62]. In a comparison of different models, the choice of model form did not significantly impact the resulting estimates of insulin sensitivity or glucose Ra. Given the expected 3 phase form of Ra, 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 for Ra (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 Ra.

Characterization of Liver and Adipose Metabolism through Model Analysis: The novel OMM* model will be used to measure insulin sensitivity and provide a quantitative characterization of glucose Ra in the 5 PCOS subjects, thereby providing insight into liver metabolism in these subjects. The following measures will be computed: mean of Ra; area under the curve describing Ra during 4 hour period associated with OGTT; time to peak Ra suppression; and time to return to basal Ra post glucose load. A similar characterization of glycerol Ra in the 5 PCOS subjects with be computed using the novel OMM* glycerol model, thereby providing insight into adipose

metabolism in these subjects. In addition, the glucose and glycerol Ra suppression associated with OGTT will be compared to those already collected from hyper-insulinemic euglycemic clamp glycerol and glucose Ra suppression from an identical subject population to identify similarities and differences associated with protocol.

Proposed Model of Glucose Absorption: A key advantage of OGTT over IV glucose delivery is the physiologic aspect of oral administration. When glucose is ingested, the physiologic pathway through the gastrointestinal tract contributes to the glucose rate of appearance in plasma. Although gastric emptying of liquids occurs exponentially in control subjects, there is evidence that gastric emptying may be delayed in certain populations, thereby impacting glucose absorption [63-65]. In order to assess possible abnormalities in glucose absorption in PCOS, we will relate a model that describes mechanisms of glucose transit through the gastrointestinal tract [60] to Ra data computed for PCOS subjects. The three-compartment model for glucose transit through the stomach and upper small intestine is described by the following system of differential equations:

$$\begin{aligned} \frac{dq_{sto1}}{dt} &= -k_{21}q_{sto1} + D\delta \\ \frac{dq_{sto2}}{dt} &= -k_{empt}(q_{sto})q_{sto2} + k_{21}q_{sto1} \\ \frac{dq_{gut}}{dt} &= -k_{abs}q_{gut} + k_{empt}(q_{sto})q_{sto2} \end{aligned}$$

where q_{sto1} and q_{sto2} are the amounts of glucose in the stomach (solid and liquid phase, respectively); δ is the impulse function associated with OGTT glucose delivery; D is the amount of ingested glucose; q_{gut} is the glucose mass in the intestine; k_{21} is the rate of grinding; and k_{abs} is the rate constant of intestinal absorption. The rate of gastric emptying, $k_{empt}(q_{sto})$, is a nonlinear function of the total amount of glucose in the stomach: $q_{sto}=q_{sto1}+q_{sto2}$. Then we compute $Ra(t) = f\ k_{abs}\ q_{gut}(t)$ where f is the fraction of intestinal absorption appearing in plasma. Model parameters will be determined by fitting to Ra and tracer data. Since model parameters reflect specific physiologic processes, they will provide additional insight into the mechanisms underlying abnormalities of glucose Ra in PCOS subjects.

Analysis of the sleep study: Respiratory parameters of interest (defined below) include arousals, apneas, hypopneas, low oxygen saturation time (SaO2T), hypercapnia time (CO2>50), and oxygen saturation nadir (SaO2N). The arousal index (AI) is the number of arousals and awakenings measured by a shift of EEG signal to > alpha range for more than 3 seconds, divided by total sleep hours as previously defined(57). Apnea is defined as an absence of airflow for two or more breath cycles. Hypopnea is a visible decrease in airflow by nasal pressure signal (or by thermistor when pressure signal was unavailable) *and* either an EEG arousal or a drop in oxygen saturation of 3% or greater. Mixed apnea is an obstructive apnea combined with a central (absent effort) apnea. The AHI is comprised of the obstructive, mixed, and hypopnea events divided by total sleep hours; central apneas (seen in normal children)) are not included in the AHI. Intermittent hypoxemia, even brief episodes of mild drops from the normal baseline, is hypothesized to be a possible cause of neuropsychological dysfunction. SaO2T is the time in minutes with oxyhemoglobin saturation less than 95% measured by pulse oximetry in order to detect mild intermittent desaturation, (not seen in normal children. SaO2N is the nadir of oxyhemoglobin saturation. Hypercapnia time (CO2>50) is time in minutes with an end-tidal CO2 level greater than 50 mm Hg. Carbon dioxide levels are generally not measured in adult sleep laboratories. The AHI in normal non-snoring children has been determined to be less than 1.0, with little hypercapnia (sleep time with CO2 > 50 mm Hg, reported to be < 10-20%.There are no established diagnostic criteria for mild, moderate, or severe sleep apnea in children. OSA in adults is defined by the AHI: the number of apnea or hypopnea (partial obstruction) events per hour. However, continuous partial obstruction is underestimated by these criteria, and is considered significant by the Pediatric Sleep Program. Therefore, normal participants with evidence of continuous partial obstruction not meeting adult criteria for OSA, an AHI >1, or hypercapnia > 10% of sleep time will be excluded. For

this protocol, mild OSA is defined as AHI 1–4, moderate OSA as AHI 5–10, and severe OSA as AHI greater than 10.

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

Melatonin offset: Salivary melatonin swabs will be analyzed to determine salivary melatonin concentrations. Concentrations will be used to calculate melatonin offset, or the time that melatonin decreases for the overnight sleeping peak, to the day time low. Offset will be determine relative to arousal time, and calculated in minutes.

VIII. Summarize Knowledge to be Gained:

Overall Expected Results*:* We anticipate that we will develop 2 new (OGTT with tracers and liver 31 MRS Phos) novel minimally invasive ways of assessing hepatic metabolism in a physiologic state. We will delineate the relationship between adipose lipolysis suppression, hepatic glucose release, and hepatic fat. We expect that PCOS girls will uniquely have glucose Ra and hepatic fat excess driven by hyperadrogenemia, and that IR will be improved in girls taking metformin but not girls taking OCP's. In addition to our protocol, these models can be utilized in other patient populations with IR and/or obesity. Further, if Glucagon or GLP-1 have a role in hepatic IR or HDNL, there are existing available therapeutic choices that later these gut hormones. This data would then provide the background evidence for future therapeutic trials.

Significance: Recent evidence in adults with PCOS indicates that 60-70% have NAFLD[7,15]. 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 protocol. 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[5,6]. Thus, understanding the relative contributions of adipose and hepatic IR to 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.

IX. References:

1. Knochenhauer ES, Key TJ, Kahsar-Miller M, Waggoner W, Boots LR, Azziz R. Prevalence of the polycystic ovary syndrome in unselected black and white women of the southeastern United States: a prospective study. J Clin Endocrinol Metab 1998;83:3078-82.
2. Azziz R, Marin C, Hoq L, Badamgarav E, Song P. Health care-related economic burden of the polycystic ovary syndrome during the reproductive life span. J Clin Endocrinol Metab 2005;90:4650-8.
3. Blank SK, Helm KD, McCartney CR, Marshall JC. Polycystic ovary syndrome in adolescence. Ann N Y Acad Sci 2008;1135:76-84.
4. Baranova A, Tran TP, Birerdinc A, Younossi ZM. Systematic review: association of polycystic ovary syndrome with metabolic syndrome and non-alcoholic fatty liver disease. Alimentary pharmacology & therapeutics 2011;33:801-14.
5. de Groot PC, Dekkers OM, Romijn JA, Dieben SW, Helmerhorst FM. PCOS, coronary heart disease, stroke and the influence of obesity: a systematic review and meta-analysis. Human reproduction update 2011;17:495-500.
6. Ehrmann DA, Liljenquist DR, Kasza K, Azziz R, Legro RS, Ghazzi MN. Prevalence and predictors of the metabolic syndrome in women with polycystic ovary syndrome. J Clin Endocrinol Metab 2006;91:48-53.
7. Gambarin-Gelwan M, Kinkhabwala SV, Schiano TD, Bodian C, Yeh HC, Futterweit W. Prevalence of nonalcoholic fatty liver disease in women with polycystic ovary syndrome. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association 2007;5:496-501.

8. De Leo V, Musacchio MC, Palermo V, Di Sabatino A, Morgante G, Petraglia F. Polycystic ovary syndrome and metabolic comorbidities: therapeutic options. Drugs Today (Barc) 2009;45:763-75.
9. Park MH, Kinra S, Ward KJ, White B, Viner RM. Metformin for obesity in children and adolescents: a systematic review. Diabetes Care 2009;32:1743-5.
10. Hoeger K, Davidson K, Kochman L, Cherry T, Kopin L, Guzick DS. The impact of metformin, oral contraceptives, and lifestyle modification on polycystic ovary syndrome in obese adolescent women in two randomized, placebo-controlled clinical trials. J Clin Endocrinol Metab 2008;93:4299-306.
11. Ibanez L, Diaz M, Sebastiani G, et al. Treatment of androgen excess in adolescent girls: ethinylestradiol-cyproteroneacetate versus low-dose pioglitazone-flutamide-metformin. J Clin Endocrinol Metab 2011;96:3361-6.
12. Lass N, Kleber M, Winkel K, Wunsch R, Reinehr T. Effect of lifestyle intervention on features of polycystic ovarian syndrome, metabolic syndrome, and intima-media thickness in obese adolescent girls. J Clin Endocrinol Metab 2011;96:3533-40.
13. Domecq JP, Prutsky G, Mullan RJ, et al. Adverse effects of the common treatments for polycystic ovary syndrome: a systematic review and meta-analysis. J Clin Endocrinol Metab 2013;98:4646-54.
14. Legro RS, Arslanian SA, Ehrmann DA, et al. Diagnosis and treatment of polycystic ovary syndrome: an endocrine society clinical practice guideline. J Clin Endocrinol Metab 2013;98:4565-92.
15. Cerda C, Perez-Ayuso RM, Riquelme A, et al. Nonalcoholic fatty liver disease in women with polycystic ovary syndrome. Journal of hepatology 2007;47:412-7.
16. Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. Alimentary pharmacology & therapeutics 2011;34:274-85.
17. Mudaliar S, Henry RR, Sanyal AJ, et al. Efficacy and safety of the farnesoid X receptor agonist obeticholic acid in patients with type 2 diabetes and nonalcoholic fatty liver disease. Gastroenterology 2013;145:574-82 e1.
18. Fabbrini E, deHaseth D, Deivanayagam S, Mohammed BS, Vitola BE, Klein S. Alterations in fatty acid kinetics in obese adolescents with increased intrahepatic triglyceride content. Obesity (Silver Spring) 2009;17:25-9.
19. Vitola BE, Deivanayagam S, Stein RI, et al. Weight loss reduces liver fat and improves hepatic and skeletal muscle insulin sensitivity in obese adolescents. Obesity (Silver Spring) 2009;17:1744-8.
20. Kim YO, Schuppan D. When GLP-1 hits the liver: a novel approach for insulin resistance and NASH. Am J Physiol Gastrointest Liver Physiol 2012;302:G759-61.
21. N. Santoro EP, B. Pierpont, S. Caprio. Hepatic De Novo Lipognesis in Youth: Role of the GCKR rs1260326 Variant. Diabetes 2013;61 Supp. 1:A1287.
22. Biddinger SB, Hernandez-Ono A, Rask-Madsen C, et al. Hepatic insulin resistance is sufficient to produce dyslipidemia and susceptibility to atherosclerosis. Cell Metab 2008;7:125-34.
23. Cai D, Yuan M, Frantz DF, et al. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. Nat Med 2005;11:183-90.
24. Arslanian SA, Lewy VD, Danadian K. Glucose intolerance in obese adolescents with polycystic ovary syndrome: roles of insulin resistance and beta-cell dysfunction and risk of cardiovascular disease. J Clin Endocrinol Metab 2001;86:66-71.
25. Ciaraldi TP, Aroda V, Mudaliar S, Chang RJ, Henry RR. Polycystic ovary syndrome is associated with tissue-specific differences in insulin resistance. J Clin Endocrinol Metab 2009;94:157-63.
26. Dunaif A, Graf M, Mandeli J, Laumas V, Dobrjansky A. Characterization of groups of hyperandrogenic women with acanthosis nigricans, impaired glucose tolerance, and/or hyperinsulinemia. J Clin Endocrinol Metab 1987;65:499-507.
27. McCartney CR, Blank SK, Prendergast KA, et al. Obesity and sex steroid changes across puberty: evidence for marked hyperandrogenemia in pre- and early pubertal obese girls. J Clin Endocrinol Metab 2007;92:430-6.

28. Bjorntorp P. The regulation of adipose tissue distribution in humans. Int J Obes Relat Metab Disord 1996;20:291-302.
29. Bjorntorp P. The android woman--a risky condition. J Intern Med 1996;239:105-10.
30. Glintborg D, Andersen M, Hagen C, et al. Evaluation of metabolic risk markers in polycystic ovary syndrome (PCOS). Adiponectin, ghrelin, leptin and body composition in hirsute PCOS patients and controls. Eur J Endocrinol 2006;155:337-45.
31. Dalla Man C, Piccinini F, Basu R, Basu A, Rizza RA, Cobelli C. Modeling hepatic insulin sensitivity during a meal: validation against the euglycemic hyperinsulinemic clamp. Am J Physiol Endocrinol Metab 2013;304:E819-25.
32. Dalla Man C, Caumo A, Basu R, Rizza R, Toffolo G, Cobelli C. Minimal model estimation of glucose absorption and insulin sensitivity from oral test: validation with a tracer method. Am J Physiol Endocrinol Metab 2004;287:E637-43.
33. Angioni S, Sanna S, Magnini R, Melis GB, Fulghesu AM. The quantitative insulin sensitivity check index is not able to detect early metabolic alterations in young patients with polycystic ovarian syndrome. Gynecol Endocrinol 2011;27:468-74.
34. Nadeau KJ, Zeitler PS, Bauer TA, et al. Insulin resistance in adolescents with type 2 diabetes is associated with impaired exercise capacity. J Clin Endocrinol Metab 2009;94:3687-95.
35. Abdul-Ghani MA, Williams K, DeFronzo RA, Stern M. What is the best predictor of future type 2 diabetes? Diabetes Care 2007;30:1544-8.
36. Sharma R, Sinha S, Danishad KA, et al. Investigation of hepatic gluconeogenesis pathway in non-diabetic Asian Indians with non-alcoholic fatty liver disease using in vivo ((31)P) phosphorus magnetic resonance spectroscopy. Atherosclerosis 2009;203:291-7.
37. Leij-Halfwerk S, van den Berg JW, Sijens PE, Wilson JH, Oudkerk M, Dagnelie PC. Altered hepatic gluconeogenesis during L-alanine infusion in weight-losing lung cancer patients as observed by phosphorus magnetic resonance spectroscopy and turnover measurements. Cancer Res 2000;60:618-23.
38. Fishbein MH, Gardner KG, Potter CJ, Schmalbrock P, Smith MA. Introduction of fast MR imaging in the assessment of hepatic steatosis. Magn Reson Imaging 1997;15:287-93.
39. Fishbein MH, Mogren C, Gleason T, Stevens WR. Relationship of hepatic steatosis to adipose tissue distribution in pediatric nonalcoholic fatty liver disease. J Pediatr Gastroenterol Nutr 2006;42:83-8.
40. Cree MG, Newcomer BR, Katsanos CS, et al. Intramuscular and liver triglycerides are increased in the elderly. J Clin Endocrinol Metab 2004;89:3864-71.
41. Perseghin G, Scifo P, De Cobelli F, et al. Intramyocellular triglyceride content is a determinant of in vivo insulin resistance in humans: a 1H-13C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents. Diabetes 1999;48:1600-6.
42. Westerterp KR. Physical activity assessment with accelerometers. Int J Obes Relat Metab Disord 1999;23 Suppl 3:S45-9.
43. Cree-Green M, West A, Brown M, et al. Assessing muscle mitochondrial function in children with 31P spectroscopy during exercise. Medicine and Science in Sports and Exercise 2014;In Press.
44. van den Boogaart A. MRUI MANUAL V. 96.3. A user's guide to the Magnetic Resonance User Interface Software Package. Delft: Delft Technical University Press; 1997.
45. Klose U. In vivo proton spectroscopy in presence of eddy currents. Magnetic Resonance in Medicine 1990;14:26-30.
46. Rico-Sanz J, Thomas EL, Jenkinson G, Mierisova S, Iles R, Bell JD. Diversity in levels of intracellular total creatine and triglycerides in human skeletal muscles observed by 1H-MRS. J Appl Physiol 1999;87:2068-72.

47. *Van Pelt RE*, *Gozansky WS*, *Hickner RH*, *Schwartz RS*, *Kohrt WM*. **Acute Modulation of Adipose Tissue Lipolysis by Intravenous Estrogens**. OBESITY 2006;14:2163-72.
48. DeFronzo RA, Simonson D, Ferrannini E. Hepatic and peripheral insulin resistance: a common feature of type 2 (non-insulin-dependent) and type 1 (insulin-dependent) diabetes mellitus. Diabetologia 1982;23:313-9.
49. Aarsland A, Wolfe RR. Hepatic secretion of VLDL fatty acids during stimulated lipogenesis in men. J Lipid Res 1998;39:1280-6.
50. **Gilker CD**, **Pesola GR**, **Matthews DE**. A mass spectrometric method for measuring glycerol levels and enrichments in plasma using $_{13}$C and $_{2}$H stable isotopic tracers. *Anal Biochem* 1992;205:172-8.
51. Wolfe RR, Chinkes DL. Isotope Tracers in Metabolic Research. Principles and Pracetice of Kinetic Analysis. Hoboken, NJ.. Wiley-Liss; 2005.
52. Schwarz JM, Neese RA, Turner S, Dare D, Hellerstein MK. Short-term alterations in carbohydrate energy intake in humans. Striking effects on hepatic glucose production, de novo lipogenesis, lipolysis, and whole-body fuel selection. J Clin Invest 1995;96:2735-43.
53. Bacha F, Pyle L, Nadeau K, et al. Determinants of glycemic control in youth with type 2 diabetes at randomization in the TODAY study. Pediatr Diabetes 2012.
54. Cree MG, Fram RY, Barr D, Chinkes D, Wolfe RR, Herndon DN. Insulin resistance, secretion and breakdown are increased 9 months following severe burn injury. Burns 2009;35:63-9.
55. Cree MG, Newcomer BR, Read LK, et al. Plasma triglycerides are not related to tissue lipids and insulin sensitivity in elderly following PPAR-alpha agonist treatment. Mech Ageing Dev 2007;128:558-65.
56. Dalla Man C, Caumo A, Basu R, Rizza R, Toffolo G, Cobelli C. Measurement of selective effect of insulin on glucose disposal from labeled glucose oral test minimal model. Am J Physiol Endocrinol Metab 2005;289:E909-14.
57. Carey PE, Gerrard J, Cline GW, et al. Acute inhibition of lipolysis does not affect postprandial suppression of endogenous glucose production. Am J Physiol Endocrinol Metab 2005;289:E941-7.
58. Steele R. Influences of glucose loading and of injected insulin on hepatic glucose output. *Ann NY Acad Sci* 1959;82:420-30.
59. Winkler B, Steele R, Altszuler N. Relationship of glycerol uptake to plasma glycerol concentration in the normal dog. Am J Physiol 1969;216:191-6.
60. Dalla Man C, Camilleri M, Cobelli C. A system model of oral glucose absorption: validation on gold standard data. IEEE transactions on bio-medical engineering 2006;53:2472-8.
61. Dalla Man C, Caumo A, Cobelli C. The oral glucose minimal model: estimation of insulin sensitivity from a meal test. IEEE transactions on bio-medical engineering 2002;49:419-29.
62. Wolfe R, Chinkes D. Isotope Tracers in Metabolic Research. 2nd ed: Wiley-Liss; 2005.
63. Basu R, Breda E, Oberg AL, et al. Mechanisms of the age-associated deterioration in glucose tolerance: contribution of alterations in insulin secretion, action, and clearance. Diabetes 2003;52:1738-48.
64. Basu R, Dalla Man C, Campioni M, et al. Effects of age and sex on postprandial glucose metabolism: differences in glucose turnover, insulin secretion, insulin action, and hepatic insulin extraction. Diabetes 2006;55:2001-14.
65. Faerch K, Pacini G, Nolan JJ, Hansen T, Tura A, Vistisen D. Impact of glucose tolerance status, sex, and body size on glucose absorption patterns during OGTTs. Diabetes Care 2013;36:3691-7.