Study Title: Immunometabolism in Pediatric Obesity

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Background and Rationale

Obesity is an evolving pediatric health crisis in the United States. Approximately 20% of children and adolescents in the US are obese[1] and consequently at increased risk for type 2 diabetes (T2D), hypertension and several cancers[2]. Previously considered as adult onset conditions, the incidence of T2D and cardiovascular dysfunction is increasing among adolescents likely due to high prevalence of obesity in children[3]. In the state of Arkansas, more than 30% of children meet the criteria for being overweight or obese[4]. The dramatic increase in the prevalence of childhood obesity[5] has enormous public health implications and, for the first time in history, may result in a reduction of the life expectancy of the current generation of children[6-9]. A better understanding of the pathophysiology of obesity and associated health complications in children is imperative for the development of effective preventative and treatment strategies, to prevent the progression into more serious health concerns, and to reduce morbidity and mortality.

Obesity is strongly linked to systemic inflammation with increased circulating pro-inflammatory cytokines and adipokines and elevated C-reactive protein (CRP) reported in obese children as young as age three[10-15]. Examining NHANES data from over sixteen thousand children, Skinner et al[15] found that prevalence of C-reactive protein (CRP) levels >1mg/L was greater in very obese children beginning at age 3 and continued through adolescence, with greater than 80% of very obese adolescents having CRP >1mg/L as compared to 18% of lean adolescents. While the dataset did not permit controlling for pubertal age, similar results were found even when very narrow age groups were compared. In a smaller study of children ages 7-14 years, Codoner-Franch et al[16] classified obese children as metabolically healthy or at risk based on well-defined criteria, and they reported CRP, Tumor necrosis factor α (TNFα) and Interleukin (IL) 6 were all increased in obese children with or without metabolic risk factors, suggesting that inflammation may appear early in obesity before metabolic alterations. Inflammation is considered pivotal to the development of obesity-related metabolic disorders including insulin resistance, T2D[17-19] and cardiovascular dysfunction[20, 21]. Modulation of inflammation in obese children provides a putative “target” for therapeutic intervention.

Suppression of inflammation is a function of regulatory T cells (T_{regs}), a subset of $CD4^+$ T cells also responsible for maintenance of peripheral tolerance and suppression of antigen-specific immune responses. However, decreased circulating T_{regs} have been reported in T2D[22] and obese[23, 24] adults. Wagner et al[24] found that reduced T_{regs} predicted a CRP level greater than 3mg/L and may therefore predict those at increased cardiovascular risk; however, a separate group reported no differences in T_{regs} between severely obese subjects with and without hypertension[23]. As in humans, T_{regs} are reduced in several mouse models of obesity, especially VAT-resident T_{regs}, with a concomitant increase in VAT-resident T effector cells[25, 26]. A critical role for T_{regs} in the development of insulin resistance has been demonstrated in these models. Winer et al[25] elegantly demonstrated that adoptive transfer of $CD4^+$T cells into lymphocyte deficient DIO $Rag1^{-/-}$ mice reduced weight gain and improved glucose tolerance. *In situ* induction of T_{regs} in HFD obese wild-type mice with anti-CD3 antibody also resulted in improved glucose tolerance and insulin sensitivity, which persisted for months despite being maintained on

HFD. Feuerer et al[26] demonstrated that depletion of adipose T_{regs} in transgenic mice expressing the diphtheria toxin receptor in T_{regs} induces insulin resistance and increased inflammatory mediators in the adipose tissue. Conversely, *in situ* induction of T_{regs} with recombinant IL-2 and IL-2 monoclonal antibody increased VAT-resident T_{regs} and improved glucose tolerance in HFD obese mice. Findings from these two landmark studies have been replicated in other mouse models of obesity[27, 28]. Targeting T_{regs} may modulate inflammation in obesity; however, whether T_{regs} are altered in early stages in obesity (e.g. overweight/obese children) remains unknown. In this study, we will fill an important research gap by testing whether obese children exhibit decreased circulating T_{regs} as compared to healthy lean children.

The past decade has seen a surge in understanding the metabolic control of immune cells, unveiling metabolic signaling pathways that may be exploited to target specific immune cell subsets. Pro-inflammatory immune cells preferentially produce ATP via glycolysis while anti-inflammatory cells primarily utilize mitochondrial oxidative phosphorylation (OXPHOS)[29-31]. Nutrient and energy-sensing signaling pathways are master regulators of cellular bioenergetics with mammalian target of rapamycin (mTOR) driving glycolysis and adenosine monophosphate activated protein kinase (AMPK) driving OXPHOS. The mTOR pathway promotes glycolysis by upregulating Glut1 trafficking to the cell surface[32], increasing glucose uptake and glycolysis. AMPK opposes the mTOR pathway and promotes glucose and lipid oxidation[33]. As depicted in **Figure 1**, T_{eff} depend upon mTOR-driven glycolysis while T_{regs} require AMPK-driven OXPHOS. Targeting these metabolic control pathways can simultaneously inhibit effector responses and promote the generation of T_{regs}, opening up a new approach for treating inflammation in obesity and preventing the progression into more serious health conditions. We will determine, for the first time, whether this immunometabolic paradigm is altered in pediatric obesity by measuring activation of AMPK and mTOR in isolated $CD4^+$ T cells. These data are necessary before the approach of immunomodulation by targeting metabolic control pathways to treat inflammation can be applied in this population.

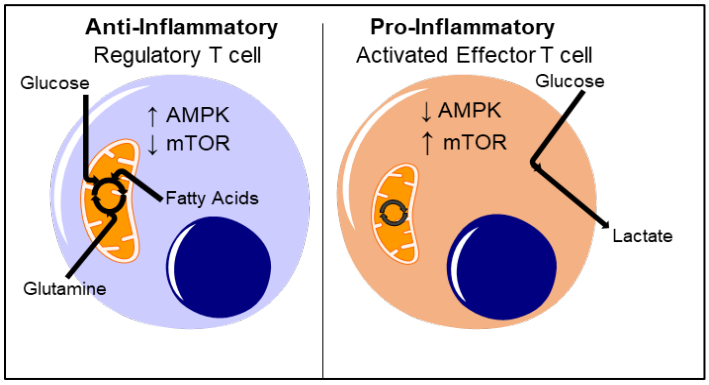

Figure 1: Distinct metabolic pathways control regulatory T cells (T_{reg}) and effector T cells (T_{eff}). *T_{regs} are characterized by high AMPK and low mTOR activation, and they primarily utilize mitochondrial oxidative phosphorylation (OXPHOS) to produce ATP. T_{eff} are characterized by low AMPK and high mTOR activation, and they primary utilize glycolysis to produce ATP.*

The AMPK activator, metformin, is the only anti-diabetic drug FDA-approved for use in children[34, 35]. In an open-label trial, metformin increased both the number and regulatory functions of T_{regs} in obese multiple sclerosis patients with metabolic syndrome[36]. In a placebo-controlled trial carried out by de Kreutzenberg et al[37], two months of metformin

treatment in pre-diabetic adults reduced mTOR activity in PBMC despite increases at the gene expression level; however, AMPK activity was not changed by metformin. Metformin's potential to induce T_{regs} has otherwise not been studied in obese individuals. We will take advantage of the fact that this AMPK activator is commonly prescribed in pediatrics and compare the immune and metabolic phenotypes of $CD4^+$ T in obese insulin resistant/T2D children before and after metformin treatment. As such, we will explore whether a potential mechanism of action of metformin is via direct modulation of $CD4^+$T cell metabolism, induction of T_{regs}, and curbing inflammation.

The main purpose of this proposed study is to determine whether the immunometabolic phenotypes of $CD4^+$T cells from obese children is skewed towards T_{eff} with mTOR-driven glycolysis and away from T_{regs} with AMPK-driven OXPHOS and whether metformin can reverse the immunometabolic phenotypes. These studies are imperative to begin testing and implementing strategies targeting the AMPK and mTOR pathways to combat obesity-associated inflammation and prevent progression into serious health complications. This translational research will move the field forward in several ways. First, we will gain insight into the pathophysiology of obesity-associated inflammation and the progression into insulin resistance and T2D. Second, we have the opportunity to uncover novel therapeutic avenues for obesity-associate inflammation and prevention of metabolic dysfunction that target immune cell metabolism. Third, we may identify an immunometabolic phenotype that correlates to risk for developing T2D and/or cardiovascular dysfunction and could be used to monitor treatment response in future treatment trials. The findings from this study will form the preliminary data for a planned R01 application to the NIDDK to be submitted in Year 2. The goal of that proposal will be to conduct additional translational studies to expand upon this study and begin testing the ability to modifying bioenergetic control pathways to combat obesity-associated inflammation.

Preliminary Data

In a current study (UAMS IRB #206164; Co-I: Rose), we have enrolled lean and obese subjects in a very narrow age range (5-9 years of age) in order to control for pubertal state. Bioenergetic profiling using the Seahorse XF96 Analyzer (Seahorse Bioscience, Inc., North Billerica, MA), a core ACRI equipment, is routinely performed by Dr. Rose[38-40]. The Seahorse measures oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in a 96-well plate of intact living cells. The sequential injection of up to four compounds into each well enables the interrogation of bioenergetics in real-time. **Figure 2A and B** presents the ATP Production Rate Assay on PBMC from lean, obese and T2D children enrolled in an ongoing study (UAMS IRB #206164; Co-I: Rose). In this assay, basal OCR and ECAR are initially measured, followed by the injection of oligomycin (oligo), an ATP synthase inhibitor, to block ATP production by the mitochondrial electron transport chain (ETC) and induce compensatory glycolysis. Next, antimycin A (AMA) and rotenone (ROT), inhibitors of ETC complex III & I, respectively, are injected together. Shutting down the ETC allows for calculating the amount of media acidification (ECAR) that was due to CO_2 production during OXPHOS. Proton efflux rate (PER) is calculated post-assay from ECAR and the buffering capacity of the assay media, and finally, validated calculations derive ATP generated by OXPHOS (mitoATP) and by glycolysis (glycoATP)[41], which is presented in **Figure 2C**. **Figure 2D** shows that the % ATP derived from glycolysis is significantly greater in PBMC in the T2D group as compared to the OB group (p=0.032). While no differences between the lean and obese group are observed in this preliminary data, the increased % glycolysis in the T2D group supports the notion that obese children with metabolic dysfunction have more pro-inflammatory cells and fewer T_{regs} in circulation. There are several important points to

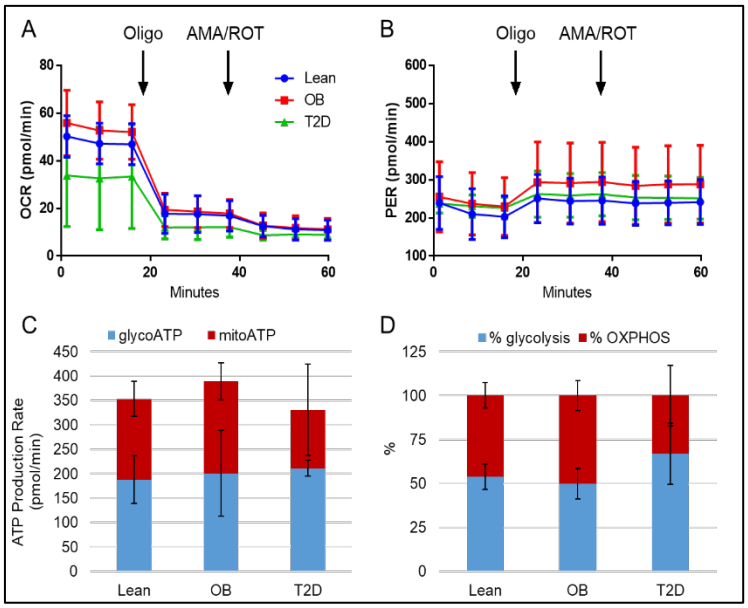

Figure 2: The ATP production rate assay demonstrates increased % glycolysis-derived ATP production in PBMC from T2D children. Oxygen consumption rate (OCR) (**A**) and Proton Efflux Rate (PER) (**B**) are plotted over time in PBMCs from lean, obese (OB) and type 2 diabetic (T2D) children. Injections of Oligomycin (oligo) and Antimycin A (AMA) and Rotenone (ROT) are indicated. (**C**) Glycolytic ATP production rate (glycoATP) and mitochondrial ATP production rate (mitoATP) are calculated from OCR and PER. (**D**) % glycolysis and % OXPHOS-derived ATP production are calculated. * p=0.032
Methods: Freshly isolated PBMC from lean (n=8), obese (OB; n=11), and treatment-naïve type 2 diabetic (T2D; n=2) children were plated at $2.5x10^6$ cells/well in RMPI 1640 with 1mM HEPES, 2mM glutamine, 1mM pyruvate and 11mM glucose on poly-D-lysine coated XF96 plates. Plates were centrifuged at 100xg with no brake to adhere cells to well bottoms. After a 30 min incubation in a 37°C non-CO2 incubator, cells were assayed in a Seahorse XF96.

note regarding this data: 1) these data are from PBMC, a mixed population of cells including monocytes and lymphocytes of various subtypes rather than purified $CD4^+$ T cells as proposed in this study; 2) the obese subjects have not been classified as metabolically healthy or unhealthy; 3) the age of the T2D subjects is greater (16.5± 0.1 years; mean±SD) than the other groups (lean: 6.8±1.1 years; obese: 7.9±1.4 years). In the studies proposed herein, we will match groups on age and subgroup obese subjects as metabolically healthy and unhealthy based on insulin sensitivity. Further, after measuring high sensitivity CRP (hsCRP), we will compare subjects with hsCRP values in the top quartile to the other groups to determine whether the % glycolysis is higher in $CD4^+$T cells from those obese subjects with inflammation.

Activation of AMPK or mTOR have not yet been evaluated in PBMC presented in Figure 2. In another study ongoing study of mitochondrial dysfunction in children with autism spectrum disorder (UAMS IRB #137162; PI: Rose), we measured PBMC bioenergetics and mTOR activation in 43 children including 38 children with autism spectrum disorder and 5 unaffected siblings. At the time of this study, the ATP production rate assay was not yet developed, and PBMC were bioenergetically phenotyped using the mitochondrial stress test[39]. mTOR activation was measured in lysates from approximately $2x10^6$ PBMC using the 11-Plex AKT/mTOR Phosphoprotein magnetic bead Kit (Millipore). As demonstrated in **Figure 3**, phosphorylated mTOR (P-mTOR) in PBMC is significantly associated with basal (**Figure 3A**) and maximal OCR (**Figure 3B**) as well as the ratio of

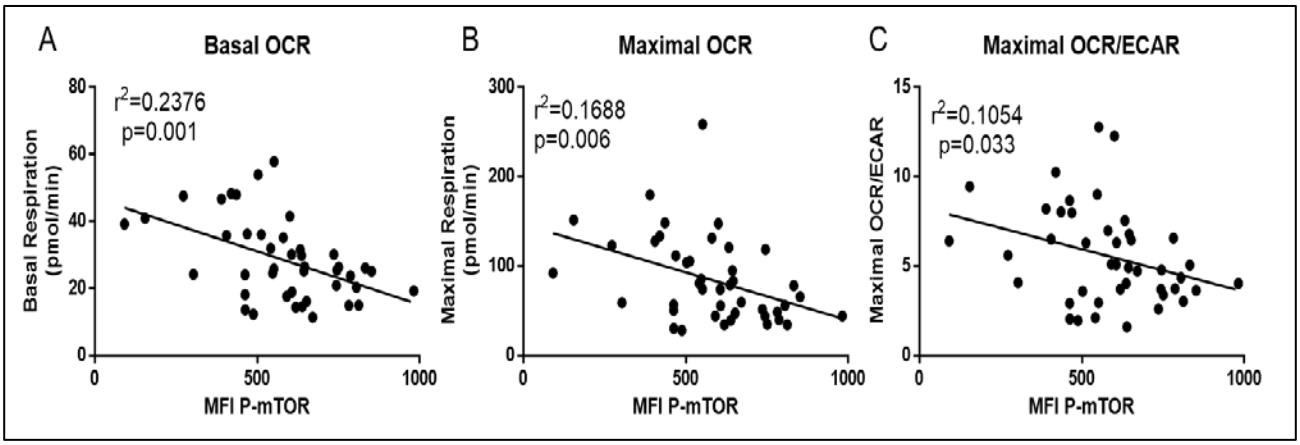

Figure 3: Activation of mTOR is associated with reduced mitochondrial respiration in PBMC from children.
Activation of mTOR (p-mTOR) is associated with reduced basal respiration (**A**), maximal respiration (**B**), and OXPHOS/Glycolysis (OCR/ECAR) (**C**).
Methods: For determination of mTOR activation, phosphorylated mTOR (Ser2448) was measured using the 11-Plex Akt/mTOR phosphoprotein magnetic bead kit (Millipore) in PBMC from 43 children ages 2-18 years. Lysates were prepared from $2x10^6$ PBMC from as directed by manufacturer. Median fluorescent intensity (MFI) was measured on a Luminex 200 and an average of 2 replicate wells is presented. For measures of Basal Respiration, Maximal Respiration and Maximal OCR/ECAR, freshly isolated PBMC were plated at $4x10^5$ cells/well of a poly-D-lysine coated XF96 plate in RPMI supplemented with 1mM pyruvate, 2mM glutamine and 25mM glucose. Plates were centrifuged at 100xg with no brake to adhere cells to well bottoms. After a 30 min incubation in a 37°C non-CO2 incubator, cells were assayed in a Seahorse XF96 using the mitochondrial stress test (Agilent). Maximal respiration was measured after the addition of oligomycin and the uncoupler, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP). Maximal OCR/ECAR represents maximal (FCCP-stimulated) respiration divided by maximal (oligomycin-stimulated) ECAR. An average of at least 3 replicate wells from each subject is presented.

OXPHOS/Glycolysis (OCR/ECAR; **Figure 3C**). These data demonstrate our ability to measure mTOR activation in small numbers of immune cells from children.

Hypothesis, Specific Aims, and Objectives

In this study, we hypothesize that compared to lean children, circulating $CD4^+$T cells from obese children with elevated biomarkers of inflammation will have fewer T_{regs} and will exhibit increased mTOR-driven glycolysis. Metformin treatment will increase T_{regs} and shift the metabolic phenotypes of $CD4^+$T cells towards AMPK-driven OXPHOS. We will test these hypotheses by executing the following Specific Aim:

Specific Aim 1. Compare the immune and metabolic phenotypes of circulating $CD4^+$T cells between lean children and overweight/obese children.

> Hypothesis**:** circulating $CD4^+$T cells from obese children with elevated biomarkers of inflammation will have fewer T_{regs} and will exhibit increased mTOR activation and % glycolysis-derived ATP production as compared to $CD4^+$T cells from lean children.

Specific Aim 2. Compare the immune and metabolic phenotypes of circulating $CD4^+$T cells between T2D children before and after metformin.

> Hypothesis: In obese T2D patients, metformin treatment will increase circulating T_{regs}, as well as AMPK activation and % OXPHOS-derived ATP production in $CD4^+$ T cells.

Study Design and Procedures

This study consists of:

1. An observational cross sectional immune and metabolic analysis of several groups of children including lean, overweight/obese, and T2D.
2. A prospective immune and metabolic analysis of newly diagnosed children with T2D or insulin resistance who will be or were recently prescribed metformin as part of their clinical care.

Children with T2D or insulin resistance who will be or were recently prescribed metformin, will be asked to complete two study visits. If completed, the second visit will occur 6 months (+/- 2 weeks) after beginning metformin as part of their clinical care. All other children, will be asked to complete only the first visit.

A prospective subject and his/her parent or legally authorized representative will come for the study visit that will last up to 2.5 hours depending on the duration of the consenting process and blood draw. A subject (and/or his/her parent) will be provided a questionnaire 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 questions relating to inclusion/exclusion criteria, 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 is revised prior to the scheduled visit, 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 have not taken seasonal allergy medication (e.g. Claritin) in the prior 72 hours and 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 not taking seasonal allergy medication in the previous 72 hours and 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, body composition by bioelectrical impedance, blood, stool, and urine collection. Attempts to repeat anthropometric measurements, and body composition by bioelectrical impedance, will occur if a reading is not given the first time or if the child is unable to comply during the first measurement. For optional sample collection (i.e., urine and stool), the parent and participant 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. 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 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 not have taken seasonal allergy medication in the previous 72 hours and 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 urine and stool 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. Anthropometrics: Anthropometric measurements including height, weight, waist circumference, hip 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.

2. Tanner (Puberty) Stage Assessment: Parents will be asked to complete a form with drawings depicting pubertal development. This form is used to determine pubertal status. The questionnaire given to parents to complete is subjective and a parent could potentially incorrectly assess Tanner Stage. Because the results from the samples may be influenced by the participant's pubertal development, pubertal development must be accounted for. 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 metabolically unhealthy 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. In order to validate the parent's assessment of Tanner Stage level, hormone tests may be conducted on the blood sample obtained.

3. 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.

4. 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.

5. 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.

6. Blood Collection: A pediatric nurse or trained phlebotomist will collect blood from the participant under fasted conditions and with no seasonal allergy medications taken in the previous 72 hours. 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, numbing spray, or a pain relieving device (Buzzy) may be used for the blood draw. Blood samples may be used for:
   a. Immunophenotyping: Mitogen-stimulated blood will be stained with combinations of fluorochrome-labeled antibodies and analyzed by flow cytometry.
   b. $CD4^+$ T cell isolations: $CD4^+$T cells will be isolated by negative selection using antibody-labeled magnetic beads for analysis of metabolic control pathway activation and bioenergetics
      i. Metabolic control pathway analysis: Using cell lysates from isolated $CD4^+$T cell lysates will be analyzed for multiple analytes in metabolic control pathways using multiplex immunoassays, ELISA's and/or western blot.
      ii. Bioenergetics analysis: $CD4^+$T cells will be bioenergetically phenotyped using the Seahorse Extracellular Flux (XF)96 Analyzer, protocols for such have been established by Rose[38, 39]. Cellular 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.
   c. Quantification of plasma CRP, pro-inflammatory and anti-inflammatory cytokines and adipokines: We will use multiplex immunoassays to quantify levels of CRP as well as cytokines and adipokines.
   d. Analyses of HbA1c, insulin, glucose, lipid levels, and other analytes including, but not limited to, redox metabolites, and hormones.
   e. Leftover blood samples collected during this study may be stored in the PI's laboratory and used for future research studies on pediatric nutrition or metabolism.

7. Urine collection (Optional): 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 fasting 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 may be used for future research studies involving pediatric nutrition or metabolism. This sample is optional and is not required for the completion of the study visit.

8. Stool collection (Optional): 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 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 involving pediatric nutrition or metabolism. This sample is optional and is not required for the completion of the study visit.

If agreed to, the second visit for children with T2D or insulin resistance who will be or were recently prescribed metformin will occur 6 months (+/- 2 weeks) after beginning metformin as part of their clinical care. This 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 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).The second study visit will include the same procedures as the first study visit. In addition, 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. As done for the first study visit, 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 the same steps as described for the re-attempt of a procedure from the initial visit followed.

A participant/parent pair will be compensated for each study visit completed. For the first and, if applicable, second study visit, a $50 gift card (or gift cards totaling $50) 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, body composition, and blood collection). Parents/participants with T2D or insulin resistance who are prescribed metformin therapy will be asked to complete two study visits and will be paid up to $100.00 in gift cards ($50.00 in gift cards at the first study visit completed and $50.00 in gift cards at the second visit completed). Other parents/participants will be asked to complete one visit and will be paid up to $50 in gift cards for this completed 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). At the first, second, or reattempt visits, participants/parents may be given up to $20 in ACRI merchandise and/or child-friendly stickers. Food and drinks may be provided during study visits following the fasting blood draw.

Study Population

110 children ages 5-17 years old will be recruited with the goal that approximately 80 subjects stratified across the following groups will complete the study: i) healthy lean (approximately n=20); ii) overweight/obese (approximately n=40, with the anticipation that approximately 20 of these children will be insulin resistant); iii) overweight/obese with T2D and prescribed Metformin (approximately n=20). Every attempt will be made to ensure that the desired number for each group is achieved and groups are balanced with respect to age, sex and ethnicity.

Inclusion Criteria:

- Age 5-17 years, inclusive
- Either healthy lean (BMI≥ 5th percentile and <85th percentile for age/sex) or overweight (BMI ≥ 85th percentile and <95th) or obese (BMI ≥ 95th percentile for age/sex)
- For those with BMI≥ 85th percentile for age/sex, parental verbal confirmation that the child had a history of BMI≥ 85th percentile for age/sex for at least six months prior to study enrollment

OR

- Age 5 years - 17 years 5 months, inclusive
- Either overweight or obese (BMI≥ 85th percentile for age/sex)
- Parental verbal confirmation that the child had a history of BMI≥ 85th percentile for age/sex for at least six months prior to study enrollment
- Diagnosed with type 2 diabetes mellitus or insulin resistance
- Prescribed metformin (either not yet taking or began taking within 3 weeks of enrollment)

Exclusion criteria:

- Having an infection (viral, respiratory, gastrointestinal) in the previous 4 weeks
- 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 for longer than 3 weeks 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 or delegated staff.

Recruitment:

Study investigators, research staff, or any qualified personnel will conduct recruitment of study participants using IRB approved advertisements and methods.

We will recruit some subjects who agreed to be contacted for future research from an ongoing human research study, Bioenergetics and Metabolism in Pediatric Populations (BMPP; UAMS IRB #206164; Rose and Carvalho, Co-PIs). In the BMPP study, we have enrolled 55 children in a period of 1 year and thus, we do not anticipate difficulty meeting this recruitment goal as the recruitment criteria are relaxed, with a wider age range, for the proposed study.

Advertisements may be distributed in the form of postcards or flyers via direct mail, at information booths or events, or to various locations for posting or distributing, including pediatricians' offices, health fairs, daycare centers, schools/universities, recreational centers, grocery stores, supermarkets, 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, newsletters, 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. Research staff may also contact parents who have signed up for the AC research registry. The research staff will educate the parents and children about the study and if interested determine potential eligibility. At the visit, study staff would obtain informed consent in person.

Study staff will also work with healthcare providers at ACH and outside of ACH to identify subjects that may qualify. Study staff may assist healthcare providers at ACH with reviewing patients who have upcoming appointments with them to identify those who may qualify based on their electronic medical records at Arkansas Children's. When a patient is identified by the study staff or healthcare provider, the healthcare providers will introduce the study to their patients (e.g., provide letter/flyer and/or briefly describe the study); if the patient is interested, the physician may obtain contact information so that the study staff can follow up with the parent or encourage the patient to contact study

staff. Alternatively, the healthcare provider may introduce their patient directly to study staff if study staff is available to meet the patient during the visit. Healthcare providers outside of ACH may provide their patient with a letter/flyer or, if the patient is interested, may use the recruitment HIPAA authorization form to obtain permission from the parent and use this form to provide their information to study staff. This form can then be sent to study staff so that they can follow-up with the parent. The only contact by study staff with those identified by healthcare providers would be to explain the study, determine potential eligibility, and schedule a visit. At the visit, study staff would obtain informed consent in person.

Study staff will also use the electronic medical records (EMR) at Arkansas Children's to identify children who may be eligible to participate. When a child is identified who has a MyChart account we will post this study's information in their MyChart account under the available studies page. This page lists all studies the child may be eligible for that have chosen to recruit using MyChart. On this page, the parent can indicate if they are interested or not. If they are interested, the system will notify the research team so they can follow-up with the parent. In addition, when a child is identified, study staff may work with the child's health care providers to inform the parent about the study. This may be done by providing/mailing the parent a letter/flyer to let the parent know about the research opportunity their child may be eligible for. If the parent is interested, the physician may obtain contact information so that the study staff can follow up with the parent or encourage the patient to contact study staff. If the parent indicates an interest in the study, study staff will proceed to determine potential eligibility and scheduled a visit where informed consent would be obtained in person.

We will protect all personal information obtained during recruitment, enrollment, and testing processes, and maintain this in a locked drawer or cabinet.

Risks and Benefits

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. 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, numbing spray, 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 stored samples do not include any personal identifiers, except for the assigned unique study code, study visit number, and the study acronym. 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

The Principal Investigator will carefully monitor study procedures to protect the safety of research subjects, the quality of the data and the integrity of the study. All study subject material will be assigned a unique identifying code or number. The electronic key to the code will be kept in a secure, access-restricted, pass-code required, location created specifically for the study. 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 location. Documents will be archived according to UAMS/ACH/ACRI 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 or hospital laboratory.

For testing to be completed by the Arkansas Children's hospital laboratory (e.g., HbA1c), sample(s) will be labeled with the participant's medical label generated through EPIC which includes the participant's name, date of birth, gender, and medical record number. This sample will be provided to the hospital laboratory where they will complete the testing, storage, and disposal of the sample per their standard operating procedures. Results will be reported through the participant's medical record.

For testing to be completed by Dr. Rose's laboratory, samples will be kept at -80°C once the initial processing and analyses have taken place. These samples will be stored in the Arkansas Children's Research Institute, Room R4017 Freezers 4017FT12 or R4017FT8 or R2108 Freezer R2108FT16. Cryopreserved samples may also be kept under liquid nitrogen in the Arkansas Children's Research Institute, Room R4017. 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, study visit number, and the study acronym. The samples may be used until they are used up for research on pediatric nutrition or metabolism.

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

Prior to any analyses, endpoints data for each group will be closely inspected for the potential presence of unusual values; effort will be made to conserve all data. Analyses will be carried out in SAS 9.4 (SAS Institute, Cary NC) at the 5% significance level; point and 95% interval estimates will be obtained for relevant metabolic group comparisons. Parametric techniques will be first choice and nonparametric equivalents if assumptions are not met. Special attention will be paid to unequal variances.

The study planned four groups of children: lean, healthy obese/overweight, insulin-resistant obese/overweight, and overweight/obese with T2D. Primary comparison of interest are these three: the average of insulin-resistant and T2D obese children compared to (*i*) lean children and (*ii*) healthy obese children; and (*iii*) lean children compared to healthy obese children. Outcomes important to this aim are proportions of T_{reg} cells among circulating $CD4^+T$ cells and $CD4^+T$ glycoATP and mitoATP production. Initially, we will use a one-factor ANOVA to analyze these outcomes (possibly with suitable transformations). Comparisons of interest will then be made with 0.05 significance level *t*-tests conducted within the ANOVA framework.

The requested sample sizes will provide about 0.80 power for detecting differences in means of size 0.8 SD for comparisons (*i*) and (*ii*) and size 0.9 SD for comparison (*iii*), where SD is the within-group standard deviation (a.k.a., root mean square error from the ANOVA). For proportions of Treg cells – a primary outcome of interest, an estimate of the SD is 2.3 percentage points (ref Table 1 of Arismendi et al,[42]). So, if the means differ by at least 1.85 percentage points in comparisons (i) & (ii) and by 2.10 percentage points for comparison (iii), we will be able to detect these differences with a reasonably high probability (0.80 or higher).

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 participants for this study will be provided a consent form describing this study and providing sufficient information in language suitable for participants/parent/legally authorized guardian 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 participants/parent/legally authorized guardian 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 guardian. Written assent will be obtained from participants aged 7-17 years, inclusive. A copy of the

signed consent will be given to the participants/parent/legally authorized guardian, 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. We request a waiver of consent for subjects who turn 18 while their samples are still available for use and who may still have data collected from their medical records, for the purposes of this study and/or for future research studies if consent obtained for this purpose. If the waiver is approved by the institutional review board and samples are still available for use/data is still being collected when a participant turns the age of 18, the participant will not be re-contacted to sign a consent form. However, these participants can contact study staff after they turn 18 to ask that any remaining samples be removed and data collection stop. In addition, participants may be re-contacted about participating in future research studies.

Initially, samples will be stored at Arkansas Children's Hospital and/or Arkansas Children's Research Institute. The sample provided to the Arkansas Children's Hospital laboratory will be tested, stored, and disposed of per their standard operating procedures and will not be used for future research studies. The information collected at the study visit, urine, and stool samples, and remaining blood samples may be stored indefinitely and may be used for future research studies on pediatric nutrition or metabolism. 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, study visit 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.

A summary of the study visit will be provided to the parent indicating the participant's anthropometric and body composition measurements and the CES-DC results from the study visit. The parent will be directed to follow-up with their child's PCP if they have concerns. If the parent has concerns and the child does not have a PCP, research staff will assist the parent with identifying a PCP. If participant scores a 15 or greater on the CES-DC, suggesting that the participant expresses symptoms of depression, research staff will follow-up with the parent to explain these results, discuss the need to follow-up with the child's PCP, and provide the parent with the 'Resources to Seek Help for Depression in Children' form. If the child does not have a PCP, research staff will assist the parent with identifying a PCP.

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.

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