Study Title: Bioenergetics and Metabolism in Pediatric Populations

Sponsor: ABI Discovery Acceleration Initiative and NIH Grants

Principal Investigator: Eugenia Carvalho, Ph.D.
UAMS Department of Geriatrics
Arkansas Children's Research Institute
13 Children's Way, Slot 317
Little Rock, AR 72205
Office phone (8 am-5 pm): (501) 364-3057
Email: ECarvalho@uams.edu

Co-Investigator: Shannon Rose, Ph.D.
UAMS Department of Pediatrics
Arkansas Children's Research Institute

Study locations: Arkansas Children's
1 Children's Way
Little Rock, AR 72202

Background and Rationale

This research aims to better understand bioenergetics and metabolism in childhood obesity and type 2 diabetes (T2D). Obesity is a chronic condition that can lead to several serious health conditions. The prevalence of obesity in U.S. children has increased dramatically over the past several decades, and as a result, T2D is being diagnosed in a growing number of young children[1]. Oxidative stress and mitochondrial dysfunction have been implicated in obesity and the development of insulin resistance in adults[2-5]. However, the exact molecular mechanisms and their contribution to T2D development are not well understood and have been understudied in children. A better understanding of the pathophysiology of obesity and the development of insulin resistance in children is imperative for the development of effective prevention and treatment strategies, to prevent the progression into more serious health concerns and to improve the lives of children.

An imbalance between cellular antioxidant defenses and endogenous reactive oxygen species (ROS) production results in oxidative stress. Numerous markers of oxidative stress are altered in obese children and adolescents[2, 6-10]. Glutathione (GSH) is the major intracellular antioxidant/redox buffer against macromolecular oxidative damage, while cysteine, the rate limiting amino acid for GSH synthesis, and the oxidized form, cystine, serves as the major redox couple in the plasma for circulating immune cells. Limited evidence indicates reduced GSH antioxidant/redox capacity in obese adults[11-14]. Mitochondrial function in skeletal muscle is essential for insulin-stimulated glucose uptake and fatty acid metabolism. In adults, studies of muscle mitochondrial function have shown decreased activities in key enzymes[15-21]. In addition, reduced skeletal muscle mitochondria size and numbers have been reported in obese and to a greater extent in T2D adults, and the expression of nuclear encoded ETC genes in skeletal muscle was also decreased[22-24].

While mitochondrial dysfunction is known to play a role in obesity and the development of T2D in adults, a major research gap remains in obese children irrespective of insulin resistance or T2D. Decreased resting energy expenditure (REE) has been shown to predispose to obesity and T2D in adults[25], but relatively little is known about REE in pediatric populations. Reduced or inefficient fatty acid oxidation (FAO) is also associated with obesity and insulin resistance[5, 26]; however, this has not been studied in a pediatric population. Given advances in stable isotope technologies, it is now possible to perform non-invasive FAO studies in children.

Circulating blood cells are an easily obtainable source to examine bioenergetics and would be a preferred non-invasive alternative to collecting tissue biopsies, especially in children. Bioenergetic profiling of circulating cells has been used as a measure of systemic mitochondrial fitness, physical function and inflammation[27-29]. While circulating blood cell bioenergetics has not been compared between lean and obese individuals, peripheral blood monocyte bioenergetics was compared in morbidly obese adults before and after bariatric surgery, with observed post-surgery increase in basal and maximal mitochondrial respiration that was also correlated with increased skeletal muscle maximal respiration[30]. Another recent report indicated that the monocyte and platelet maximal respiratory capacity correlated with the bioenergetics in both skeletal and cardiac muscle[31]. These studies suggests that bioenergetic profiling of circulating cells largely reflects the bioenergetic capacity of other tissues.

The main purpose of the proposed investigation is to fill a critical research gap and gain insight into the pathophysiology of obesity and insulin resistance in childhood by establishing, for the first time, whether obese insulin resistant children exhibit reduced antioxidant/redox capacity and abnormal bioenergetics in circulating blood cells as compared to normal weight and obese insulin sensitive children, and whether REE and FAO are correlated with abnormal redox status and bioenergetics. In addition, not all obese subjects are metabolically unhealthy (MUO) and body mass index (BMI) may not be the best determinant of metabolic health. Fat mass, on the other hand is correlated with insulin resistance, particularly visceral adiposity. Metabolically healthy obese (MHO) have no signs of the metabolic syndrome, (cardiometabolic risk, hypertension and dyslipidemia), including children. Therefore, a repeated measures study at 12 months post baseline on obese children will help establish not only the degree of change in the studied outcomes, but also their predictive value for T2D development [32, 33]. The findings from these studies will lay the foundation needed for future investigations into identifying the cellular and molecular mechanisms of insulin resistance, and the development of a non-invasive biomarker in children at an increased risk of developing insulin resistance.

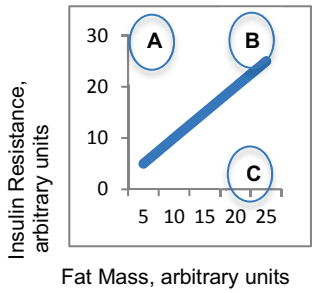

Figure 1: A: Metabolically unhealthy (MU), lipodystrophy; B: MU, obese; C: Metabolically healthy (MH) obese

Hypothesis and Specific Aims

We hypothesize that when compared to normal weight or obese insulin sensitive children, obese insulin resistant children will exhibit altered circulating blood cell bioenergetics and a more oxidized plasma redox state and that these alterations will be associated with decreased resting energy expenditure and impaired fatty acid oxidation (FAO). Furthermore, we hypothesize that poor oxidative capacity over time may distinguish between metabolically healthy obese (MHO) and metabolically unhealthy obese (MUO) phenotypes, as well as be predictive of T2D development. We also hypothesize that bioenergetics, REE and FAO will be improved in obese T2D or insulin resistant children after 6 months of metformin therapy that will be prescribed as part of their clinical care. To test these hypotheses, we will evaluate the following Specific Aims:

Specific Aim 1: Determine whether peripheral blood mononuclear cell (PBMC) and platelet bioenergetics (oxidative phosphorylation, glycolysis and fatty acid oxidation) are impaired and whether plasma glutathione-mediated redox/antioxidant capacity is decreased in obese insulin resistant children as compared to normal weight and obese insulin sensitive children.

Specific Aim 2: Determine whether resting energy expenditure (REE) and fatty acid oxidation (FAO) are decreased in obese insulin resistant as compared to lean and obese insulin sensitive children and whether REE and FAO are correlated with bioenergetic and redox profiles.

Specific Aim 3: Determine whether the bioenergetic oxidative capacity measured in circulating cells, PBMCs/platelets, and FAO are valuable predictive markers of T2D in obese children, in addition to distinguishing MUO from MHO subjects.

Specific Aim 4: Determine whether circulating blood cell bioenergetics and whole-body REE and FAO are modulated by metformin therapy that will be prescribed as part of clinical care in

obese children with T2D or insulin resistance.

Study Design and Procedures

This study consists of:

1. An observational cross sectional bioenergetic and metabolic analysis of several groups of children including lean, obese, and T2D.
2. A longitudinal bioenergetic and metabolic analysis of obese children that will be followed for 12 months, repeated measures will be taken at 12 months post-baseline.
3. A prospective bioenergetic and metabolic analysis of obese insulin resistant children (Aim 3) and newly diagnosed children with T2D or insulin resistance who will be prescribed metformin as part of their clinical care (Aim 4).

A prospective subject and his/her parent will come for the study visit that will last up to 4 hours. A subject (and/or his/her parent) will be asked questions regarding the subject's medical history that will be reviewed by study staff to identify patients who fulfill inclusion/exclusion criteria. In addition, study staff may review medical records from ACH or other healthcare practitioners (such as physicians or pharmacists) outside ACH in order to confirm the inclusion/exclusion criteria. Information from medical records may be collected from the time of consent up until the time that data analysis for this study is complete and results have been published. Study staff will ask parents about demographic information including biologic gender, date of birth, race/ethnicity, and parental occupations as well as grade in school.

Prior to the study visit, study staff may contact the parent and ask for medications that the participant is currently taking and may ask of any new health diagnoses. In the event that the participant is taking medications or has a new health diagnosis that may cause him/her to be ineligible to participate in the study visit, the study visit may be rescheduled or canceled. Study staff may contact a parent and send the IRB approved consent form and information regarding the scheduled visit to the parent(s) prior to the scheduled date. If the consent form has been revised since the last informed consent discussion, the new IRB approved consent form may be sent to parents. If applicable, the parent will be informed of the blood draw and advised that if the parent wishes their child to complete the blood draw at the visit, the child will need to be fasting including no food or liquid after 12 am, except for water and medications the day of the visit.

At the initial study visit eligible participants will be met by study staff at the designated study location. Participants will arrive after an overnight fast of no food or liquid after 12 am, except for water and medications the day of the visit. The study visit may include collection of anthropometric measurements, tanner stage assessment, physical activity questionnaire, depression scale, resting energy expenditure, body composition by bioelectrical impedance, saliva, buccal swab, blood, stool, and urine collection. Attempts to repeat anthropometric measurements, body composition by bioelectrical impedance, and resting energy expenditure will occur if a reading is not given the first time or if the child is unable to comply during the first measurement. The parent and subject will not have to stay at the research location for the entire

visit in order for the samples to be obtained. Instructions will be given to the parents for collection of samples if the parent and child decide not to remain at the research location for the entire visit.

If a procedure(s) (see below) is not successfully completed, the parent and, if warranted, the participant may be asked to return to the study site for an additional visit to re-attempt the procedure. The decision to ask the parent/participant, to return for another attempt will be made by the principal investigator and/or co-investigator. When a decision is made to re-attempt the procedure, study staff will contact the parent and explain what procedure they would like to re-attempt and if the parent is willing, schedule the visit. If a blood draw or fat oxidation estimation will be re-attempted, the parent will be re-advised that if the parent wishes their child to complete the procedure, the child will need to be fasting including no food or liquid after 12 am, except for water and medications the day of the visit. The study visit will be conducted in the same manner as the initial visit, with only the desired procedure(s) being re-attempted.

The collection of stool, saliva, and buccal swab samples is optional, and these samples may not be collected if the subject or parent does not want the sample(s) to be used for future research.

Study Procedures to be conducted at each visit:

1. Intake of Food and Liquid: A parent will be asked by the study staff about the food and drink that the subject eats and drinks the day of and day following the visit (up until the last urine sample is collected for fat oxidation estimation). This is important because the diet will influence the fat oxidation estimation.

2. Anthropometrics: Anthropometric measurements including height, weight, waist circumference, blood pressure, and pulse will be obtained using standardized techniques. BMI-for-age will be calculated and plotted on CDC growth charts to confirm lean/obese stratification.

3. Tanner (Puberty) Stage Assessment: Parents will be asked to complete a form with drawings depicting pubertal development. This form will be used to determine pubertal status. A physician may examine the subject for Tanner Stage (if the parent agrees) during the visit in order to confirm the parental report and for purposes of analyzing study results. The questionnaire given to parents to complete is subjective and a parent could potentially incorrectly assess Tanner Stage. While the inclusion/exclusion criteria prevents the inclusion of those with puberty development, if a parent incorrectly assesses his/her child's Tanner Stage, then this may alter the results or may cause the exclusion of many potential participants based on the parents response, especially in obese participants. Because the results from the samples are reliant on the participants being pre-pubertal with no signs of maturation, conclusions may be incorrectly drawn from the study data if a child has pubertal development. At the beginning stages of puberty, insulin levels rise dramatically showing insulin resistance. Thus, if the study determines the child to have insulin resistance but insulin resistance is actually due to development of puberty instead of being MUO then this

could alter the results and conclusions made from the study. Because of this, it is important to the study and the results to have the correct Tanner Stage level for each participant.

4. Physical Activity Questionnaire (Elementary School): A parent or participant will complete a form indicating the level of physical activity the participant had during the previous week in order to provide general estimates of physical activity levels for each subject during a 7-day period prior to the study. In addition, the parent or participant will be asked about the participant's physical activity from the previous months leading up to the study visit.

5. Depression Scale: With assistance from the parent, a child will complete a form (Center for Epidemiological Studies Depression Scale for Children (CES-DC)) used as a depression scale. This scale will be used to account for depression as a confounding factor that could affect oxidative and bioenergetics parameters.

6. Saliva and Cheek Cells: We will ask each participant if he/she is willing to provide a sample of saliva and have his/her buccal mucosa swabbed. Cheek cells will be collected by study staff using a soft swab to gently swipe the inside of the child's cheek bilaterally for approximately one minute each, per cheek. The swabs and saliva will be labeled with the study ID number of the participant and the study acronym. These samples will remain on ice or in a refrigerator until transported to the PI's laboratory. After the sample has been processed, the sample will be stored at -80°C in the PI's laboratory. This sample will be used for future research studies on pediatric nutrition and may be retained until it is used up. This is optional and is not a requirement for completion of the study visit.

7. Blood Collection: A pediatric nurse or trained phlebotomist will collect blood from the participant under fasted conditions. Depending on the child's weight, up to the maximum allowed per the guidelines provided by Arkansas Children's for the maximum allowable single blood draw volumes will be collected. The volumes are derived using a conservative 3.0 ml/kg estimate as well as a 5% of total body volume estimate. Numbing cream or a pain relieving device (Buzzy) may be used for the blood draw. Blood samples may be used for:
   a. Isolation of PBMC and platelets for bioenergetics analysis using the Seahorse Extracellular Flux (XF)96 Analyzer, protocols for such have been established by Rose and Frye [34, 35]. Platelets and PBMC bioenergetics may be analyzed immediately, or cells may be cryopreserved and analyzed at a later time. Leftover cells may be cryopreserved for repeated bioenergetic profiling.
   b. Analyses of insulin, glucose, lipid levels, and other analytes including, but not limited to, redox metabolites, hormones and inflammatory markers.
   c. Leftover blood samples collected during this study may be stored in the PI's laboratory and used for future research studies on pediatric nutrition.

8. Urine collection: Urine collection may occur at the study location or the sample may be collected at home and brought back to study staff. When possible, attempts will be made to collect the first-morning urine. Urine collection will be attempted in a sterile specimen cup or in a hat and then transferred to a specimen cup. Samples collected during the study visit will be placed on ice, in an ice bath, or in a refrigerator until they can be delivered to the research site laboratory. Samples collected by subjects/parents after the study visit may be stored in a tightly closed specimen cup in a refrigerator until they are able to return to the research site, to occur within 6 weeks of collection. Samples delivered to the research site will be stored in a refrigerator until they can be transferred to the research laboratory and stored at -80°C. This sample will be used for this study and may be used for future research studies involving pediatric nutrition.

9. Stool collection: Stool collection may occur at one of the study locations or the sample may be collected at home and brought back to study staff. Attempts will be made to collect the entire sample using a hat and then transferring it to a sterile specimen cup(s). Samples collected during the study visit will be placed on ice, in an ice bath, or in a freezer until they can be delivered to the research site laboratory. Samples collected by subjects/parents after the study visit may be stored in a tightly closed specimen cup in a freezer until they are able to return to the research site, to occur within 6 weeks of collection. Samples delivered to the research site will be stored in a freezer until they can be transferred to the research laboratory and stored at -80°C. This sample will be used in future research studies on pediatric nutrition. This sample is optional and is not required for the completion of the study visit.

10. Fat oxidation estimation: Fat Oxidation may be measured by administering deuterated palmitate. Breakfast, lunch and snacks may be provided during this visit to the participant to ensure all participants are consistent. After obtaining a baseline urine sample, the stable isotopes will be administered. Follow up urine samples will be obtained up to 3 times within 48 hours following the administration of the stable isotopes. Recommended collection times for the 3 samples will be approximately 2 (± 30 minutes), 6 hours (± 1 hour) post dosing, and the morning following the study visit, depending on the compliance of the child and parent. The urine samples will be obtained to measure the labeling in total body water that may be used to estimate dietary fat oxidation. The stable tracer ($^{2}H_{31}$-palmitic acid) may be administered orally at a dose of 15 mg/kg in a drink and/or mixed with food (i.e. yogurt, etc.). The parents will be asked to collect some of these samples at home. They will be given thorough instructions about how to collect, label, record the time and store these samples. A specimen collection bowl (hat) may be used for collection of urine. The use of these stable isotopes in children to measure fat oxidation has been previously approved (IRB# 202870, 202954, 203770). If a baseline urine sample is not collected, then the stable isotopes will not be administered and the study visit will not be completed.

11. Body composition may be assessed by bioelectrical impedance analysis using the Tanita Body Composition Analyzer. A device (InBody 570 Body Composition

Analyzer) similar to the Tanita, is routinely used in the clinic. The Tanita requires participants to stand still with bare feet on the weighing platform while a small amount of electrical current is used to measure impedance.

12. Resting energy expenditure by indirect calorimetry: A metabolic cart (Microlife Medgem Analyzer may be used to make gas exchange measurements (i.e. volume of oxygen consumed and volume of carbon dioxide produced by the participant). Measurements will be performed in a quiet, temperature-monitored room. Children will be required to lie still without falling asleep for the duration of the measurement. The data obtained will be used to assess whole-body substrate oxidation rates.

Participants in the obese insulin-resistant and newly prescribed metformin groups may be asked to participate in additional visits. The additional study visits will include the same procedures as the initial visit. For the newly prescribed metformin group, participants/parents will be asked to verify their compliance with metformin by bringing their medication to the visit to show study staff and asking the parent/participant to report their compliance. In addition, if a procedure(s) is not successfully completed, the parent and, if warranted, the participant may be asked to return to the study site for an additional visit to re-attempt the procedure. The decision to re-attempt the procedure(s) will be made by the principal investigator and/or co-investigator and the same steps as described for the re-attempt of a procedure from the initial visit followed.

- Participants in the obese-insulin resistant group may be asked to complete one additional study visits to occur at 12 months (± 2 weeks) after the initial study visit. This is to follow changes in bioenergetic and metabolic parameters over time to assess validity and will help establish not only the degree of change in the studied outcomes, but also their predictive value for T2D development, in addition to distinguish between metabolically healthy obese (MHO) and metabolically unhealthy obese (MUO) phenotypes. We may request that all obese insulin resistant participants complete one additional study visit; however, this additional study visit is optional as we do not want to discourage potential enrollment due to the possibility of another visit or lengthy visit. We anticipate roughly 43% of the 70 obese insulin resistant participants will decline a 2nd study visit with a goal of 40 subjects completing a second visit at 12 months (± 2 weeks).
- Participants in the newly prescribed metformin group who are prescribed metformin as part of their clinical care will be asked to complete a second study visit to occur six months (± 2 weeks) after beginning metformin therapy prescribed as part of their clinical care. We may request that all 20 participants in this group complete the additional study visit; however, the additional study visit is optional as we do not want to discourage potential enrollment due to the possibility of multiple visits or lengthy visits. We anticipate 50% of the 20 participants in this group will decline a 2nd study visit with a goal of 10 subjects completing a 2nd visit at 6 months (± 2 weeks).

A participant/parent pair will be compensated $100.00 in gift cards for each study visit completed. Completion of the study visit may include participation in parental study forms, anthropometric

measurements, fat oxidation estimate measured by deuterated palmitate, blood sample, urine samples, body composition assessment, and resting energy expenditure by indirect expenditure. Because the parent and participant do not have to stay at the study location for the entire visit, completion of the study visit would occur once the samples are obtained by the research personnel. Parents/participants who are healthy with a normal weight and also healthy obese children, will be given a one-time $100.00 in gift cards for participation in the research study visit. Parents/participants with T2D or insulin resistance who are prescribed metformin therapy at their normally scheduled doctor visit or part of the obese with insulin resistance group will be asked to complete two study visits and will be paid up to $200.00 in gift cards ($100.00 in gift cards at the first study visit and $100.00 in gift cards at the second visit). For each study visit, a $50 gift card will be provided to the study participants/parents after completion of the procedures to be completed at the research site (e.g., anthropometric measurements, Tanner Stage Assessment, physical activity questionnaire, depression scale, and blood collection) and another $50 gift card will be given to the study participants/parents upon return of the study samples and intake of food and liquid form from the visit. Those participants/parents who are asked to return to re-attempt procedure(s) not successfully completed at a study visit (initial or second) will be given a $25 gift card after re-attempting to complete the procedure(s). Participants/parents may be given up to $20 in ACRI merchandise. Breakfast, lunch and snacks may be provided during study visits.

Study Population

Study investigators, research staff, or any qualified personnel will conduct recruitment of study participants using IRB approved advertisement. Advertisements may be distributed in the form of postcards or flyers via direct mail or to various locations, including pediatricians' offices, health fairs, daycare centers, schools/universities, recreational centers, child retail stores, websites (ACH, ACRI, ACNC, UAMS, and others as applicable) and churches. Also, print or digital ads may appear in newspapers, magazines, social media and circulars. On-hold phone messages, BoomText messages, screensavers, and radio/television advertisements may be used. In addition, research staff may contact parents who expressed an interest in our studies or who previously agreed to be contacted regarding future studies. The research staff will educate the parents and children about the study. We will also work with physicians at ACH to identify subjects that may qualify. They will provide study staff with contact information for the patient, and study staff will call and educate the parents and children about the study. The physicians will introduce the study to their patients; if the patient is interested the physician will obtain contact information so that the study staff can follow up and talk to the parents and children about the study. In this case, the only contact by study staff would be to introduce the study followed by informed consent in person. We will protect all personal information obtained during recruitment, enrollment, and testing processes, and maintain this in a locked drawer or cabinet.

175 pre-pubertal children ages 5-9 years old or T2D/insulin resistant children ages 5-17 years old who have been newly prescribed metformin will be recruited and may enroll for this study with the goal that 130 subjects stratified across the following groups will complete the study: i) healthy lean (n=20); ii) healthy obese (n=20); iii) obese insulin resistant (n=70); obese with new

metformin prescription(n=20) (children with T2D or insulin resistant who have been newly prescribed metformin therapy as part of their clinical care). Every attempt will be made to ensure that groups are balanced with respect to sex and ethnicity.

Inclusion Criteria:

- Age 5-9 years and Tanner stage as reported by parent no greater than stage 1 OR Age 5 years - 17 years 5 months, diagnosed with type 2 diabetes mellitus or insulin resistance and prescribed metformin (not yet taking)
- Either healthy lean (BMI≥ 5th percentile and <85th percentile for age/sex) or obese (BMI ≥ 95th percentile for age/sex)
- For those with BMI≥ 95th percentile for age/sex, parental verbal confirmation will be obtained that the child had a history of BMI≥ 95th percentile for age/sex for at least six months prior to study enrollment

Exclusion criteria:

- Genetic or physical conditions impacting mobility over past year as determined by the PI
- Having known chronic illnesses/disorders that may independently affect study outcome measures: type 1 diabetes mellitus, neurologic (e.g. epilepsy), developmental (developmental delay, autism spectrum disorder), endocrine (thyroid, Cushing's), hepatic, autoimmune, cardiac and renal disorders. Also, chronic lung disorders except well controlled asthma that does not require permanent use of inhaled/oral steroids
- Taking any of the following medications that can affect study outcome: antipsychotics, thyroid hormone replacement therapy, inhaled/oral steroids, insulin, anabolic drugs (growth hormone replacement therapy and oxandrolone) and stimulants
- Taking metformin prescribed as part of their clinical care at the time of enrollment (may begin metformin therapy prescribed as part of their clinical care while enrolled in the study)
- BMI<5th percentile for age/sex (classified as underweight based on CDC growth charts)
- Subjects determined ineligible by the PI.

Risks and Benefits

We do not foresee any adverse events associated with this protocol. There is the potential risk of loss of confidentiality to study participants. Measures to protect the confidentiality of study participants will be implemented by applying the appropriate steps to secure the collected data as described in the Data Handling and Recordkeeping section below. The proposed stable isotopes to measure whole body fat oxidation is non-radioactive and presents no risk to humans. There is a small risk that participants will encounter bruising and/or infection after having blood taken, however, the use of well-established blood taking techniques, and sterile procedures, by trained phlebotomists or nurses, will ensure the risk is minimal. Also, numbing cream and/or a

pain relieving device (Buzzy) may be used to minimize the pain associated with the blood draw. There is the risk that researchers could develop ways in the future to link genetic information back to the subjects even though the samples do not include any personal identifiers, except for the assigned unique study code and the study acronym. Thus, we do not anticipate any adverse effects associated with the study. However, any adverse effects or unanticipated problems will be reported to the study PI, the IRB, and the study sponsor in accordance with IRB Policy 10.2. There will be no direct benefits to the study participants; however, knowledge gained from the study could potentially benefit patients in the future.

Data Handling and Recordkeeping

All study subject material will be assigned a unique identifying code or number. The electronic key to the code will be kept on a secure sharepoint site designed specifically for the study. Sharepoint is beneficial in that UAMS Techsource controls access for the site so that only select individuals are able to view the site. Only the PI and research personnel will have access to the code and information that identifies the subject in this study.

The PI and research study personnel will complete and maintain appropriate CITI training. Source documents and CRFs will be stored in a locked drawer. Documents will be archived according to UAMS/ACH/ACNC policies regarding destruction of research records.

Data will be entered into a research database. Access to the study database(s) is password protected and will be limited to study personnel and regulatory authorities.

At the time of collection, samples will be kept on ice or refrigerated (with the exception of stool which will be kept in a freezer) in the pediatric clinical research unit or in Dr. Rose's laboratory (Rm 4015 of ACRI) until they can be transported to the research laboratory. Samples will be kept at -80°C once the initial processing and analyses have taken place. Samples will be stored in Room N2002 Freezer N2002FT6 or N2002FT18 of ACRI in Dr. Carvalho's laboratory. These are monitored continuously for proper temperature and working condition. None of a subject's personal identifiers will be present on any biological sample, except the unique subject ID and the study acronym. The samples may be used until they are used up for research on pediatric nutrition.

At any time if the subject decides that he/she does not want to participate anymore, that subject's data will be included as part of the planned analysis of study data. No more information will be collected after withdrawal, however all data and samples collected prior to withdrawal will still be used.

Information pertaining to this study will be made publically available through the ClinicalTrials.gov website.

Data Analysis

Statistical and Power Analyses: 175 pre-pubertal children ages 5-9 years old will be recruited for this study assuming that 130 subjects stratified across the following groups will complete the study: i) healthy lean (n=20); ii) healthy obese (n=20); iii) obese insulin resistant (n=70); obese

with new metformin prescription (n=20). A one-factor analysis of variance (ANOVA) will be used to compare mean outcome of interest across the groups of children (healthy lean, healthy obese, obese insulin resistant and obese T2D). Pair-wise comparisons using Tukey-Kramer post-hoc tests will be used to identify groups whose means differ significantly while retaining the family-wise error rate at 5%. Outcomes will be also checked to verify that the assumptions of the parametric tests are met. When substantial deviation from assumptions are encountered, and if suitable transformation are not found, groups will be compared using the nonparametric Kruskal-Wallis rank test, followed by Bonferroni corrected Wilcoxon rank-sum tests to identify groups that differ. Generalized linear model (GLM) will also be used to incorporate important covariates and effect modifiers into the analysis.

For Aim 3: A paired t-test will be used to compare the mean change in the various outcomes described in SA1 and SA2 from baseline to 12 months for pre-pubertal insulin resistant obese children. If the assumptions for the paired t-test are not met, the non-parametric Wilcoxon matched-pairs signed-ranks test will be used. Alternatively, generalized linear mixed models (GLMM) will be used for Aim 3. GLMM account for the dependence between measurements from the same individual taken over time and allow the inclusion of fixed and time varying covariates.

For Aim 4: A paired t-test will be used to compare the effects of metformin on the various outcomes on obese T2D/insulin resistant children with a new metformin prescription. If the assumptions for the paired t-test are not met, the non-parametric Wilcoxon matched-pairs signed-ranks test will be used. Alternatively, generalized linear mixed models (GLMM) will be used for Aim 4. GLMM account for the dependence between measurements from the same individual taken over time and allow the inclusion of fixed and time varying covariates.

Sample Size Justification**:**

For Aims 1 and 2, statistical power was computed based on a one-factor ANOVA with a total of 110 children (70 obese insulin resistant, 20 healthy obese, 20 healthy lean). Assuming 80% power and $\alpha = 0.05$, the minimum detectable Cohen's f effect size (ES(f)) is 0.33. This is a medium effect size and should provide valuable information regarding whether a large study is warranted.

For Aim 3, statistical power was computed based on a paired t-test with a total of 40 children. Assuming 80% power and $\alpha = 0.05$, the minimum detectable Cohen's d effect size (ES(d)) is 0.45 for those who completed the study. These are medium effect sizes and should provide valuable information regarding whether a large study is warranted.

For Aim 4, statistical power was computed based on a paired t-test with a total of 10 children. Assuming 80% power and $\alpha = 0.05$, the minimum detectable Cohen's d effect size (ES(d)) is 1.0. Although this effect size is large, it should provide valuable information regarding whether a large study is warranted.

Ethical Considerations

This study will be conducted in accordance with all applicable government regulations and University of Arkansas for Medical Sciences research policies and procedures. This

protocol and any amendments will be submitted and approved by the UAMS Institutional Review Board (IRB) to conduct the study.

The formal consent of each subject, using the IRB-approved consent form, will be obtained before that subject is submitted to any study procedure. All subjects for this study will be provided a consent form describing this study and providing sufficient information in language suitable for subjects to make an informed decision about their participation in this study. The person obtaining consent will thoroughly explain each element of the document and outline the risks and benefits, alternate treatment(s), and requirements of the study. The consent process will take place in a quiet and private room, and subjects may take as much time as needed to make a decision about their participation. Participation privacy will be maintained and questions regarding participation will be answered. No coercion or undue influence will be used in the consent process. This consent form will be signed by the parent or legally authorized representative. Written assent will be obtained from participants age 7-17. A copy of the signed consent will be given to the participant, and the informed consent process will be documented in each subject's research record. A signed copy of the consent form may be placed in patients' medical record. If consent is given for samples to be used for future research studies and if samples are still being used when a participant turns the age of 18, subjects will not be re-contacted to sign a consent form and consent will be waived. However, subjects can contact study staff after they turn 18 to ask that any remaining samples be removed. In addition, participants may be recontacted about participating in future research studies.

Initially, samples will be stored at Arkansas Children's Research Institute. The information collected at the study visit, saliva, blood, urine, stool samples may be stored indefinitely and may be used for future research studies on pediatric nutrition. Prior to the information collected at the study visit and samples being used for future research studies, the PI will assess the ethics and scientific merit of the proposed research with the samples, and proposed future research will be reviewed by the IRB as may be required. The samples and health information collected for the study visit may be shared with researchers at the University of Arkansas for Medical Sciences, Arkansas Children's Hospital, or Arkansas Children's Research Institute. The samples may be shared with an outside group. The samples will only have a study ID number and study acronym to maintain confidentiality. If participants decide they no longer want us to use their samples for future research studies, they may ask the study staff that the sample be removed. If the sample has been shared or if publication of results has occurred, then we may not be able to remove the sample. In addition, research results from the depression scale may be shared with the child's PCP or if the child is actively followed at ACH the depression scale results may be shared with the child's ACH physician if found to be significant and more information may be provided. If the child does not have a PCP or is not actively followed at ACH, then a referral will be made to a PCP for follow-up. For example, a score of 15 or greater on the CES-DC suggests that the participant expresses symptoms of depression. In this scenario, the child's PCP or ACH physician would be notified of the depression scale result. If the child did not have a PCP, then a referral would be made to a PCP near the child's home and the research results from the depression scale would be shared. In addition, the parent would be given information of resources to seek help for the child.

Dissemination of Data

Results of this study may be used for presentations, posters, or publications. The publications will not contain any identifiable information that could be linked to a participant.

References

1. Mokdad AH, Ford ES, Bowman BA, Nelson DE, Engelgau MM, Vinicor F, Marks JS: Diabetes trends in the U.S.: 1990-1998, Diabetes care 2000, 23:1278-1283
2. Vincent HK, Innes KE, Vincent KR: Oxidative stress and potential interventions to reduce oxidative stress in overweight and obesity, Diabetes, obesity & metabolism 2007, 9:813-839
3. Civitarese AE, Ravussin E: Mitochondrial energetics and insulin resistance, Endocrinology 2008, 149:950-954
4. Hesselink MK, Schrauwen-Hinderling V, Schrauwen P: Skeletal muscle mitochondria as a target to prevent or treat type 2 diabetes mellitus, Nature reviews Endocrinology 2016,
5. Patti ME, Corvera S: The role of mitochondria in the pathogenesis of type 2 diabetes, Endocrine reviews 2010, 31:364-395
6. Codoner-Franch P, Pons-Morales S, Boix-Garcia L, Valls-Belles V: Oxidant/antioxidant status in obese children compared to pediatric patients with type 1 diabetes mellitus, Pediatric diabetes 2010, 11:251-257
7. Faienza MF, Francavilla R, Goffredo R, Ventura A, Marzano F, Panzarino G, Marinelli G, Cavallo L, Di Bitonto G: Oxidative stress in obesity and metabolic syndrome in children and adolescents, Hormone research in paediatrics 2012, 78:158-164
8. Marin MT, Dasari PS, Tryggestad JB, Aston CE, Teague AM, Short KR: Oxidized HDL and LDL in adolescents with type 2 diabetes compared to normal weight and obese peers, Journal of diabetes and its complications 2015, 29:679-685
9. Matusik P, Prokopowicz Z, Norek B, Olszanecka-Glinianowicz M, Chudek J, Malecka-Tendera E: Oxidative/Antioxidative status in obese and sport trained children: a comparative study, BioMed research international 2015, 2015:315747
10. Vehapoglu A, Turkmen S, Goknar N, Ozer OF: Reduced antioxidant capacity and increased subclinical inflammation markers in prepubescent obese children and their relationship with nutritional markers and metabolic parameters, Redox report : communications in free radical research 2016, 1-10
11. Albuali WH: Evaluation of oxidant-antioxidant status in overweight and morbidly obese Saudi children, World journal of clinical pediatrics 2014, 3:6-13
12. Codoner-Franch P, Boix-Garcia L, Simo-Jorda R, Del Castillo-Villaescusa C, Maset-Maldonado J, Valls-Belles V: Is obesity associated with oxidative stress in children?, International journal of pediatric obesity : IJPO : an official journal of the International Association for the Study of Obesity 2010, 5:56-63
13. Paltoglou G, Fatouros IG, Valsamakis G, Schoina M, Avloniti A, Chatzinikolaou A, Kambas A, Draganidis D, Mantzou A, Papagianni M, Kanaka-Gantenbein C, Chrousos GP, Mastorakos G: Antioxidation improves in puberty in normal weight and obese boys, in positive association with exercise-stimulated growth hormone secretion, Pediatric research 2015, 78:158-164

14. Santillan LD, Moyano M, Frau M, Flores O, Siewert S, Zirulnick F, Ramirez DC, Gimenez MS: Reduced blood nrf-2 mRNA in local overweight boys at risk of metabolic complications: a study in San Luis City, San Luis, Argentina, Metabolic syndrome and related disorders 2013, 11:359-365
15. Simoneau JA, Colberg SR, Thaete FL, Kelley DE: Skeletal muscle glycolytic and oxidative enzyme capacities are determinants of insulin sensitivity and muscle composition in obese women, FASEB journal : official publication of the Federation of American Societies for Experimental Biology 1995, 9:273-278
16. Simoneau JA, Bouchard C: Skeletal muscle metabolism and body fat content in men and women, Obesity research 1995, 3:23-29
17. Simoneau JA, Kelley DE: Altered glycolytic and oxidative capacities of skeletal muscle contribute to insulin resistance in NIDDM, J Appl Physiol (1985) 1997, 83:166-171
18. Simoneau JA, Kelley DE, Neverova M, Warden CH: Overexpression of muscle uncoupling protein 2 content in human obesity associates with reduced skeletal muscle lipid utilization, FASEB journal : official publication of the Federation of American Societies for Experimental Biology 1998, 12:1739-1745
19. Kelley DE, Goodpaster B, Wing RR, Simoneau JA: Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss, The American journal of physiology 1999, 277:E1130-1141
20. Ritov VB, Menshikova EV, He J, Ferrell RE, Goodpaster BH, Kelley DE: Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes, Diabetes 2005, 54:8-14
21. Ritov VB, Menshikova EV, Azuma K, Wood R, Toledo FG, Goodpaster BH, Ruderman NB, Kelley DE: Deficiency of electron transport chain in human skeletal muscle mitochondria in type 2 diabetes mellitus and obesity, American journal of physiology Endocrinology and metabolism 2010, 298:E49-58
22. Kelley DE, He J, Menshikova EV, Ritov VB: Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes, Diabetes 2002, 51:2944-2950
23. Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn CR, Mandarino LJ: Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1, Proceedings of the National Academy of Sciences of the United States of America 2003, 100:8466-8471
24. Sreekumar R, Halvatsiotis P, Schimke JC, Nair KS: Gene expression profile in skeletal muscle of type 2 diabetes and the effect of insulin treatment, Diabetes 2002, 51:1913-1920
25. Ten S, Bhangoo A, Ramchandani N, Mueller C, Vogiatzi M, New M, Lesser M, Maclaren N: Resting energy expenditure in insulin resistance falls with decompensation of insulin secretion in obese children, Journal of pediatric endocrinology & metabolism : JPEM 2008, 21:359-367
26. De Pergola G, Pannacciulli N, Minenna A, Martina RA, Cannito F, Giorgino R: Fuel metabolism in adult individuals with a wide range of body mass index: effect of a family history of type 2 diabetes, Diabetes, nutrition & metabolism 2003, 16:41-47
27. Chacko BK, Kramer PA, Ravi S, Benavides GA, Mitchell T, Dranka BP, Ferrick D, Singal AK, Ballinger SW, Bailey SM, Hardy RW, Zhang J, Zhi D, Darley-Usmar VM: The Bioenergetic Health Index: a new concept in mitochondrial translational research, Clin Sci (Lond) 2014, 127:367-373

28. Kramer PA, Chacko BK, Ravi S, Johnson MS, Mitchell T, Darley-Usmar VM: Bioenergetics and the oxidative burst: protocols for the isolation and evaluation of human leukocytes and platelets, Journal of visualized experiments : JoVE 2014,
29. Tyrrell DJ, Bharadwaj MS, Van Horn CG, Marsh AP, Nicklas BJ, Molina AJ: Blood-cell bioenergetics are associated with physical function and inflammation in overweight/obese older adults, Experimental gerontology 2015, 70:84-91
30. Nijhawan S, Richards W, O'Hea MF, Audia JP, Alvarez DF: Bariatric surgery rapidly improves mitochondrial respiration in morbidly obese patients, Surgical endoscopy 2013, 27:4569-4573
31. Tyrrell DJ, Bharadwaj MS, Jorgensen MJ, Register TC, Molina AJ: Blood cell respirometry is associated with skeletal and cardiac muscle bioenergetics: Implications for a minimally invasive biomarker of mitochondrial health, Redox biology 2016, 10:65-77
32. Bervoets L, Massa G: Classification and clinical characterization of metabolically "healthy" obese children and adolescents, Journal of pediatric endocrinology & metabolism : JPEM 2016, 29:553-560
33. Munoz-Garach A, Cornejo-Pareja I, Tinahones FJ: Does Metabolically Healthy Obesity Exist?, Nutrients 2016, 8:
34. Rose S, Frye RE, Slattery J, Wynne R, Tippett M, Melnyk S, James SJ: Oxidative stress induces mitochondrial dysfunction in a subset of autistic lymphoblastoid cell lines, Translational psychiatry 2014, 4:e377
35. Rose S, Frye RE, Slattery J, Wynne R, Tippett M, Pavliv O, Melnyk S, James SJ: Oxidative stress induces mitochondrial dysfunction in a subset of autism lymphoblastoid cell lines in a well-matched case control cohort, PloS one 2014, 9:e85436