Title: Post-Prandial Liver Glucose Metabolism in Polycystic Ovarian Syndrome (PCOS) and understanding Standard of Care Medications

Version Date: 8/17/2017

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COMIRB Protocol

COLORADO MULTIPLE INSTITUTIONAL REVIEW BOARD
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Project Title: Post-Prandial Liver Glucose Metabolism in Polycystic Ovarian Syndrome (PCOS) and understanding Standard of Care Medications

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Principal Investigator: Melanie Cree Green, MD, PhD

I. Hypotheses and Specific Aims:

HYPOTHESIS:
Obese girls with hepatic steatosis (HS) will have hepatic glucose metabolism that is upregulated towards de novo lipogenesis (DNL) via multiple intrahepatic pathways, compared to PCOS without HS and obese controls without HS. HS will be related to relative post-prandial hyperglycemia. Post-prandial hyperglycemia will be made worse or remain unchanged with oral contraceptives and better with metformin.

Specific Aims:
Aim 1: In obese girls with PCOS, post-prandial hepatic substrate metabolism will differ by HS status and treatment regimen and can be assessed with just an oral tracer protocol combined with NMR isotopmer analysis.
Hypothesis: Girls with HS will have upregulated post-prandial hepatic non-glycogenic metabolism compared to those without HS. This will be unchanged by oral contraceptives, and decreased with metformin.
Rationale: A marker of whole body DNL was increased in girls with HS, and preliminary work from my K23 project indicates that direct measures of DNL are elevated, but upstream alterations in intrahepatic metabolism are unclear. Oral contraceptives do not mitigate metabolic risk, whereas metformin decreases HS.
Methods: A 6.5 hour OGTT with an oral U-C_{13}glycerol tracer will be paired with NMR isotopomer analysis of serum samples to describe flux through the hepatic pentose phosphate pathway, TCA cycle and DNL pathways in girls with PCOS receiving lifestyle only, metformin or oral contraceptive treatment, and obese girls with regular menses receiving lifestyle therapy.

Aim 2: Describe the optimal timing for identifying metabolomics profiles associated with risk for T2D and HS.
Hypothesis: Metabolomics profiles between girls with HS and worse IR will differ the most between 3-6 hours.
Rationale: Altered glucose and fat metabolism is most apparent in the post-prandial state, in terms of insulin resistance, β-cell failure, hyperglycemia and DNL. Our preliminary data indicates that glucose and insulin concentrations are most different between 3-4 hours following a glucose load. The optimal timing post-drink to describe metabolomics differences is not known.
Methods: Targeted metabolomics profiles will be performed at intervals during the OGTT to determine time points that provide the biggest difference in girls with HS, hyperglycemia, inadequate β-cell response and IR. Specific targets will likely be free fatty acid and DNL markers, and will be determined from on ongoing pilot study.

II. Background and Significance:

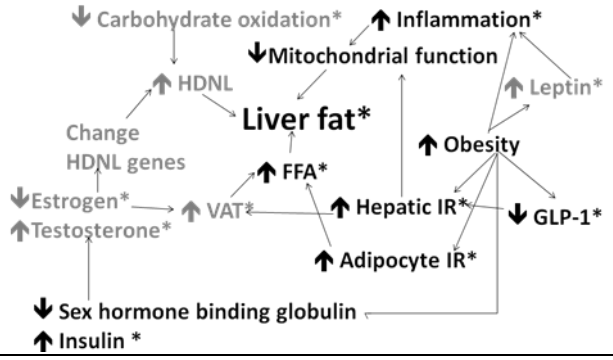
a) Overall background:

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 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. It is possible that in the girls with PCOS and HS, the energy production from the TCA and pentose phosphate pathways are shifter towards DNL, whereas in girls without PCOS they may be shifted towards glycogenolysis. *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.*

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

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. In our current study, we are addressing this by developing an OGTT model of hepatic glucose suppression, lipolysis suppression and peripheral IR, in conjunction with directly measuring DNL. However, this study does not allow for measuring intrahepatic processes and detailed metabolomic analysis. This type of analyses is needed as there are several new medication classes that may be useful in PCOS. *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

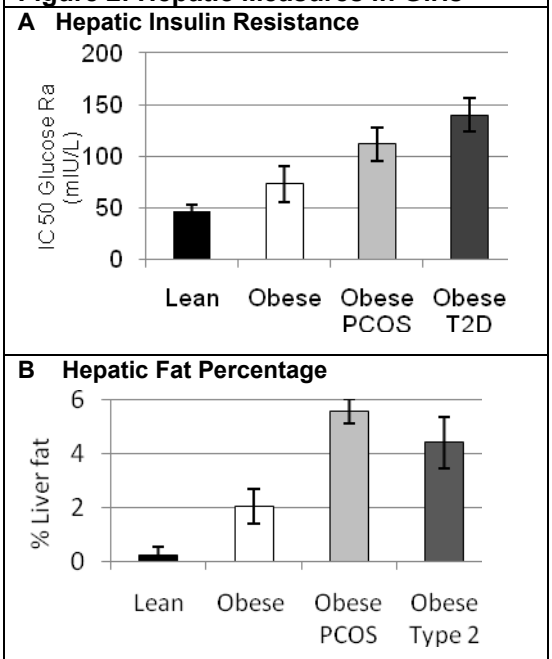


Table 1: Serum Markers in Obese Girls

	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

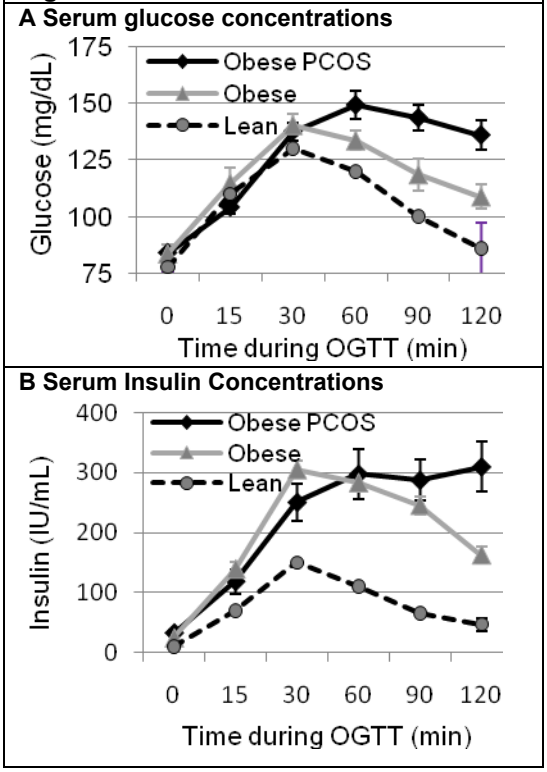
Pilot Data on Liver fat and Insulin Resistance in youth with PCOS: Obese girls with PCOS have moderate adipose, hepatic (Figure 2A) and muscle IR relative to obese controls, but are not as IR as T2D girls. Adipokines such as adiponectin are similarly low in PCOS and T2D girls, whereas leptin is higher 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:

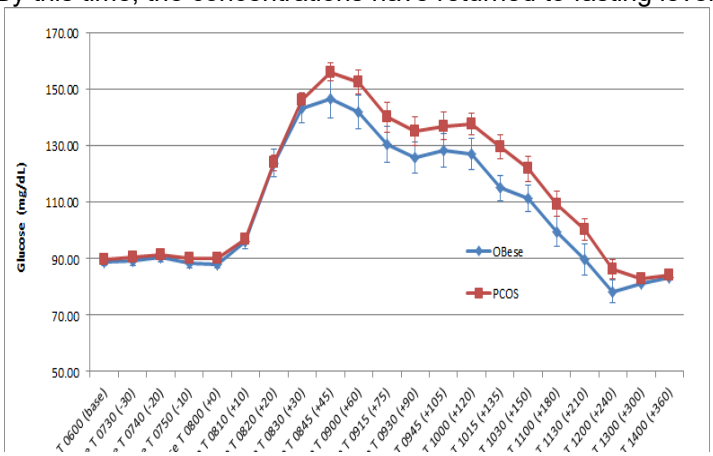
Obese girls with PCOS have high 2 hour oral glucose tolerance test (OGTT) glucose and insulin concentrations (Figure 3 A and B). Of note, the PCOS OGTT insulin concentrations are higher than those achieved during the clamp 245±40IU/ml). Despite the relative hyperinsulinemia, they have poor insulin secretion 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 additional oral models.

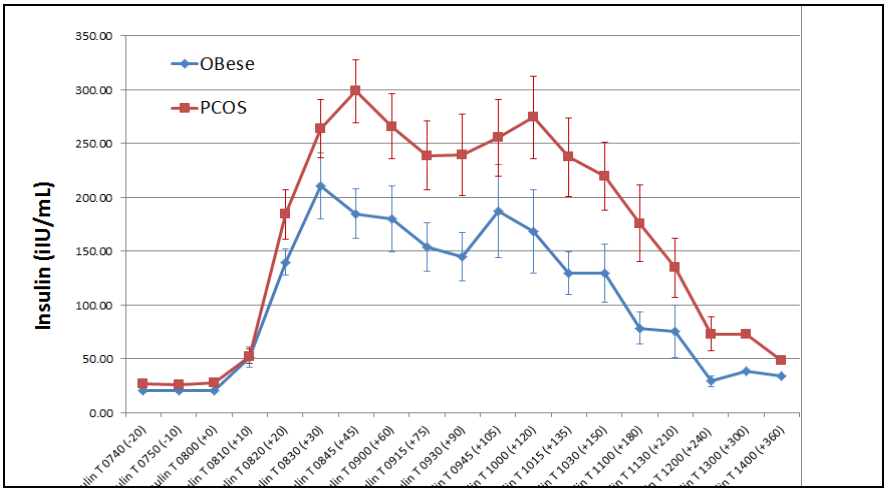
Figure 3: OGTT Plasma Results



Prolonged OGTT in obese girls:

In our current protocol, we initially started with an OGTT with 4 hours of post-prandial monitoring. However, we found that at 4 hours, the insulin and glucose concentrations were still not returned to fasting levels. We thus amended our protocol and are now conducting monitoring out to 6.5 hours. By this time, the concentrations have returned to fasting levels.





Additionally, in the initial work describing this method in healthy lean men, serum metabolite profiles at 4 hours have not yet returned to baseline, and thus especially in our patients we will need to follow their metabolites out to 6.5 hours.

Preliminary data from polysomnograms in girls with PCOS:

We had a pilot study to perform a limited number of inpatient polysomnograms in another of our current protocols, 14-0542, which has identical inclusion criteria. Just analyzed results from 14-0542 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 with protocol 14-0542, 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): Activity ratios of the hepatic pentose phosphate pathway, TCA cycle and TG synthesis.

Secondary Outcome Measure(s): Peripheral IR as assessed with a 6.5 hour OGTT, measures of SNS activity, Hepatic fat content, hepatic phosphate profile. Targeted glucose and fat metabolism metabolomics, and untargeted exploratory analysis, gut microbiota profile.

Likely contributors to above measures: Lipid/glucose markers:(fasting C-peptide and lipid panel, HbA1c); hepatic markers:(c-reactive protein, glucagon, GLP-1 and leptin at baseline, 5 min and 30 min post glucose load to 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[36-39]), Whole body fat oxidation at rest and following glucose ingestion as measured with a metabolic cart; Physical activity/ diet:(accelerometer, activity survey (3DPAR); Food frequency survey).Questionnaires for presence of obstructive sleep apnea. Questionnaires for perceived mental strengths and difficulty (note there is no assessment of suicidality on this tool). Obstructive Sleep Apnea, to be assessed with an overnight sleep study via WatchPAT.

B. Description of Population to be Enrolled:

Study staff aims to enroll 20 obese girls with regular menstrual cycles, 20 girls with un-treated PCOS, 20 girls with PCOS treated with at least 6 months of Metformin and 20 girls with PCOS treated with at least 6 months of oral contraceptives. The PCOS groups will be compared to 20 obese non-PCOS controls. This is the number of participants needed to be completed statistically, thus more subjects may be enrolled, to allow for screen failures and dropouts. Total enrollment will be up to 100 participants.

Ethnic Categories	**Gender**		
	Females	Males	Total
Hispanic or Latino	40	0	**40**
Not Hispanic or Latino	60	0	**60**
Ethnic Categories: Total of All Subjects	**100**	**0**	**100**
Racial Categories			
American Indian/Alaska Native	5	0	**5**
Asian	10	0	**10**

Native Hawaiian or Other Pacific Islander	10	0	**10**
Black or African American	32	0	**32**
White	43	0	**43**
Racial Categories: Total of All Subjects*	**100**	**0**	**100**

Inclusion Criteria:

1) Female

2) Ages 12-21

3) Sedentary- less than 2.5 hours of moderate (jogging, swimming etc) exercise a week.

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

5) 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.

6) For PCOS groups: patients un-treated or currently treated with either Metformin 1500-2000 mg a day or oral contraception (30-35 mcg ethynyl estradiol a day) for at least 6 months, with > 80% adherence confirmed via refill frequency from pharmacy.

7) For non-PCOS groups: regular menstrual cycles at least 1.5 years after menarche and no clinical evidence of hyperandrogenism

Exclusion Criteria:

1. Use of medications known to affect insulin sensitivity: oral glucocorticoids within 10 days, atypical antipsychotics, immunosuppressant agents, HIV medications. Nexplanon, Depo-Provera or Mirena progesterone only contraceptives. Dermal patch or vaginal ring contraception methods. For controls only: metformin or oral contraception.
2. Currently pregnant or breastfeeding women. Development of pregnancy during the study period will necessitate withdrawal from the study.
3. Severe illness requiring hospitalization within 60 days
4. Diabetes, defined as Hemoglobin A1C > 6.4%
5. BMI percentile less than the 90th percentile for age and sex. Weight >325 lbs or <84 lbs.
6. Anemia, defined as Hemoglobin < 10 mg/dL
7. Diagnosed major psychiatric or developmental disorder limiting informed consent
8. Implanted metal devices that are not compatible with MRI
9. Use of blood pressure medications
10. Known liver disease other than NAFLD or AST or ALT >150 mg/mL

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 and Sleep assessments			X
DEXA Scan (located in Leprino building)			X
Oral Glycerol Tracer			X
WatchPAT sleep study			X
EndoPat and Dynapulse		X	
MRI of abdomen and liver,		X	
P MRS of Liver (pending scheduling)		X	
Metabolic Cart			X
Total Time of visit (approximately)	2 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 2 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	Oral Glycerol tracer	DEXA
Untreated PCOS	25	20	20	20	20
PCOS w/ Metformin	25	20	20	20	20
PCOS w/ OCP's	25	20	20	20	20
Obese Control	25	20	20	20	20
Total	100	80	80	80	80

VISIT 1 (SCREEN VISIT)
Pediatric CTRC Outpatient unit: Participants will begin with a medical screening and physical exam for inclusion/exclusion criteria evaluation. During this visit, patients will review and complete consent documents, have demographics and medical history confirmed, assess allergies and further evaluation of inclusion/exclusion criteria, have blood samples drawn, and have anthropometrics completed. HbA1c, ALT, AST, CBC panel and testosterone panel will be drawn at the beginning of the visit after consent in all 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 > 6.4% subject to be excluded
ALT, AST	Ensure no severe liver disease, if >150 IU 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 criteria 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
17-hydroxyprogesterone	Rule out late onset congenital adrenal hyperplasia
DHEAS	Rule out adrenal tumor
Prolactin	Rule out prolactin secreting brain tumor

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 is optional pending scheduling. It 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 and Heart Rate Variability will be assessed with Endopat and Dynapulse, which take approximately 30 minutes.

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

MRS Data acquisition: Imaging and MRS will be performed on a Seimens 2.9 Tesla MRI magnet. A custom ^{1}H/^{31}P abdominal coil will be used for imaging and MRS (Clinical MR Solutions, Brookfield, WI) as in our previous ^{31}P work[40]. 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[38]. 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 25 grams of fructose 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]. Hepatic fibrosis will be measured with a fibroscan sequence.

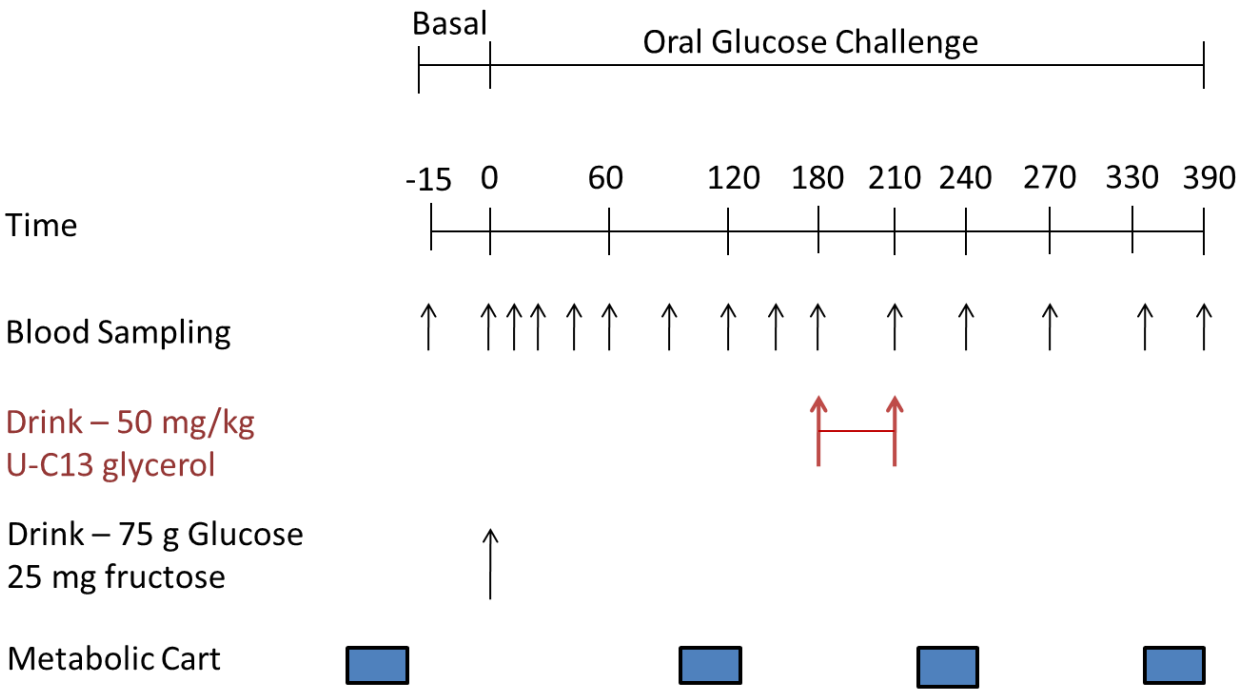
MRS Data Analysis: For the ^{31}P data, peak positions and areas of interest [phosphocreatine (PCr), inorganic free phosphate (Pi), β-ATP(3 peaks), α-ATP(2 peaks), γ-ATP(2 peaks), and PME] will be determined by time domain fitting with jMRUi [41,42], utilizing AMARES (A Method of Accurate, Robust and Efficient Spectral fitting), a nonlinear least-square-fitting algorithm using our previously built prior knowledge files [43]. 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 #'s 10-1288 and 14-0542).

Gut Bacteria Collection: The gut microbiome consists of the microorganisms, predominantly bacteria, that inhabit the gastrointestinal tract and are estimated to outnumber mammalian cells by up to a factor of 10, and their genes outnumber human genes by a factor of over 100 [69]. It is possible that gut microbiota contribute to the development of NAFLD, and this has not been explored in PCOS. We will collect a sample to define the microbiota in girls who are already undergoing extensive metabolic profiling. The gut microbiota will be collected with BBL culture swabs (Becton, Dickinson and Company, Sparks, Maryland) one week prior to visit 3. Fecal samples will be collected from the first bowel movement of any day the week before visit 3 and stored in the freezer. Fecal samples are being collected for present and future research markers in the mircrobiome. Fecal samples are routinely collected in research and pose little risk to 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.

Accelerometer: One week prior to visit 3, the subject will be provided two accelerometers (GT3X BT by Actigraph and ActiWatch by Philips Respironics) to be worn for seven days to measure level of habitual physical activity, which affects insulin sensitivity, and sleep patterns. Accelerometers are effective tools for the objective measurement of physical activity [44] because they have the ability to continuously record physical activity data and such data can be used to estimate METs of activity. They provide more detailed information than pedometers, which only measure walking steps, and help get around the recall bias of questionnaires. We are currently using the GT3X BT Actigraph in adolescents in our other diabetes studies; therefore, we are familiar with their use in this population and have the necessary computer software and interpretation skills. The Actiwatch is being used as a tool for objective measurement of sleep patterns. The Actiwatch is fitted with a LED monitor that detects multiple spectrums of light to better assess sleep patterns in this population.

One week prior to visit 3, participant will receive via mail the accelerometer, sleep diary and stool sample collection kit (Gut bacteria collection)

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. The labelled glycerol is given anywhere from 3 to 3.5 hours after the glucose load, as hepatic glucose output needs to be high enough to detect the incorporated carbon from the glycerol.

VISIT 3 (ORAL GLUCOSE TOLERANCE TEST WITH ORAL GLYCEROL TRACER)

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. Subjects will be questioned regarding changes in concomitant medications and medical history, height, weight, BP.

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 care physician for follow-up.

The following morning, a blood sample for baseline metabolic labs will be drawn the modified OGTT will be completed. DNA extract-and-hold samples for future genetic analysis associated with hepatic steatosis will also be drawn and stored at this time. Additional blood will be drawn and stored for future inflammatory markers as well as for targeted and untargeted metabolomics analysis.

WatchPAT: During the hospital overnight stay, trained study staff will place the WatchPAT sleep monitor. The primary measures will be for oxygen saturation and apnea hypopnea index (AHI). Each participant will wear the watch with a one-time use finger cuff as recommended by the FDA..The watch will be placed by 8 PM, and will be removed the following morning.

Details of Stable Glycerol Isotope Tracer studies with an OGTT: After the overnight fast at approximately 730AM, subjects will consume a 75 grams glucose load with an additional 25 grams of fructose to stimulate hepatic de novo lipogenesis. An 3 hours later, following consumption of the glucola/fructose mixture, 50 mg/kg of an oral $^{U}C_{13}$-glycerol tracer will be consumed mixed with water. Analysis of serum glucose isotopomers will be performed by our collaborator Craig Malloy at UT Southwestern, using NMR isopomomer analysis and pathway modeling software. Lipolysis will be modeled using FFA concentrations and whole body IR by a time modified version of the Matsuda model.

Using a metabolic cart and hood, resting VO2 (ml/kg/min) and VCO2 (ml/kg/min) measurements (REE) will be collected the morning of the OGTT prior to the start of the OGTT, as well a, 30 min after the start of the OGTT, 3 hours and 30 min after the start of the OGTT and 5 hours and 30 min after the start of the OGTT. This is required to determine what portion of ingested carbohydrates are subject to oxidative and non-oxidative glucose disposal[45].

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 2 meals of 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 [46].

Purpose for lab test to be drawn:

OGTT Labs	Purpose
Lipids and glycerol samples for tracer analysis	Determination of hepatic metabolism flux
Glucose	Determination of IR
Insulin	Determination IR
FFA	Measure of lipolysis

Glycerol	Measure of lipolysis
Glucagon	Gut hormone known to influence hepatic IR
GLP-1	Gut hormone known to influence hepatic IR
CRP	Marker of inflammation, know to effect IR
Leptin	Gut hormone known to influence hepatic IR
Adiponectin	Adipokine thought to influence adipose IR
Estradiol	Known to effect IR and HDNL
Progesterone	Demonstrate the subject is in the follicular phase of cycle, required for publication
Metabolomics	Correlation with hepatic metabolism flux
1 sample for genetic analysis	Measure for common polymorphisms associated with hepatic steatosis[47,48]
Stored blood	Future markers of glucose and fat metabolism, CVD or hormones related to PCOS

Purpose for questionnaires being done:

Questionnaire	**Description**	**Study Validity**
Adolescent Sleep Hygiene Scale	Measurement of sleep patterns/habits	Cronbach's alpha ranges from .46-.74; total scale alpha = .80 LeBourgeois et al. 2005. *The Relationship Between Reported Sleep Quality and Sleep Hygiene in Italian and American Adolescents*
Center for Epidemiological Studies Depression (CES-D)	Measuring for depression	See Table 3 below, adapted from: Stockings et al. 2015. *Symptom screening scales for detecting major depressive disorder in children and adolescents: A systematic review and meta-analysis of reliability validity and diagnostic utility*
Cleveland Adolescent Sleepiness Questionnaire	Measurement of sleepiness during a typical week	alpha = .89 Spilsbury et al. 2007. *The Cleveland Adolescent Sleepiness Questionnaire: A New Measure to Assess Excessive Daytime Sleepiness in Adolescents*
SDQ: Strengths and Difficulties Questionnaire	Behavioral screening questionnaire	The internal reliability of the various self report scales was assessed using Cronbach's alpha coefficient. This was 0.82 for the total difficulties, 0.75 for emotional symptoms, 0.72 for conduct problems, 0.69 for hyperactivity, 0.65 for prosocial behaviour, and 0.61 for peer problems. Goodman et al. 2003. *The Strengths and Difficulties Questionnaire: a pilot study on*

		the validity of the self-report version
Sleep Disturbances Scale for Children	Gain understanding of sleep-wake rhythm and any problems in sleep behavior	Internal consistency ranged from .71-.79l test-retest reliability r = .71 Bruni et al. 1996. *The Sleep Disturbance Scale for Children (SDSC)Construct ion and validation of an instrument to evaluate sleep disturbances in childhood and adolescence*
3DPAR: Activities Scale	Measuring activity in the 3 days previous as a typical activity score	Interrater and test-retest reliability was 0.99 and 0.98, respectively (P < 0.01). The correlation between relative energy expenditure from the PDPAR (kcal.kg-1.l.d-1) and pedometer and Caltrac counts was 0.88 (P < 0.01) and 0.77 (P < 0.01), respectively. The correlation between percentage heart rate range (HRmax-HR-rest) and mean energy expenditure from the PDPAR was 0.53 (P < 0.01). The correlation between 1-min heart rates > 50% HRR sustained for 20 min and the number of 30-min blocks with a relative energy expenditure of at least four metabolic equivalent tasks (MET) was 0.63 (P < 0.01). The PDPAR provides valid and reliable estimates of physical activity and also accurately identifies bouts of moderate to vigorous activity. Weston et al. 1997 *Validation of an instrument for measurement of physical activity in youth*
Food Frequency Questionnaire	Measuring typical food intake over previous seven days.	The mean correlations, adjusted for measurement error, of food groups and nutrients between the FFQ and true usual intake were 0·41 and 0·38, respectively, with 57 % of food groups and 70 % of nutrients exhibiting correlations >0·35. Correlations were high for low-fat dairy (0·80), sugar-sweetened beverages (0·54), cholesterol (0·59) and saturated fat (0·51), while correlations were poor for high-fibre bread and cereal (0·16) and folate (0·11). Reliability of FFQ intake based on two FFQ administrations was also reasonable, with 54 % of Pearson correlation coefficients ≥0·5. Reliability was high for low-fat dairy (0·7), vegetables (0·6), carbohydrates, fibre, folate and vitamin C (all 0·5), but less than desirable for low-fat poultry and high-fibre bread, cereal, rice and pasta (0·2-0·3). Liese et al. 2015 *Relative validity and*

		reliability of an FFQ in youth with type 1 diabetes First described in 2006: Mayer-Davis et al. 2006. *Search FFQ Dietary Intake among Youth with Diabetes: The SEARCH for Diabetes in Youth Study*
Optional 7 Day Food Log	Optional record of 7-day diet to aid in food recall. Added per participant request to help fill out food frequency questionnaire.	N/A
Actigraphy Daily Sleep Diary	Recording bedtime/wake time during actigraphy. Needed to corroborate watch collected data	N/A

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

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

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

LIFESTYLE PRESCRIPTION

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. 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 and 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. A results letter will be provided to the family about clinically relevant results obtained during the study. Participants will be encouraged to contact study staff with questions regarding the results letter.

Follow-up from Sleep study results:

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

Pediatrics:

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

Adults

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

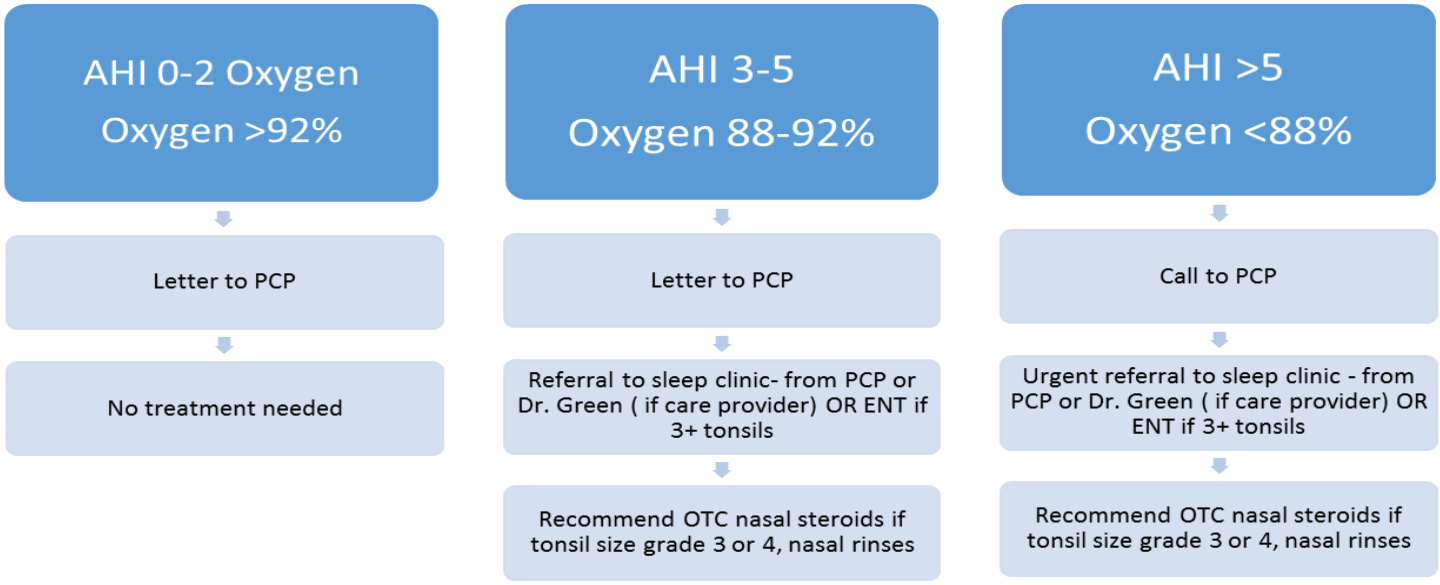
A. Ages for Which Pediatric Respiratory Scoring Rules Apply

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

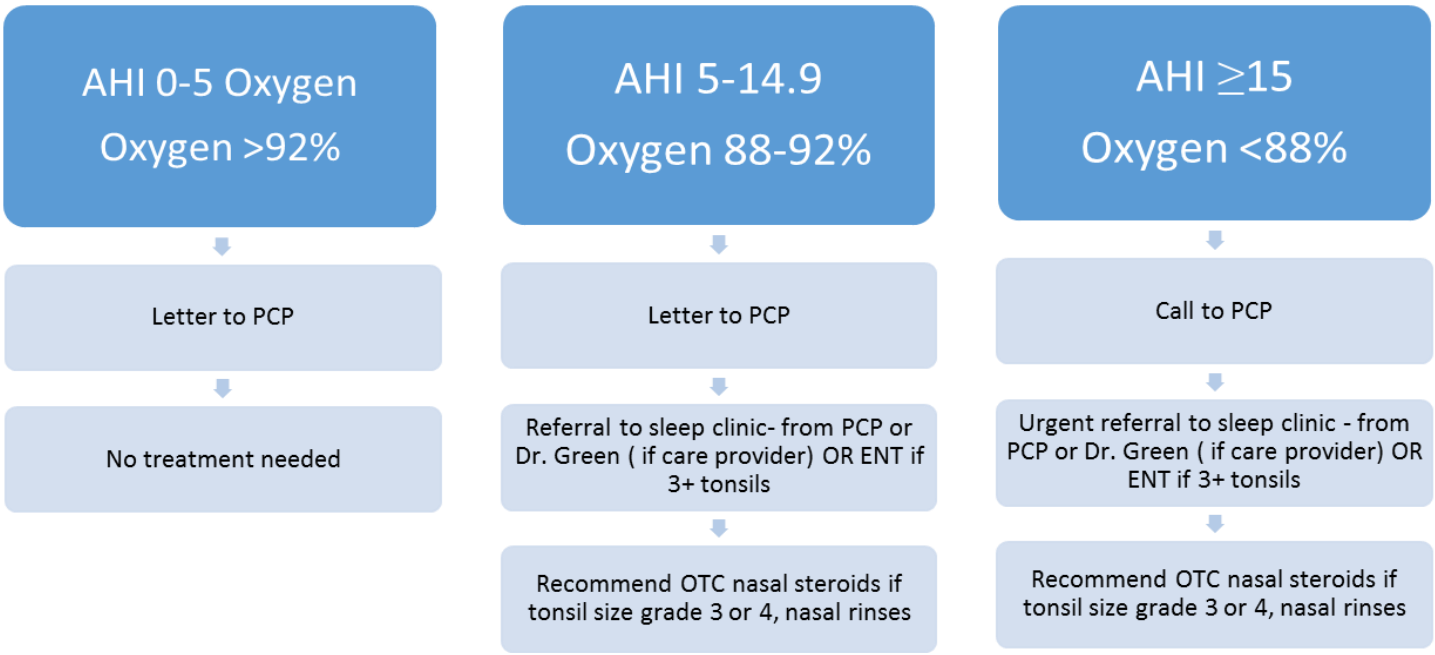
Note 1. Several studies suggest that the apnea hypopnea index (AHI) will be higher in adolescent patients when using pediatric compared to the adult rules presented in the 2007 version of the AASM scoring manual. As adult hypopnea rule 1A and pediatric hypopnea rules are similar, there may now be less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

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

For participants 12-17 years of age:



For participants ≥18 years of age:



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 60 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. Our past studies have consented participants and asked if they'd like to be re-contacted for future research studies. Furthermore, after the conclusion of our previous studies in PCOS (this protocol, 14-0542 and 10-1288), participants may have been prescribed treatment with OCP or Metformin, therefore study staff may aim to re-contact them had those participants agreed to be re-contacted and still meet inclusion criteria of the current study.

2. **Informed Consent Plan**

Appropriately qualified and informed personnel who have completed the COMIRB and HIPPAA course requirements will fully explain the study protocol and consent form to the 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 consent form will also be translated into Spanish. The qualified personnel mentioned above will then obtain written consent from the guardian and assent from the 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 and lab draw and the 2nd for the MRI will result in a $50 gift card each. The 3rd and final visit consisting of the overnight portion and questionnaires will reward a $150 gift card. Compensation for all completed visits will total $250.

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

1. Blood Sampling

Description: Blood will be drawn for Complete Blood Count (CBC), 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 5 ml/kg over a 4 week period. Our baseline visit will include 11.5 ml of blood (HbA1c, Hb, Cr, AST, ALT) and 25.5 ml of blood for PCOS patients (additional draw for T4, TSH, prolactin, LH, FSH, 17OH progesterone and DHEAS). The OGTT visit includes up to 300 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 9 ml/kg in 6-8 weeks and within Children's Hospital Colorado's institutional guidelines of 5 ml/kg. In addition, by study design, 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. 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: One peripheral IV will be placed during the OGTT for drawing blood samples.
Risk: There is temporary discomfort when the needle goes in and 10% of the time there is a small amount of bleeding under the skin that may produce a bruise. Rarely, there is a risk of a blood clot forming or infection. We will use a low dose of a medication called heparin to try to prevent blood clotting.
Justification/Minimization: These studies involve sampling blood at multiple time points. Thus, an IV is needed, so as to avoid multiple needle sticks. These studies are focused on measured rates of change which necessitates the sampling of the same test over time. Proper sterile technique will be used with blood draws and IV placement to decrease the infection risk. EMLA cream will be used if subject desires to minimize pain of IV.

3. Oral Glucose Tolerance Test (OGTT):
Description: An OGTT will be performed with multiple blood draws over 6.5 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+fructose 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[49]. 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: Oral stable isotope tracer of glycerol will be utilized to determine rates of intrahepatic substrate flux. These are substances normally present or produced in the body, and thus pose no more risk than typical glucose infusions. Measurements of these metabolic processes are only able to be made with the utilization of stable isotope tracers.
Risk: We are utilizing an isotope which already exists in all humans, but are simply increasing the percentage. We are only giving this medication orally. These are NOT radioactive substances.
Justification/Minimization: Sterile and pyrogen-free $^{2}C_{13}$ glycerol will be obtained form the manufacturer and delivered to CHCO IDS. The IDS pharmacist will deliver the tracer to the 9th floor inpatient CTRC once ordered by the physician.

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. (Pending scheduling)

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. One-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. 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 [46].

7. Magnetic Resonance Imaging (MRI)
Description: The MRI will usually be obtained the day of admission to CHC), at the UCD Brain Imaging Center on the Fitzsimmons campus. A trained research radiographer who is supervised by Dr. Mark Brown, of UCD radiology, will perform an abdominal MRI to obtain hepatic, visceral and subcutaneous fat on a 3.0 T whole–body MRI scanner (Siemens MAGNETROM, Malverne, PA). Subjects will lie supine while these measurements are obtained, need to hold reasonably still during the scan and cannot weigh >325 lbs. A second sequence to measure the amount of fibrosis (if any present) in the liver will be performed. 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 that is not cleared by the MRI technician may not be able to have the MRI due to the type of magnet involved.
Justification/Minimization: MRI is a non-invasive and non-radiation method to assess body fat, and mitochondrial function. The risks are minimized by assuring patient comfort prior to starting the scan, placing eye goggle that plays movies on the 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 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. Measurement of heart rate variability for autonomic tone will be performed using the Endopat
Risk: This procedure may lead to mild discomfort due to the blood pressure cuff being inflated.
Justification/Minimization: Endothelial function is a novel measure in PCOS and will aid in the determination of cardiovascular dysfunction with this population.

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

11: Accelerometer:
Description subject will be provided two accelerometers (GT3X BT by Actigraph and ActiWatch by Philips Respironics) to be worn for seven days to measure level of habitual physical activity, which affects insulin sensitivity, and sleep patterns. The accelerometers will be worn on the participants wrist. The wrist position has been validated to hip position actigraphy in this population.
Risk: There is no risk involved with the accelerometer.
Justification/Minimization: Accelerometers are effective tools for the objective measurement of physical activity [44] because they have the ability to continuously record physical activity data and such data can be used to estimate METs of activity. They provide more detailed information than pedometers, which only measure walking steps, and help get around the recall bias of questionnaires. We are currently using the GT3X BT Actigraph in adolescents in our other diabetes studies; therefore, we are familiar with their use in this population and have the necessary computer software and interpretation skills. The Actiwatch is being used as a tool for objective measurement of sleep patterns. The Actiwatch is fitted with a LED monitor that records multiple spectrums of light to better assess sleep patterns in this population.

12: Metabolic Cart:
Description: The metabolic cart measures the amount of air that the 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 clear plastic 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. Furthermore, 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 visit 3. screening.
Risk: None
Justification/Minimization: Physical activity can directly affect insulin sensitivity, our primary outcome measure. The 3DPar is a well validated measure to asses 3 days of physical activity in youth, and includes a variety of youth centric activities.

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

16. WatchPAT and Questionnaires to assess for Obstructive Sleep Apnea
Description: The WatchPAT is a noninvasive portable system that measures the oxygen saturation and apnea hypopnea index. Three surveys querying signs and symptoms of obstructive sleep apnea.
Risk: No risk associated with the questionnaires and the WathchPAT,other than a mild discomfort from having to wear the watch and cuff around finger during sleep. It is possible that we will discover that the participant has obstructive sleep apnea, and will need to be referred for further clinical care.
Justification: Obstructive sleep apnea is associated with obesity, and can worsen both fatty liver and insulin resistance. Thus the presence of OSA must be accounted for when measuring either of these outcomes. These surveys selected are currently being utilized by the NIH multiple center study in obese youth at risk for diabetes, and are well validated in youth from multi-ethnic populations. If OSA is suspected during the course of the screen, the 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: 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 diseases.
Justification: Studies in obese individuals with type 2 diabetes have alterations in the gut microbiota that may be related to NAFLD. These studies have not been performed in PCOS.

18. Violation of Privacy and Loss of Confidentiality
Description: These are both risks to which research participants are exposed. The possibility of these risks increases when protected health information is collected. Every effort will be made to decrease this risk by limiting access to protected health information, storing this information in a password protected database, and identifying 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. At the screening visit, we will obtain specific consent to contact the subjects pharmacy to verify home medication (metformin or OCP only) adherence. This contact increases the risk of loss of privacy and confidentiality.
Justification/Minimization: The best way to externally assure medication adherence for home medications is by verifying that the medications have been refilled at an expected interval. Every

effort will be made to decrease the risk of loss of confidentiality by limiting access to protected health information, storing this information in a password protected database, and de-identifying study specimens.

E. Benefits of the study:

Benefits to Society:

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

Knowledge to be gained:

A better understanding of how NAFLD develops in girls with PCOS could lead to more effective treatment strategies. This understanding could ameliorate development of diabetes and heart disease for these girls as they become adults, and may also help with many of the health and social difficulties teens with PCOS experience. Since PCOS is one of the most common endocrine diseases in the US female population, improving PCOS care could have major health implications. The data to be generated from this project will be utilized to inform R01 grant applications of treatment trials with new medications. We will also begin to learn the effect of the two 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 related protocols (10-1288 and 14-0542) and received this counseling have higher rates of weight loss upon clinical follow-up than those children being seen in obesity clinics alone.

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 signs of obstructive sleep apnea, they will be referred to the sleep clinic for appropriate evaluation and treatment.

2) All of the subjects enrolled must be sedentary, with less than 2.5 hours a week of physical activity. This is less than the time recommended by the US Preventive Task Force and the American College of Sports Medicine for this age group. This lifestyle puts them at risk for several diseases including diabetes and cardiac disease later in life, even if they don't have evidence of disease at this time. At the end of the study, all subjects will receive counseling on how to increase their activity levels by trained study staff. Increased activity has been shown to reverse the risk for diseases later in life.

3) All 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 may also be re-contacted once by phone call to follow-up on their study results and exercise recommendations. They may also be followed up in a clinical setting if PI notes further benefit of clinical follow up. Sedentary 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 or fibrosis. The blood tests done for screening can detect alterations in blood glucose, pubertal sex hormones, as well as abnormalities in fasting lipids. The risk of all of these endpoints is increased in sedentary 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 2.5 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.

4) 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 available is scarce in this age group and it is critical to understand the pathophysiology of PCOS in its developing stages.

IV. Potential Scientific Problems:

Limitation of Method Development:

The protocol as described includes the entire subject set, which allow for comparisons between obese girls with and without PCOS. OGTT with an oral tracer will be conducted in all subjects. The specific points that are to be evaluated are listed below:

1) OGTT with glycerol tracers
   a. Purpose: Develop an oral stimulus model to assess hepatic substrate flux
   i. Question 1. Is 6.5 hours post oral load long enough to model hepatic and adipose insulin sensitivity?
      1. Rationale: In our current study (14-0542), we have found that 6 hours are required for metabolites to return to normal, we do not anticipate this to change greatly with the addition of the glycerol tracer.
      2. Measurement. Serum glucose and insulin will be measured post-glucose load for 6.5 hours in the first 6 subjects. If 6.5 hours post glucose values are within 20 mg/dL for glucose and 20 IU/mL for insulin relative to baseline values, 6.5 hours will be considered sufficient.

2) Measurement of Hepatic Substrate flux in youth.
   a. Purpose: Optimize a model to asses hepatic substrate flux in youth looking at shifts from pentose phosphate pathway (PEP), TCA cycle and fatty acid synthesis
   i. Rational: utilizing and oral glycerol model with NMR isotopmer analysis is a newer technique. It has not been validated in youth.
   ii. Measurement: NMR isotopomer analysis with pathway flux calculations and modeling
   iii. Revision plan if model not correct: 1) increase the amount of tracer given 2) increase the frequency of blood draws in the later sampling period.

Overall Project Limitations:

1) 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 and PCOS 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 (Redcap).

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

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

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

VI. Data and Safety Monitoring Plan

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

No protected health information will be collected until the appropriate HIPAA forms are completed. The protected health information that will be collected will include: Name and phone number, demographic information (DOB, sex, ethnicity, address, etc.), diagnosis (es), history and physical, laboratory or tissue studies, radiology studies, procedure results, survey/questionnaire results, research visit records, and portions of previous Medical Records that are relevant to this study. This information will be accessible only by the study investigators, Federal agencies overseeing human 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 OGTT is a standard procedure used in a large number of research studies and settings. Adverse events are uncommon when the procedure is done by experienced personnel in an appropriate setting.

1. Adverse Event Definition

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

a) Adverse Event Reporting

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

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

B. Serious Adverse Events (SAE)

1. Serious Adverse Event Definition

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

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

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

- Infection related to blood draw or IV placement

a) Serious Adverse Event Reporting

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

C. 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 >150 IU, HbA1C>6.4%, Hg <10 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 study staff prior to continuing study visits with subjects. The PI and RSA will consult about the significance of the SAE's and make a recommendation about subject continuation in the study.

VII. Data Analysis Plan:

Overall Project Statistical Plan:

Power calculation: The primary outcome is hepatic substrate flux pathway percentages measured as PEP, TCA and TG synthesis pathways. Power analysis is based on publications utilizing similar methods, with the Primary Aim of understanding differences in hepatic metabolism in obese girls with PCOS who either have or do not have hepatic steatosis. The reference manuscript included 13 men without HS, and 6 men with HS[50]. They found that during hyper-insulinemia, glycerol related gluconeogenesis was increased, as was the PEP cycle in individuals with HS compared to those without. Utilizing the PEP value, from this paper, the difference between the group means is 3 µmol.min.kg FFM, and the standard deviation is 2 µmol.min.kg FFM. 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. With 15 untreated PCOS with HS and 15 untreated PCOS without HS, we would have a power of 0.97 to detect an alpha of 0.05. Since the reference population is men who have more uniform hormone profiles, I am choosing to be overpowered should the variability in our population be greater. As the measurement is in kg of fat free mass, we will not have to do adjustments with BMI or weight. However, as African Americans are less likely to have HS, and Hispanics more likely to have HS, if ethnicity percentages are different between groups, we will need to adjust for ethnicity in our calculations.

Secondary Aims: a) For comparison of the PCOS to control groups, individuals will be matched in terms of HS status, so that the comparison is just between the hormonal state. I am assuming that there will be less of a difference between these groups, so with a difference of 2 Umol.min.kg FFM, and the standard deviation is 2, with 20 PCOS and 20 controls, we would have a power of 0.87 to detect a alpha of 0.05. b) For comparison of treatment with either metformin or OCP's, we will compare the untreated group to the treatments individually. We will not compare the two treatments to each other. I am assuming that there will be less of a difference between these groups, so with a difference of 2 Umol.min.kg FFM, and the standard deviation is 2, with 30 PCOS and 15 in a treatment group, we would have a power of 0.87 to detect a alpha of 0.05.

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

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

Non-Isotope OGTT calculations: The Matsuda model of IR will be used but adapted for the 6.5 hour OGTT, and B-cell function [insulinogenic index (ΔI30/ΔG30) and (ΔC30/ΔG30) and disposition index (1/IFasting × ΔI30/ΔG30) and (1/CFasting ×ΔC30/ΔG30) will be calculated[38,49,51,52].

Differences between groups will be evaluated with and without controlling for activity and sleep status.

Fitting glucose and glycerol concentrations curves: Using computed data points, glycerol and glucose concentrations will be fitted with an appropriate model. In control subjects, concentration typically shows 3 phases in OGTT [53]. Cubic polynomial, piecewise-linear, spline, and dynamic models have been used for fitting Ra in previous work[54,55]. We will assume a cubic polynomial model; we will validate estimates obtained with the cubic polynomial model by computing comparable estimates using at least one other model form. In previous work comparing different models (piecewise-linear, cubic polynomial, dynamic), results have been relatively unaffected by choice of model. If we find that this is not the case for our data, then we would certainly consider the advantages of adopting a more complex approach, such as the state-space, for describing concentration changes.

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

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

VIII. Summarize Knowledge to be Gained:

Overall Expected Results*:* We anticipate that we will determine which specific areas of hepatic metabolism are upregulated in girls with hepatic steatosis. This data would then provide the background evidence for future therapeutic trials. We will also refine this protocol and identify if metabolomics can be utilized to measure this type of hepatic energy flux.

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 protocols. The majority of NAFLD studies have focused on males, and none have been performed in multi-race cohorts of girls with PCOS, despite this high prevalence. NAFLD has been described as the primary driver of worsening metabolic syndrome and CVD in obesity across populations, and can progress to cirrhosis and liver failure[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.

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