PROTOCOL TITLE: Influence of carbohydrate availability on skeletal muscle and circulating microRNA expression

SECTION A: RESEARCH TEAM AND LOCATIONS

A1. RESEARCH TEAM

Study Role	Institution/Company and Contact Information
Sponsor	*Organization/Institution/Company:* US Army Research Institute of Environmental Medicine (USARIEM) Military Nutritoin Division (MND) *Address:* 10 General Greene Ave, Bldg 42, Natick, MA 01760 *Point of Contact:* *Name and Degree:* Scott J Montain, PhD *Title:* Division Chief *Phone Number:* 508-233-4564 *Email:* scott.j.montain.civ@mail.mil
Principal Investigator	*Name, Rank, and Degree:* Lee M Margolis, PhD, RD *Title:* ORISE Post Doctoral Fellow *Institution:* USARIEM / Military Nutrition Division *Address:* 10 General Greene Ave, Bldg 42, Natick MA 01760 *Phone Number:* 508-233-4591 *Email:* lee.m.margolis.ctr@mail.mil
Associate Investigator(s)	*Name, Rank, and Degree:* Andrew J. Young, PhD *Title:* Nutritional Physiologist, ORSIE Fellow *Institution/Company:* USARIEM / Military Nutrition Division *Address:* 10 General Greene Ave, Bldg 42, Natick, MA 01760 *Phone Number:* 508-233-5141 *Email:* Andrew.j.young.ctr@mail.mil *Name, Rank, and Degree:* Scott J. Montain, PhD *Title:* Division Chief *Institution/Company:* USARIEM / Military Nutrition Division *Address:* 10 General Greene Ave, Bldg 42, Natick, MA 01760 *Phone Number:* 508-233-4564 *Email:* scott.j.montain.civ@mail.mil *Name, Rank, and Degree:* James P. McClung, PhD *Title:* Deputy Chief *Institution/Company:* USARIEM / Military Nutrition Division *Address:* 10 General Greene Ave, Bldg 42, Natick, MA 01760 *Phone Number:* 508-233-4979 *Email:* james.p.mcclung8.civ@mail.mil

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A2. ROLES AND RESPONSIBILITIES

A2.1 Key Research Personnel

Name(s): Lee M Margolis
Research Role: Principal Investigator
Study Responsibilities: The Principal Investigator is responsible for the safe and scientifically sound conduct of the study. He will oversee all aspects of study, ensure safety and ethical treatment of participants; maintain required documentation for the study and obtain required approvals; and will have primary responsibility for data analysis, interpretation, and publication. Dr. Margolis will also be actively involved in the volunteer brief, obtaining consent, data collection, performing muscle biopsies, catheterization, phlebotomy, and exercise testing and interventions. Drs. Andrew Young, James McClung, Scott Montain, or Stefan Pasiakos will be alternate PI in the event of Dr. Margolis' absence and per his written direction. Dr. Pasiakos will act as the first alternative PI in the event of Dr. Margolis' absence.

Name(s): Andrew J Young, Scott J Montain, James P McClung
Research Role: Associate Investigators
Study Responsibilities: Protocol concept development; formulation of protocol questions, hypotheses, experimental approach and design. Assist PI with volunteer briefs, obtain consent, data collection, management, and analysis and manuscript preparation.

Name(s): Nicholas D. Barringer
Research Role: Associate Investigators
Study Responsibilities: Protocol concept development; formulation of protocol questions, hypotheses, experimental approach and design. Assist PI with volunteer briefs, obtain consent, aerobic exercise testing, data collection, management, and analysis and manuscript preparation.

Name(s): Stefan M Pasiakos
Research Role: Associate Investigators
Study Responsibilities: Protocol concept development; formulation of protocol questions, hypotheses, experimental approach and design. Assist PI with volunteer brief, obtain consent, perform muscle biopsies, catheterization, phlebotomy, exercise testing and interventions, data collection, management, and analysis and manuscript preparation.

Name(s): Marques Wilson
Research Role: Project Coordinator
Study Responsibilities: Supervise, manage, and coordinate study logistics and biological data collection. He will be involved with protocol development and study implementation. He will actively participate in data collection to include catheterization, phlebotomy, exercise testing and interventions, and DEXA scans. He will assist in management, analysis and interpretation of data, as well as preparation of manuscripts and technical reports for publication.

Name(s): Claire Whitney and Adrienne Hatch
Research Role: Study Dietitian
Study Responsibilities: Baseline diet assessments, study diet development, and prepare and administer test diets to volunteers. They will actively participate in data collection to include aerobic exercise testing and interventions, and DEXA scans. She will assist in management, analysis and interpretation of data, as well as preparation of manuscripts and technical reports for publication.

A2.2. Others Involved in the Research, as applicable

Name(s): Nancy Murphy
Research Role: Biological Sample Coordinator
Study Responsibilities: Supervision, management, and coordination of logistics and biological data collection. She will be involved with protocol development, study implementation. Data collection responsibilities will involve sample processing.

Name(s): Christopher Carrigan
Research Role: Research Assistant
Study Responsibilities: Assist with data collection and biological sample processing. Data collection responsibilities will involve with DEXA measurements, phlebotomy, and catheterization.

Name(s): Nicholas Armstrong
Research Role: Research Dietitian
Study Responsibilities: Assist with baseline diet assessments, study diet development, and prepare and administer test diets to volunteers. Assist with DEXA measurements and data collection during exercise testing.

Name(s): Laura Lutz
Research Role: Research Dietitian
Study Responsibilities: Assist with baseline diet assessments, study diet development, and prepare and administer test diets to volunteers. Assist with data collection during exercise testing

Name(s): Anthony Karis
Research Role: Research Assistant
Study Responsibilities: Assist with phlebotomy, catheterization, and sample processing.

Name(s): Rasha Hammamieh and Aarti Gautam
Research Role: Consultants
Study Responsibilities: Drs. Hammamieh and Gautam will receive and analyze coded, de-identified muscle RNA for miR Sequencing and bioinformatics analysis. They will not interact or intervene with study volunteers or have access to personal identifiable information. ***A coded specimen transfer agreement is included with this submission.***

A3. RESEARCH LOCATIONS

USARIEM, Natick MA: The U.S. Army Research Institute of Environmental Medicine (USARIEM) is a DoD research facility within the U.S. Army Medical Research and Materiel Command. It is the Institute responsible for conducting basic and applied research to determine the effects of exposure to environmental extremes, occupational tasks, physical training, deployment, operational stress and nutritional factors on the health and performance of military personnel. The facility contains environmental chambers for controlling temperature and humidity, an environmentally controlled hypobaric chamber, a water immersion laboratory, as well as several dry and wet laboratories for animal and human experimentation. The dry laboratories are capable of a broad range of experiments, including biomechanical analysis, body composition, energy expenditure, muscle strength and endurance. The wet laboratories include general clinical chemistry analyzers, as well as equipment for ELISA, RIA, histology, and molecular biology assays. Each investigator at the facility has a personal computer with software for data management, analysis, presentation and report generation. Their computers are interfaced with a network server for easy, secure data handling and transfer. Pre-study screening and baseline testing will take place at USARIEM for active duty military participants.

SECTION B: RESEARCH METHODOLOGY

B1. ABSTRACT

Understanding how carbohydrate metabolism is altered during exercise and post-exercise with recovery nutrition is necessary for the design and implementation of nutrition interventions aimed to maintain carbohydrate (e.g., glycogen) stores during military operations. The primary objective of this investigation is to determine the influence of carbohydrate availability (e.g., glycogen depletion and repletion) on skeletal muscle microRNA expression, and if changes in circulating microRNA are reflective of changes in skeletal muscle microRNA. microRNA are small non-coding RNA that have been identified as regulators of skeletal muscle plasticity in response to exercise, and have recently been found to be measurable stable analytes within circulation. Little is known regarding the influence of substrate availability, particularly carbohydrate on microRNA expression in skeletal muscle and circulation. This randomized cross-over trial will evaluate the influence of exercise-induced (cycle ergometry) depletion and diet-induced repletion of skeletal muscle carbohydrate stores (e.g., glycogen) on the expression of microRNA in skeletal muscle and circulation in

12 healthy, non-obese, recreational active male and female participants between the ages of 18-39. After a glycogen depletion protocol, volunteers will drink a carbohydrate (CHO: 1.0 $g \cdot kg^{-1} \cdot hr^{-1}$ body mass) or an energy free control (CON) beverage during the 3-hr post-exercise recovery phase to assess the initial phase of glycogen repletion on microRNA expression. For the remainder of the day participants will consume meals ready-to-eat (MRE) components designed to adequately (6.0 $g \cdot kg^{-1} \cdot d^{-1}$ carbohydrate) or inadequately (1.5 $g \cdot kg^{-1} \cdot d^{-1}$ carbohydrate) replenish glycogen stores. Volunteers will return to the laboratory the following day to assess how modified daily carbohydrate intake following glycogen depletion influences microRNA expression. At this time, volunteers will complete 80-min of steady-state (60% VO_{2peak}) cycle ergometry, while consuming carbohydrate at a rate of 1.8 $g \cdot min^{-1}$ to determine of how initiation of exercise with adequate or low glycogen stores effects exogenous carbohydrate efficiency. Following a minimum 7-d washout period participants will return to the laboratory to complete the second arm of the investigation. All testing will take place at the USARIEM research labs in Natick, MA.

B2. BACKGROUND AND SIGNIFICANCE

Carbohydrate Availability and Physical Performance: Carbohydrate is a readily available fuel source in skeletal muscle, providing necessary energy to sustain physical performance (1). The body's ability to store carbohydrate as glycogen is however limited, and can be altered daily by dietary intake, as well as exercise type, intensity and duration (2). Depletion of glycogen stores with prolonged strenuous physical activity is associated with fatigue, resulting in reduced work capacity (3). If glycogen stores are not sufficiently replenished during recovery from exercise, physical performance may be diminished during subsequent exercise bouts. As military personnel often engage in sustained physical activity, with mission requirements limiting time available to eat, service members can experience degraded ability to sustain strenuous physical activity during military operations (4). Understanding how carbohydrate metabolism is altered during exercise and with post-exercise recovery nutrition is crucial for development of nutrition interventions aimed to maintain glycogen stores during military operations to mitigate diminished physical performance.

Molecular Regulation of Carbohydrate Metabolism: The molecular regulation of carbohydrate metabolism in skeletal muscle is a complex network influenced by multiple factors, such as glucose, energy availability, muscle contraction, and hormonal environment (e.g., insulin) (5). Modulation in one or more of these factors results in a shift in the cellular environment, which initiates activation (e.g., phosphorylation / dephosphorylation) of signaling pathways that control glucose uptake and storage (6). Specific to exercise-induced glycogen depletion, diminished glycogen stores stimulate the upregulation of adenosine monophosphate-activated protein kinase (AMPK) to enhance glucose uptake and oxidation. Activation of AMPK phosphorylates AKT substrate 160 (AS160) resulting in glucose transporter 4 (GLUT4) translocation to the cell membrane to increase glucose uptake (7). Upregulation of AMPK also stimulate glucose breakdown (i.e., glycolysis) through elevated hexokinase activity to increase energy availability in the cell (8). Along with AMPK, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is considered a key regulator of energy metabolism (9). When energy availability is limited within the muscle, PGC-1α is upregulated (9). Activation of PGC-1α modulates the activity of its downstream targets, including peroxisome proliferator-activated receptors (PPARs) and estrogen-related receptorα (ERRα), which govern fatty acid β-oxidation and carbohydrate oxidation through the tricarboxylic acid cycle (10). When there is an increased concentration of circulating glucose and insulin with carbohydrate consumption, AMPK and PGC-1α are downregulated to inhibit carbohydrate oxidation and increase carbohydrate storage (11). Elevated insulin concentrations result in insulin binding to its receptor at the membrane of cell, resulting in phosphorylation of insulin receptor substrate 1 (IRS1) which stimulates GLUT4 translocation to enhance glucose uptake and storage (12). Together, these molecular processes tightly regulate glucose uptake, glycolysis, and fatty acid oxidation to ensure adequate energy availability to support cellular function.

microRNA and Carbohydrate Metabolism Recently, a new class of small noncoding RNAs, termed microRNA, has been identified as critical regulators of energy homeostasis and carbohydrate metabolism (13). Through negative inhibition, microRNA can influence molecular processes by binding to target mRNA, resulting in post-transcriptional modifications that repress the translation of protein (14). microRNA-dependent gene regulation is a complex process, as one microRNA can regulate hundreds to thousands of genes (15). The ability for one microRNA to inhibit the expression of a large number of genes allows a single microRNA to repress several genes in a common biological pathway, resulting in robust regulation of an entire molecular process (16). Additionally, one gene can be targeted by multiple microRNA, resulting in cooperative / redundant regulation of a signal molecular process (15). This tight regulation that microRNA exert on cellular networks (17) suggests microRNA have may be an important mechanism controlling skeletal muscle plasticity (18).

Emerging evidence, suggest that altered expression of microRNA targets specific proteins that regulate carbohydrate metabolism. In cell culture experiments, miR-451 has been shown to target an upstream activator of AMPK, resulting in an inhibition of the AMPK signaling cascade (19). Elevation in miR-451 expression appears to be sensitive to glucose availability. When glucose concentrations are low, miR-451 expression is repressed, allowing for increased activation of AMPK (20). Conversely, with high glucose concentrations miR-451 expression is increased, resulting in diminished AMPK (20). Similarly microRNA have been shown to alter normal insulin signaling processes. Cell culture experiments have identified miR-33a/b and miR-126 as inhibitors of IRS (21, 22). Over expression of these microRNA reduce IRS protein content, resulting in insulin resistance and impaired glucose uptake (21, 22). Furthermore, in disease states that altered metabolic demands, such as diet-induced obesity and diabetes, dysregulation of microRNA expression has been observed, with specific microRNA (miR-29a, miR-34a, miR-103, miR-107, and miR-375) predicted to inhibit signaling proteins associated with insulin signaling upregulated (13). Findings from cell culture experiments and disease models indicate that microRNA contribute to regulation of carbohydrate metabolism.

Our group recently found that microRNA which are highly enriched in skeletal muscle (i.e., myomiR) are sensitive to differing exercise modes with or without essential amino acid plus carbohydrate supplementation (unpublished data). In this investigation we found that weighted endurance-type exercise diminished miR-1-3p, miR-206, miR-208a-5p, and miR-499 expression, while conventional cycle ergometry endurance exercise increased microRNA expression. However, when cycle ergometry was combined with ingestion of essential amino acids plus carbohydrate, microRNA expression was either downregulated or remained the same immediately post and during the recovery phase, compared to baseline values. Divergent microRNA were all identified to target insulin signaling, with miR-206, miR-208-5p, and miR-499 were all predicted to target Akt, an intermediate signaling protein between IRS1 and AS160. These findings suggest that diminished microRNA expression immediately following exercise may have a functional role in mediating glucose uptake to replenish glycogen stores. Corroborating our findings, previous investigations have reported the microRNA acutely altered by exercise. Following an acute bout of endurance exercise there is diminished expression of miR-23, while mRNA expression of PGC-1α and its downstream targets are upregulated (23, 24). When miR-23 is overexpressed in skeletal muscle there is a reduction in total PGC-1α mRNA and protein content that results in reduced efficiency of mitochondrial function, an organelle crucial to cellular energy production (23). Furthermore, endurance exercise also downregulates miR-494, another inhibitor of PGC-1α, allowing for increased gene expression of PGC-1α and mitochondrial transcription factor A (25). While interventions have been conducted examining the relationship of microRNA expression to energy sensing genes in response to endurance exercise, no study has investigated the influence of altered glycogen availability in response to dietary manipulation on microRNA expression. Understanding how glycogen availability alters expression of microRNA will enable determination of their functional role in regulating carbohydrate metabolism to support energy availability to fuel physical performance.

Circulating microRNA: Beyond their regulatory function within tissue, microRNA have been reported to be stable and reproducible analytes present in circulation (26). From the cytoplasm, microRNA can be released into the circulation in membrane-derived vesicles (exosomes), complexed with proteins, lipoproteins (cholesterol), or apoptotic bodies (plasma membrane fragments) (27). Once released from the cell, circulating microRNA can function in cell-to-cell communication, indicating an endocrine-like function (28). Though the exact mechanism by which circulating microRNA influence cellular processes is still unknown, it has been observed that alterations in circulating microRNA profiles reflect the underlying physiological state of the tissue (29). In disease states that negative impact skeletal muscle, multiple investigations have shown alterations in circulating microRNA profiles are reflective the underlying physiological condition of skeletal muscle (30-32). In healthy individuals, our preliminary findings indicate that circulating microRNA profiles can be used as a predictive tool to determine anabolic signaling in response to acute resistance exercise in skeletal muscle (33). Use of advanced statistical and bioinformatics analysis identified IGF-1 and mTORC1 signaling as the top canonical pathways upregulated post-exercise. These findings were supported by activation (e.g., phosphorylation) status of mTORC1 signaling in skeletal muscle following resistance exercise. Western blotting results determined that an upregulation in upstream (p-Akt^{Ser473}) and downstream (p-70S6K1^{Thr389}) targets of mTORC1, were positively associated with increased expression of specific circulating microRNA that have been reported to target this pathway. Though these and other recent (34, 35) findings suggest that circulating microRNA profiles are altered by acute exercise, little is known regarding the influence of substrate availability, particularly carbohydrate, on circulating microRNA expression. As microRNA regulate metabolic pathways within skeletal muscle, alterations in circulating microRNA expression profiles may be used as a marker for underlying molecular adaptions within tissue. Though assessment of microRNA expression in skeletal muscle can yield valuable insight into their functional role in energy metabolism, this type of data collection requires invasive muscle biopsies, which is difficult to perform during military field operations. Identification of circulating microRNA as non-invasive markers of carbohydrate metabolism in skeletal muscle has the capacity to signal nutritional readiness of service members.

B3. MILITARY RELEVANCE

Military personnel often engage in sustained physical activity while time to eat and availability of food is limited (4, 36, 37). Under such conditions, it is likely that skeletal muscle glycogen stores are depleted, reducing the service member's ability to maintain an optimal level of physical performance. However, during 'real-world' military training operations there is limited knowledge regarding altered carbohydrate metabolism at the skeletal muscle level, as invasive investigations requiring muscle biopsies cannot be conducted during training operations. Identifying the mechanism, as well as determination of non-invasive markers of molecular pathways regulating carbohydrate metabolism is critical to move findings from controlled laboratory settings to field operations. Modeling of circulating microRNA may allow for a more comprehensive understanding of nutrient adequacy during military operations, allowing for implementation of targeted nutrition interventions to optimize military performance. Support for this protocol comes from Defense Health Program (DHP) Research funds, under Joint Program Committee-5 Nutrition and dietary supplements working group.

B4. OBJECTIVES/SPECIFIC AIMS/RESEARCH QUESTIONS

Objectives

1. Determine the influence of carbohydrate availability (e.g., glycogen depletion and repletion) on skeletal muscle microRNA expression, and if changes in circulating microRNA are reflective of changes in skeletal muscle microRNA.

2. Determine how initiation of exercise with adequate or low glycogen stores effects exogenous carbohydrate efficiency.

Hypotheses

1. Expression of microRNA in skeletal muscle will be influenced by alterations in glycogen status, and that these changes in skeletal muscle microRNA will be associated with modulation in circulating microRNA.

2. Exogenous carbohydrate efficiency will be greater when exercise is initiated with lower glycogen stores. Increased efficiency will be associated with higher phosphorylation of IRS-1 and AMPK.

B5. RESEARCH PLAN

B5.1 Research Design

This study will be a randomized cross-over placebo control trial.

B5.2 Research Subjects/Population(s)

B5.2.1 Subject Population(s)

Subject population will be representative of active duty male and female service members, being in good health and recreationally active.

B5.2.2 Number of Subjects, Records, and/or Specimens

To complete testing on the 12 volunteers necessary to reach statistical power, we estimate we will need to consent 44 individuals. All screening will stop once complete data has been collected on 12 volunteers. Records and specimen collection are described in the Research Procedures and Data Collection sections. During briefings and consenting potential participants will be informed that even though they may be eligible and want to participate, if we are able to obtain enough data from preceding subjects, they may not ultimately be tested.

B5.2.3 Inclusion Criteria

- Men and women aged 18 – 39 years
- Weight stable (±5 lbs) for at least 2 months prior to the start of the study
- Body mass index (BMI) between 18.5-30 kg/m^2
- Recreationally active based on assessment of physical activity history 2-4 days per week aerobic and/or resistance exercise
- Refrain from taking any NSAIDS (i.e., aspirin, Advil®, Aleve®, Naprosyn®, or any aspirin-containing product for 10 days before and at least 5 days AFTER each muscle biopsy. (*Tylenol® or acetaminophen is ok to use if needed for discomfort)
- Refrain from the use of alcohol and nicotine for the duration of the study
- Females must be on contraception (e.g., oral birth control, NuvaRing®, Depo Provera®, etc.)
- Supervisor approved leave status for federal civilian employees working within the US Army Natick Soldier Systems Center

B5.2.4 Exclusion Criteria

- Metabolic or cardiovascular abnormalities, gastrointestinal disorders (i.e., kidney disease, diabetes, cardiovascular disease, etc.)
- Disease or medication (i.e., diabetes medications, statins, corticosteroids, etc) that affects macronutrient utilization and/or the ability to participate in strenuous exercise
- Allergies or intolerance to foods (including but not limited to lactose intolerance/milk allergy), vegetarian practices, or medications (including, but not limited to, lidocaine or phenylalanine) to be utilized in the study
- Anemia (HCT < 38) and Sickle Cell Anemia/Trait
- Abnormal PT/PTT test or problems with blood clotting
- Present condition of alcoholism, use of nutritional/sports supplements, anabolic steroids, or other substance abuse issues
- Musculoskeletal injuries that compromise the ability to exercise
- Blood donation within 8 weeks of beginning the study
- Pregnancy

B5.3 Research Procedures

Study Design

Following a 10-hr overnight fast, a baseline blood draw will be conducted. A baseline muscle biopsy, obtained from the lateral portion of the vastus lateralis, will be collected under local anesthetic (lidocaine). Participants will rest for 15-min before beginning a 5-min warm-up on a cycle ergometer at 50% peak power output (determined from screening VO_{2peak} test). A glycogen depletion protocol (38) will then be initiated with participants cycling at various intensities until failure. A second muscle biopsy will be performed immediately after completion of glycogen depletion. This biopsy will allow for confirmation of glycogen depletion and allow for the assessment of the initial impact of exercise-induced glycogen depletion on microRNA expression. Participants will then consume a carbohydrate (CHO: 1 $g \cdot kg^{-1} \cdot hr^{-1}$) or an energy free control (CON) beverage matched for taste and color during the first 3-hr post glycogen depletion. Blood will be drawn from a catheter every 30-min post-exercise to assess changes in serum glucose and insulin. At 3-hr post-exercise a third muscle biopsy will be conducted to assess acute effects of carbohydrate consumption on microRNA during the initial phase of glycogen resynthesis during the early phase recovery from exercise. For the remainder of the day participants will consume meals ready-to-eat (MRE) components designed to adequately or inadequately replenish glycogen stores. On the study day in which participants receive the carbohydrate beverage, daily intake will be provided as 6.0 $g \cdot kg^{-1}$ carbohydrate, 1.2 $g \cdot kg^{-1}$ protein, 1 $g \cdot kg^{-1}$ fat. On the study day in which participants receive the placebo beverage, daily intake will be provided as 1.5 $g \cdot kg^{-1}$ carbohydrate, 1.2 $g \cdot kg^{-1}$ protein, 3.0 $g \cdot kg^{-1}$ fat. Following a 10-hr overnight fast, participants will return to the laboratory for a fourth muscle biopsy. As 24-hrs is sufficient time to replenish glycogen stores (1), the fourth muscle biopsy with modified daily carbohydrate intake will allow for isolation of the effect of carbohydrate availability on microRNA expression. Volunteers will then complete 80-min of steady-state (~60% VO_{2peak}) cycle ergometry consuming carbohydrate at a rate of 1.8 $g \cdot min^{-1}$ enriched with U-^{13}C-glucose and U-$^{13}C_6$-D-fuctose to determine exogenous carbohydrate efficiency. A final biopsy will be taken at the conclusion of the exercise bout. This fifth biopsy will be conducted to assess the impact of initiating steady-state exercise with adequate or low glycogen stores on molecular markers of glucose uptake and substrate utilization. Following a minimum 7-d washout period volunteers will return to the laboratory to complete the second arm of the investigation. To ensure glycogen stores are similar between volunteers on testing day, 48-hrs prior to testing all volunteers will complete a glycogen depletion protocol and then consume a diet providing 6.0 $g \cdot kg^{-1}$ carbohydrate to replenish glycogen stores.

Figure 1. Study Design

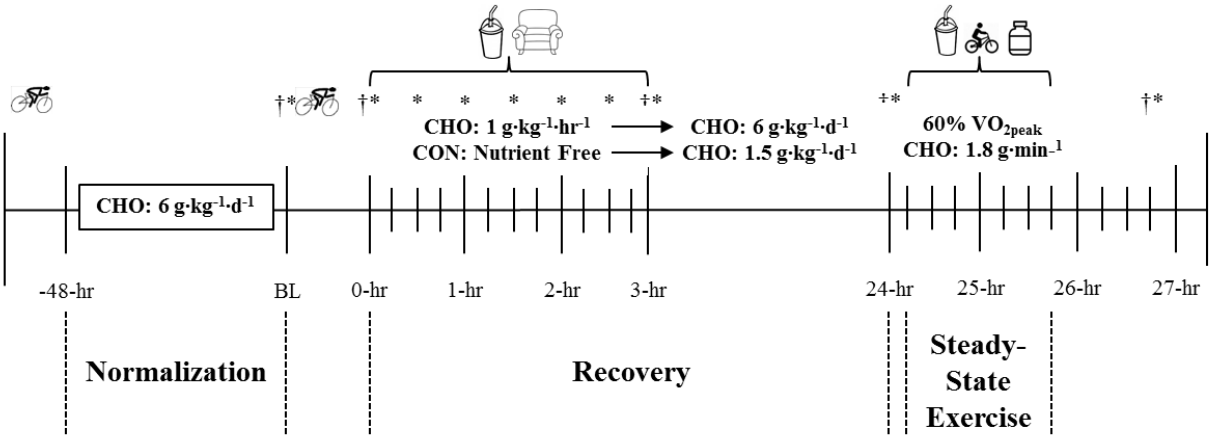

Figure Legend:
BL = Baseline
CHO = Carbohydrate
CON = Control
= Glycogen Depletion
= Carbohydrate or control drink
† = Muscle Biopsy
* = Blood Draw
= Steady-state (60% VO2peak) exercise
= ^{13}C-glucose / $^{13}C_6$-D-fructose
= Resting

Research Procedures

Diet Intervention

Volunteers will complete a baseline 3-d diet record (**Appendix A**) and a 3-d activity log (**Appendix B**) according to instructions provided by study team dietitians (39). The data will be analyzed using Food Processor SQL™ (Salem, OR Version 10.0) and the American College of Sports Medicine (ACSM) Compendium of Physical Activities, respectively. Information for these forms will be collected to estimate volunteers total daily energy requirements. To ensure all volunteers begin the protocol day with similar glycogen stores, following glycogen depletion 48-hrs before testing, volunteers will be provided with food and beverages (except water) containing 6.0 g carbohydrate ·kg^{-1}·d^{-1} to replete glycogen stores. Meals will be derived from military combat rations (MRE) and supplemental food items. Example of energy and macronutrient content will be consistent with ACSM dietary guidelines (**Table 1**) (40).

On glycogen depletion protocol days, volunteers will receive standardized meals derived from MRE components and supplemental carbohydrate-based food items. Meals will either provide adequate carbohydrate (6.0 g·kg^{-1}·d^{-1}) to replenish glycogen stores or provide low carbohydrate (1.5 g·kg^{-1}·d^{-1}) so glycogen stores remain below baseline concentrations (**Table 1**). Study dietitians will prepare and administer meals to volunteers. In this cross-over study the order of carbohydrate intake amount will be randomized to avoid order bias.

Table 1. Energy and Macronutrient Intake for Lead-in Diet and Protocol Days

	Lead in Diet	Protocol Days	
		Adequate CHO	Low CHO
Energy ($kcal \cdot d^{-1}$)	3024	3024	3024
Protein ($g \cdot kg^{-1} \cdot d^{-1}$, $kcal \cdot d^{-1}$)	1.2 (384)	1.2 (384)	1.2 (384)
Carbohydrate ($g \cdot kg^{-1} \cdot d^{-1}$, $kcal \cdot d^{-1}$)	6.0 (1,920)	6.0 (1,920)	1.5 (480)
Fat ($g \cdot kg^{-1} \cdot d^{-1}$, $kcal \cdot d^{-1}$)	1.0 (720)	1.0 (720)	3.0 (2160)

Values based on an 80 kg individual

Volunteers will be instructed to maintain their normal physical activity during the study. However, volunteers will be required to refrain from any additional physical activity outside of the study (resistance and endurance exercise) 48-hrs before the glycogen depletion protocol days. Restriction of physical activity will minimize complications and the potential for carryover effects that may influence study outcomes.

Anthropometric Data

Anthropometrics, performed using standardized techniques and equipment, will be used to determine volunteer eligibility and characterize study participants. Height will be measured to the nearest 0.1 cm using a stadiometer at screening. Body mass will be measured, semi-nude and after an overnight fast (≥ 8 hr), using a calibrated digital scale to the nearest 0.1 kg at screening. Body mass will be measured at baseline, the morning of and the morning after glycogen depletion protocol days.

Body composition will be determined using dual energy x-ray absorptiometry (DEXA, DPX-IQ, GE Lunar Corporation, Madison, WI). The DEXA technique allows for the non-invasive assessment of soft tissue composition by region with a precision of 1-3% (41). The volunteer will lay face-up on the DEXA densitometer table in shorts, t-shirts, and stocking feet. Volunteers will be asked to remain motionless for the 8-10 min scan. These data will be used to calculate total body mass, fat-free mass, and fat mass. Calibration to external standards will be performed before actual data collection. The operator remains in the room with the volunteer during the scan.

Determination of Peak Oxygen Uptake

Peak oxygen uptake (VO_{2peak}) will be determined by a cycle ergometer test using a computer-based metabolic system (True Max 2400, Parvomedics, Sandy, Utah, USA). Volunteers will be instructed to fast overnight (≥ 8 hr) before testing. Volunteers will be clothed in appropriate athletic attire and perform this assessment at standard ambient indoor temperature (20-22°C) and humidity conditions (30-80%). Following instructions on testing procedures, the volunteer will be allowed a 5-min warm-up pedaling at 70W. At the start of testing, the volunteer will put on a nose clip and a mouthpiece connected to a 2-way respiratory valve, which is attached to a head piece to hold it in place. Every minute, workload intensity will be progressively increase by 30W until the volunteer is fatigued or unable to maintain a pedaling rate that either maintains or increases O_2 consumption. Heart rate will be monitored using a heart-rate monitor (Polar Electro Inc, Oulu, Finland) the last 30 seconds of each workload. The test will be stopped immediately if the subject reports angina-like symptoms, exertional syncope, shows signs of poor perfusion (i.e., light-headedness, confusion, ataxia, pallor, cyanosis, nausea, or cold and clammy skin), or if testing equipment fails.

Glycogen Depletion Protocol

The glycogen depletion protocol will be completed on a cycle ergometer, with intensity based on results from VO_{2peak} assessment. Volunteers will begin with a 5-min warm-up at 70 watts before beginning the glycogen depletion protocol. After the warm-up period volunteers will complete 2-min of high-intensity

cycling (work period) at 90% VO_{2peak}, followed by 2-min recovery period where volunteers will cycle at 50% VO_{2peak}. This work-to-recovery ratio will be maintained until volunteers are no longer able to complete 2-min of cycling at 90% VO_{2peak}, determined as the inability to maintain a cycling cadence of 60 rpm for 15-sec. Cycling intensity will then be lowered to 80% VO_{2peak}. When the volunteer is unable to complete 2-min of cycling at 80% VO_{2peak}, cycling intensity will be lowered to 70% VO_{2peak}. Once the volunteer can no longer complete 2-min of cycling at 70% VO_{2peak}, cycling intensity will be lowered to 60% VO_{2peak}. The glycogen depletion protocol will be terminated once the volunteer is unable to complete 2-min of cycling at 60% VO_{2peak}. For each drop in the intensity of the work period, the cycling intensity during the recovery period will be maintained at 50% VO_{2peak} for 2-min. This exercise protocol will maximally deplete muscle glycogen stores (38). Prior to the protocol day, volunteers will perform two practice sessions to ensure they are familiar with the glycogen depletion protocol procedures. Following familiarization volunteers will complete four glycogen depletion protocols, two 48-hrs before testing and two testing days.

Volunteers will be allowed to consume water *ad libitum* during the glycogen depletion protocol. Exercise will be conducted in a temperature controlled room. Heart rate will be monitored using a heart-rate monitor and the session will be terminated if the subject reports any discomfort (i.e. angina-like symptoms or exertional syncope) or shows signs of poor perfusion, or if there is an equipment failure.

Muscle Biopsy

Percutaneous muscle biopsy will be performed to obtained skeletal muscle samples for assessment of muscle glycogen, microRNA expression and molecular pathways regulating carbohydrate metabolism. Muscle samples will be taken from the vastus lateralis using a 5-mm Bergstrom needle with manual suction while the participant is under local anesthesia (1% lidocaine) according to the approved USARIEM SOP (42, 43). Multiple passes with the biopsy needle may be necessary to obtain adequate sample with each biopsy. Muscle biopsies will be conducted immediately before and after the muscle glycogen depletion protocol. After the second muscle biopsy volunteers will rest and consume either a carbohydrate beverage or a matched placebo. Three hours following the conclusion of the glycogen depletion protocol a third biopsy will be obtained to assess the acute influence of carbohydrate intake. Volunteers will be allowed to leave at this point. Volunteers will return the following morning for a fourth muscle biopsy taken at a new incision site. In this same incision a fifth biopsy will be taken after 80-min of steady state exercise to determine the influence of adequate or low glycogen storage on exogenous carbohydrate uptake and oxidation during exercise. Following a minimum 7-d washout period, volunteers will return to the laboratory to complete the second arm of the investigation. A total of ten muscle biopsies will be conducted per volunteer.

Assessment of Resting Respiratory Quotient

Respiratory Quotient (RQ) will be measured under resting conditions before the 24-hr post glycogen depletion muscle biopsy, using open circuit indirect calorimetry (Parvo Medics). Assessment of RQ will allow us to determine the influence of glycogen storage on carbohydrate and fat oxidation. Measurements will occur between 0500-0800-hrs following a minimum 10-hr overnight fast. Volunteers will rest in the supine position for approximately 30-min prior to each measurement in a quiet and dim, temperature regulated room. To minimize error, volunteers will be instructed to minimize movement once the mouthpiece is inserted, or the mask/hood is placed over their heads to collect expired air. The test will be discontinued when 10-min of steady state oxygen consumption (VO_2) and carbon dioxide production (VCO_2) are recorded.

Steady-State Exercise

After the fourth muscle biopsy volunteers will complete 80-min of steady-state exercise on the cycle ergometer at 60 ± 5% of their $VO_{2peak.}$ During exercise VO_2 and VCO_2 will be measured for 4-min using a metabolic cart (Parvo Medics) at approximately 0, 15, 30, 45, 60, and 75-min to determine substrate oxidation. HR (measured continuously; Polar Electro) will be recorded at the same times as the metabolic

cart measurements. Breath samples will be collected at approximately 0, 20, 40, 50, 60, 65, 70, 75, and 80-min to determine exogenous carbohydrate oxidation using single-patient breath collection bags (Quin-Tron Instrument Company, Milwaukee, WI, USA). Breath will be transferred to 20 mL evacuated tubes.

Carbohydrate Supplementation

After the post glycogen depletion muscle biopsy, volunteers will drink a CHO (glucose and fructose in a 2:1 ratio) or CON beverage (Combat Feeding, Natick, MA. Carbohydrate will be provided at 1 $g \cdot kg^{-1} \cdot hr^{-1}$ in accordance with current American College of Sports Medicine Guidelines (40). The CON beverage will be sugar free and void of other nutrients to allow for assessment of the independent effect of carbohydrate availability on skeletal muscle and circulating microRNA expression. Beverages will be consumed immediately following the post glycogen depletion muscle biopsy and then every 30 min thereafter (5 times) until the 3-hr post glycogen depletion muscle biopsy.

Additionally, all participants will consume the CHO beverage at 1.8 $g \cdot min^{-1}$ during steady-state exercise to examine the impact of glycogen availability on exogenous carbohydrate oxidation efficiency. Volunteers will consume the beverage immediately before the start of the exercise and then again at 20, 40, and 60-min during the exercise bout. The CHO beverage consumed during exercise will be enriched with 200 mg of U-^{13}C-glucose and U-$^{13}C_6$-D-fructose (Cambridge Isotope Laboratory, Andover, MA, USA) to significantly increase the isotopic enrichment above natural levels and optimize the measurement of exogenous carbohydrate oxidation.

B5.4 Data Collection

Blood Sampling

Blood samples will be collected using intravenous access by a USARIEM credentialed phlebotomist on glycogen protocol days at baseline (before glycogen depletion), immediately following and every 30 min after the glycogen depletion protocol to assess circulating glucose and insulin (**Figure 1**). Baseline and 24-hr post glycogen depletion blood sampling will occur after a 10-hr overnight fast. A total of 120 mL of blood will be sampled from each volunteer during each glycogen depletion protocol day study (**Table 2**). Blood samples will be used to assess nutrient status (glucose and insulin) and circulating microRNA.

Biochemical indices of metabolic homeostasis will be measured from stored samples at USARIEM. Unused serum will be retained for future use if subject provide permission in the informed consent document. Samples will be stored at USARIEM using subject identification numbers.

Table 2. Volume of Blood Sampled for Glycogen Protocol Day

Tube	Number of Tubes	Total Volume (mL)
10 mL Red Top Tube	5	50
7 mL Red Top Tube	10	70
Total	**15**	**120**

Breath Sample Processing for Isotopic Enrichment

Measurement of $^{13}C/^{12}C$ in expired CO_2 will be measured using isotope-ratio mass and cavity ring-down spectroscopy (Metabolic Solutions, Inc., Nashua, NH).

Calculations of Carbohydrate and Fat Oxidation

Fat and CHO oxidation will be calculated from VO_2 and VCO_2 measured using open circuit indirect calorimetry (4 min collection), neglecting the contribution of protein oxidation to the energy yield (44):

Fat oxidation (g/min) = 1.695 x VO_2 (L/min) – 1.701 x VCO_2 (L/min)

Glucose oxidation (g/min) = 4.585 x VCO_2 (L/min) – 3.226 x VO_2 (L/min)

Calculations of Exogenous and Endogenous Glucose Oxidation

Exogenous glucose oxidation can be calculated:

Exogenous glucose (g/min) = VCO_2 [(Rexp - Rref)/(Rexo - Rref)]/k

where VCO_2 is in L/min, Rexp is the observed isotopic composition of expired CO_2, Rref is the isotopic composition of expired CO_2 at rest before ingestion of the first dose of ^{13}C-glucose and U-$^{13}C_6$-D-fructose, Rexo is the isotopic composition of the exogenous glucose ingested, and k (0.747 L/g) is the volume of CO_2 provided by the complete oxidation of glucose. Endogenous glucose oxidation can be calculated by subtracting exogenous glucose oxidation from total CHO oxidation. The first 40 min of steady-state exercise will allow for equilibration between the $^{13}C/^{12}C$ in expired CO_2 and the $^{13}C/^{12}C$ in CO_2 produced in tissues (45). Thus, endogenous glucose oxidation will only be calculated from samples obtained in the last 40 min of steady-state exercise (40 to 80 min).

Muscle Glycogen

Approximately 20 mg of muscle will be dehydrated in a freeze dryer. Samples will then be ground to powder and visible connective tissue will be removed. Powdered muscle will be placed in 500 µl 2 N HCl. Samples will then be placed in an incubator for 120 min at 100˚C. Following incubation samples will be neutralized with 1500 µl 0.67 N NaOH. Muscle glycogen will be in solution at this point. Glycogen will be quantified by a fluorometric assay (Sigma-Aldrich, St. Louis, MO, USA).

Intramuscular Triglyceride Concentrations

Intramuscular triglyceride concentrations will be determined using the Folch method (46). Dehydrate muscle will be homogenized in a 2:1 chloroform-to-methanol solvent. Following extraction, samples will be saponified in ethanolic KOH at 60 °C, and glycerol content will determined using a commercially available colorimetric assay (Sigma)

mRNA and microRNA Expression

Total RNA will be isolated from approximately 25 mg of muscle using a mirVana™ miRNA isolation kit (Invitrogen, Carlsbad, CA, USA). Quantity and quality of RNA will be assessed using a Nanodrop ND-1000spectrophotometer (Nanodrop, Wilmington, DE, USA). Equal amounts of total RNA will be synthesized into cDNA for analysis of mRNA (iScript™ Advanced cDNA Synthesis Kit; Bio-Rad) and a TaqMan® microRNA RT kit (Applied Biosystems, Foster City, CA, USA). Individual primers will be used to determine the mRNA expression of known intracellular targets regulating muscle metabolism, to include but not limited to PGC-1α, SIRT1, ACC, AMPK, PDK4, IRS1, and GLUT4. miRNA (20-40 bp) will be size-selected from total RNA using Illumina's protocol (US Army Center for Environmental Health Research, Frederick, MD). The sorted miRNA will be tagged and assayed using the Illumina NextSeq instrument. Briefly, 500 ng total RNA will be used to construct sequencing libraries. Subsequently, the samples will be amplified using index-tagged primers to facilitate multiplexing. The amplified cDNA constructs will be size-selected, quality controlled before loading to the NextSeq instrument to generate 50 base reads. Image analysis and base calling will be performed using the Illumina pipeline.

Preprocessing of raw base calls, sample de-multiplexing, trimming and filtering will be similar to that described in 'Analysis of sequencing output'. For the miRNA quantification/enrichment, reads are first aligned to the Rfam RNA database to filter and profile other small RNA species in the samples. Then reads are mapped for small RNA annotation against reference zebrafish mature mature RNA sequences from the miRBASE database. The miRNA read count matrix for each sample will be generated using open source tools such as PICARD.

Following identification of skeletal muscle microRNA that had a significant change, Taqman® probes (Applied Biosystems) will be used to assess the expression of these microRNA in serum to determine their potential use as noninvasive markers of altered carbohydrate metabolism following glycogen depletion and repletion. Total circulating miRNA will be extracted from 200 µL serum using miRNeasy Serum/Plasma kit, which allows for extraction and purification of small (< 200 nt) cell-free RNA (Qiagen, Valencia, CA, USA). Exosomal circulating miRNA will also be extract from 1000 µL serum using miRCURY™ RNA Isolation Kit to measure miRNA. To avoid introduction of potentially contaminating material, prior to RNA extraction serum samples will be centrifuged for 10 min at 4°C to remove cellular debris. Supernatant will be removed and transferred to a new tube without disturbing the pellet. Due to the small amount of RNA in the serum, 3.5 µL of a Spike-In Control (C. elegans miR-39; Qiagen) will be added to all samples prior to extraction of RNA to determine the yield of template recovered. After extraction 3 µl of serum RNA will reverse transcribed using the TaqMan® microRNA RT kit (Applied Biosystems) with miRNA-specific stem-loop RT primers pooled in 1X-Tris-EDTA (TE) buffer for a final dilution of 0.05X. A pre-amplification step will be performed after reverse transcription to increase cDNA template using a primer pool of 20 X Taqman® Small RNA Assays (Applied Biosystems) for miRNA of interest at 0.05X concentration in 1X TE buffer. All serum miRNA will be normalized to the geometric of external (Spike-In Control C. elegans miR-39) and internal controls to allow for both technical and inter-individual normalization (47). Geometric mean of controls will be used to correct for possible outlying values and abundance differences between the different controls (48).

All reverse transcription for mRNA and miRNA, and pre-amplification of serum miRNA will be conducted in a T100™ Thermal Cycler (Bio-Rad, Hercules, CA). A StepOnePlus™ real-time PCR system (Applied Biosystems) will be used to perform all mRNA and miRNA analysis. Fold changes will be calculated using the ΔΔ cycle threshold ($\Delta\Delta C_T$) method as described below in statistical analysis section.

Bioinformatics Analysis

microRNA with significant changes in their expression will be uploaded to DNA Intelligent Analysis (DIANA)-miRPath 3.0 (Alexander Fleming Biological Sciences Research Center [BSRC], Athens, Greece; http://diana.cslab.ece.ntua.gr) to determine potential molecular pathways that these microRNA have previously been reported to regulate. Relevant Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) pathways will be identified using experimentally verified targets from TarBase 7.0 (Alexander Fleming BSRC). Based on findings from this analysis, relevant gene and protein expression of relevant targets will be assessed.

Western Blotting

Approximately 30 mg of muscle will be homogenized in ice-cold buffer (1:10 wt/vol) containing 50 mM Tris–HCl (pH 7.5), 5 mM Na-pyrophosphate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 10% glycerol (v/v), 1% Triton-X, 1 mM DTT, 1 mM benz-amidine, 1 mM PMSF, 10 µg mL−1 trypsin inhibitor and 2 µg mL−1 aprotinin. Homogenate will be centrifuged for 15 min at 10,000 × g at 4°C. Protein concentration of supernatant (lysate) will be determined using 660 nm Protein Assay (ThermoFisher Scientific, Waltham, MA, USA). Phosphorylation status and total protein expression of molecular markers associated with carbohydrate metabolism will be determined using Western blot. Muscle lysates will be solubilized in Laemmli buffer, with equal amounts of total protein (15 µg) separated by SDS-PAGE using precast Tris·HCl gels (Bio-Rad). Proteins will be transferred to polyvinylidene fluoride (PVDF) membranes and exposed to commercially available primary antibodies of intracellular markers involved with muscle metabolism to include but not limited to Akt, p-Akt^{Ser473}, AMPKα, p- AMPKα^{Thr172}, PGC-1α , SIRT1, ACC, p-ACC^{Ser79}, PDK4, IRS1, p-IRS1 ^{Ser302}, GSK-3β, p-GSK-3β^{Ser9}, GSK-3α , p-GSK-3α ^{Ser21}, GLUT4 and PEPCK (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. Labeling will be performed using secondary antibody (anti-rabbit IgG conjugate with horseradish peroxidase; Cell Signaling Technology), and chemiluminescent reagent will be applied (Super Signal, West Pico Kit; Pierce Biotechnology, Rockford, IL, USA). Blots will be quantified using a phosphoimager (ChemiDoc XRS; Bio-Rad) and Image

Lab software (Bio-Rad). To confirm equal protein loading per well a normalizing protein such as GAPDH or *β*-actin will be assessed.

Citrate Synthase Activity

Homogenate from muscle samples prepared for Western blotting will be used to assess citrate synthase activity. Enzyme activity will be determined using a colorimetric assay analyzed on an SpectraMax® M Series Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA), by combining 10 µl of diluted (1:10; 0.1 M Tris HCl pH 8.1) sample to 150 µl of reaction master mix (1 mL DNTB, 3 mg Acetyl CoA, and 8 mL 0.1 M Tris HCl pH 8.1). The reaction was initiated when 10 µl of 10 mM oxaloacetate was added to each well (49). Samples were read at 412 nm. Data were normalized to protein content.

Any use of the data or samples outside of this defined research plan will be submitted as a protocol amendment or a new protocol.

B5.5 Managing Data and/or Human Biological Specimens for this Research

All data and medical information obtained will be considered privileged and held in confidence. Study volunteers will be assigned unique subject identification (ID) numbers that will not contain any personal identifiers such as name, social security number, address, date of birth, zip code, etc. This study subject ID number will be used on all data collection instruments, to include questionnaires, data collection forms(**Appendix D-F**), computer records, etc. A number will be assigned as each volunteer is medically cleared for participation. A master list linking the volunteers' names and ID numbers will be kept in a separate locked file in the principal investigator's or project manager's office, or kept in a computer file with password-protected access restricted to the principal investigator and project manager. When the results of the research are published or discussed in conferences, no information will be included that would reveal identity. De-identified muscle RNA samples for miR sequencing will be shipped USACEHR and breath samples for isotopic analysis will be shipped to Metabolic Solutions via FedEx and stores in these laboratories until analyzed. Once analyzed, there will be no remaining sample for storage. All other study samples will be analyzed at USARIEM and stored in n -80ºC freezer at USARIEM in room 322 or 304. All samples will be stored using the subject identification number.

Only personnel assigned to the research study by the principal investigator will have access to the data. Hard copy data records will be stored for a minimum of three years from the time the study is completed. Electronic data records will be maintained for a period of at least five years after the study has been completed.

B5.6 Managing Data and/or Human Biological Specimens for Future Research

Any use of the samples outside of this defined protocol will be submitted as a protocol amendment or a new protocol. Samples will be retained for future analyses once the protocol is closed if the subject provided permission in the informed consent document.

B5.8 Statistical Analysis

B5.8.1 Sample Size Estimation

Statistical power and sample size were determined using the minimum fold change required for a physiologically relevant difference in microRNA expression. Variance (standard deviation) was based on recent publications assessing change in microRNA expression in skeletal muscle (18, 50, 51) and circulating (34, 35, 52) in response to an acute bout of exercise. Using this information the sample size necessary to determine statistical significance between intervention arms, with a mean fold

change of 1.5 ± 1 is 9 per group, with an alpha of 0.05 and 80% power. In the present study, 12 volunteers will be studied in a repeated measures crossover design to account for potential greater inter-individual variability.

B5.8.2 Data analysis

Statistical analyses will be conducted using either SPSS (IBM Corp. Armonk, NY), SAS 9.3 (SAS Institute Inc., Carey, NC), or equivalent. Common descriptive statistics will be used to describe volunteer characteristics. Shapiro-Wilk tests will be used to determine normality of data. Mixed model repeated measures ANOVA will be performed to determine main effects of time (baseline, immediately, 3-hr, and 24-hr post glycogen depletion), carbohydrate intake (6.0 $g \cdot kg^{-1} \cdot d^{-1}$ versus 1.5 $g \cdot kg^{-1} \cdot d^{-1}$) and time-by-carbohydrate interactions for skeletal muscle and circulating microRNA, mRNA expression, and markers of nutritional status. If interactions are significant, Bonferroni correction will be used to examine these relationships. Correlation coefficients and multiple regression analysis will be used to evaluate relationships between study outcome measures. The alpha level will be adjusted for multiple comparisons, with the level for statistical significance set at $P < 0.05$.

SECTION C: HUMAN RESEARCH PROTECTIONS

C1. RECRUITMENT AND CONSENT

C1.1 Identification and Selection of Subjects

Potential volunteers will be recruited from the U.S. Army Natick Soldier Center pool of military volunteers during a face-to-face briefing as described in the below *Recruitment Process* section. Additional volunteers will be recruited from civilian population and active duty military stationed at the U.S. Army Natick Soldier Center, as well as civilians from the local surrounding area using flyers and electronic posting (**Appendix G**)

C1.2 Recruitment Process

Superiors of Service members (e.g., unit officers, senior NCOs, and equivalent civilians) / supervisors of DoD civilians (e.g., military and civilian supervisors or anyone in the supervisory structure) shall not be present at any recruitment sessions or during the consent process in which members of units under their command / personnel under their supervision are afforded the opportunity to participate as human subjects of research.

For Soldiers in the U.S. Army Natick Soldier Center pool of military volunteers, the Principal Investigator will furnish a copy of the consent form to the Human Research Subjects Program Coordinator or designee. The Coordinator will schedule the consent briefing for the military human research volunteer platoon and will serve as ombudsman during the briefing. Civilians and permanent party military will be recruited for the study using posted or electronically distributed or though informational briefs. Principal Investigator or Project Coordinator will receive and respond to inquiries submitted from these recruitment materials, and will schedule informed consent briefings for these potential volunteers. Potential volunteers not in the U.S. Army Natick Soldier Center pool of military volunteers will be briefed one-on-one by the Principal Investigator or Project Coordinator.

C1.3 Eligibility

All potential volunteers will complete the background questionnaire (**Appendix H**). Volunteers must be medically cleared by the Office of Medical Support and Oversight (OMSO) for participation in accordance with USARIEM procedures outlined for screening volunteers for research involving exercise. Potential military and civilian participants will undergo the same clearances used at USARIEM for research involving maximal aerobic exercise testing. In addition, volunteers will be screened for problems with blood clotting, including prothrombin time (PT)/ partial thromboplastin time (PTT), which is a specific criterion for research involving muscle biopsies. Health problems identified during the screening process will be documented and a copy provided to the volunteer. The volunteer will be encouraged to make an appointment with their primary care provider for a full evaluation of the problem. Volunteers with evidence of any physical, mental, and/or medical conditions that would make the proposed studies relatively more hazardous will be excluded. Any personal health information collected during this screening process will be destroyed at the time of study withdrawal or at the completion of the study.

All females will be given a urine pregnancy test during the initial screening and the morning of or at least 24 hours prior to each DEXA scan. The test will be read by a female member of the study team and the results will be shared with the volunteer. If the pregnancy test is positive, the volunteer will be excluded from further participation in this study.

All volunteers must be willing to consume only food and beverages provided by study staff during the 48-hr normalization period and on glycogen depletion protocol days. Additionally, volunteers must be willing to refrain from any addition exercise during this period.

C1.4 Consent Process

Informed consent documents will be provided to each prospective volunteer in writing, as well as in an oral group presentation by the principal investigator or his designee. The purpose of this study, procedures involved, risks, and expectations of volunteers will be explained. The principal investigator or designee will answer all group and private questions. Interested participants will sign the informed consent form prior to undergoing initial screening for which they will have blood drawn for study-specific clearance. If they meet all the medical selection criteria after completing the screening health assessment, and attending the consent information meeting, they will begin the pre-study assessments including measurements of VO_{2peak}, DEXA, and dietary and activity logs. A copy of the informed consent will be provided to the volunteer with the original kept for study documentation. No study procedures will occur prior to the volunteer giving informed consent. Volunteers who have already consented will be informed of any new information or changes to the protocol that may affect their willingness and ability to continue participation in the study using an approved consent addendum.

C1.4.1 Research involving subjects with cognitive impairment or who lack capacity to provide informed consent: N/A

C1.4.2 Research involving non-English speaking subjects: N/A

C1.4.3 Research involving a waiver of the requirement to obtain informed consent OR alteration of the elements of informed consent: N/A

C1.4.4 Research involving a waiver of the requirement for investigator to obtain a signed consent form: N/A

C1.4.5 Waivers of assent or parental permission when the research involves children: N/A

C1.4.6 Research involving data collection for the USAMRMC Volunteer Registry Database

It is the policy of USAMRMC that data sheets are to be completed on all volunteers participating in this research for entry into the U.S. Army Medical Research and Materiel Command Volunteer Registry Database. The information to be entered into this confidential database includes name, address, social security number, study name, and dates. The intent of the database is twofold: first, to readily answer questions concerning an individual's participation in research sponsored by the USAMRMC; and second, to ensure that the USAMRMC can exercise its obligation to ensure research volunteers are adequately warned (duty to warn) of risks and to provide new information as it becomes available. The information will be stored at the USAMRMC for a minimum of 75 years.

C2. COMPENSATION FOR PARTICIPATION

Military and civilian personnel will receive $35 for each successful blood draw. There are 20 blood draws during the two glycogen depletion protocol days (10 per protocol day). Volunteers completing all 20 draws will receive $700. If a volunteer does not complete the entire study, they will be compensated for the number of successful blood draws they did complete. Volunteers will not be eligible for any other form of compensation during this study.

C3. WITHDRAWAL FROM RESEARCH PARTICIPATION

Participants will be allowed to withdraw at any time without penalty or loss of benefits to which they would otherwise be entitled. An investigator may stop an individual's participation in the study if the participant is unwilling or unable to complete study procedures. An investigator or the medical monitor may also withdraw a participant if the individual becomes ill or injured or it would not be in the participant's best interest to continue.

C4. PRIVACY FOR SUBJECTS

To protect the participant's privacy, any of their research-related records will be labeled or "coded" with an assigned research participant number that will not include their name or social security number. Dr. Lee M. Margolis will keep the link between participant number and the participant's research records in a locked cabinet. Any documents that will require the participant's name, such as the consent form, will be kept in a locked cabinet separate from any research documents that contain the participant's ID number. The principal investigator is the only person who will be able to match the research participant number with any of their personal identifying information.

When the results of the research are published or discussed in conferences, no information will be included that would reveal the participants identity to others. If photographs, videos, or audio-tape recordings of you will be used for educational purposes, your identity will be protected or disguised. All identifiable or recognizable information (e.g., names and faces) will be covered in any photographs unless volunteers agree to sign a photo release form.

C5. CONFIDENTIALITY PROCEDURES FOR RESEARCH RECORDS, DATA, HUMAN BIOLOGICAL SPECIMENS

All data and medical information obtained will be considered privileged and held in confidence. Study volunteers will be assigned unique subject identification (ID) numbers that will not contain any personal identifiers such as name, social security number, address, date of birth, zip code, etc. This study subject ID number will be used on all data collection instruments, to include questionnaires, data collection forms, computer records, etc. A number will be assigned as each volunteer is medically cleared for participation. A master list linking the volunteers' names and ID numbers will be kept in a separate locked file in the principal investigator's or project manager's office, or kept in a computer file with password-protected access restricted

to the principal investigator and project manager. When the results of the research are published or discussed in conferences, no information will be included that would reveal identity. All samples will be stored using the subject identification number. Samples will be shipped for analysis to USACEHR and Metabolic Solutions. Remaining samples will be stored at USARIEM in a -80ºC freezer in room 322 or 304. The volunteers name or other identifiable information will not be included on any data, data collection sheets, specimens, or other research records.

Only personnel assigned to the research study by the principal investigator will have access to the data. Hard copy data records will be stored for a minimum of three years from the time the study is completed. Electronic data records will be maintained for a period of at least five years after the study has been completed.

C6. RISKS OF HARM, MEASURES TO REDUCE THE RISKS OF HARM, AND BENEFITS OF PARTICIPATION

C6.1 Risks of Harm

Research Procedure Name: DEXA Scan
Research Procedure Description: Volunteer will lay face-up on the DEXA densitometer table in shorts, t-shirts, and stocking feet. Volunteers will be asked to remain motionless for the 8-10 min scan.
Research-related Risks: The DEXA scan is an X-ray and is considered to be a no greater than minimal risk procedure. The radiation dose of the whole-body DEXA scan is 0.1 mrem. This dose is equivalent to approximately 1/250 of normal annual background radiation, 1/9 of the radiation received in a transatlantic flight, or 1/30 of the radiation received in a chest X-ray.
Measures to Minimize Risks of Harm: (Precautions, safeguards): A quality assurance check will be completed on the DEXA each day prior to its use; the software will not allow the use of the DEXA densitometer if the quality assurance check fails. All females will be required to have a pregnancy test the day before, or the day of testing. If you are pregnant you will not be scanned, nor will you be able to continue the study.

Research Procedure Name: Indwelling Catheters
Research Procedure Description: A 18-20 g x 1.25 inch needle will be used to guide a catheter into the antecubital vein of the volunteer. The catheter will be attached to saline to keep the line patent for multiple blood draws.
Research-related Risks: The risks of blood sampling are small and usually limited to local bruising or swelling. Also sometimes volunteers feel faint or may faint, and in very rare cases experience a seizure during or right after a blood draw. If the volunteer has had problems with fainting during blood draws in the past, they may be more prone to them during future procedures. If the catheter, the tube that is left in the arm after the needle is removed, becomes clogged at any time during the protocol, we will have to replace this to continue blood sampling and therefore the study. This will require another needle to be inserted into your arm.
Measures to Minimize Risks of Harm: (Precautions, safeguards): Trained technicians will use sterile techniques to place the catheter; however, in spite of being careful there is a chance that the site may become infected. Volunteers should not give blood for eight weeks before or after this study.

Research Procedure Name: Venipuncture
Research Procedure Description: A 21 g x 0.75 inch needle will be used for single blood draws of the antecubital vein.
Research-related Risks: Venipuncture is a routine clinical procedure the medical community commonly uses to obtain blood samples. The immediate complications may be slight pain during the entry of the needle into the skin, possible dizziness, syncope, and in very rare cases experience a seizure. Dizziness or syncope constitutes no long-term harm, and immediate relief is achieved by having the subject put their

head down between their knees or lie down. Additionally, a hematoma may result from the venipuncture, but this is more unsightly than risk producing. Late complications might include thrombosis of the vein due to trauma or infection. These complications are extremely rare.
Measures to Minimize Risks of Harm: (Precautions, safeguards): Participant monitoring, aseptic technique, including sterile disposable blood collection apparatus and adherence to standard medical precautions reduce risk. Trained technicians will perform all venipuncture.

Research Procedure Name: Lidocaine Injection
Research Procedure Description: Roughly 8-10 mL of Lidocaine will be injected using a 25 g needle at the site of the incision, superficially (i.e., skin) and within the vastus lateralis.
Research-related Risks: Slight pain at the site of injection might occur. Although rare, anaphylactic reactions may also occur following administration of lidocaine. Unlikely, but possible side effects could include: dizziness, confusion, shakiness, visual changes, nausea, and unusually slow heartbeat.
Measures to Minimize Risks of Harm: (Precautions, safeguards): Volunteers will be instructed to Notify study coordinator or PI immediately if an allergic (i.e., swelling, itching, rash, hives, difficulty swallowing, or difficulty breathing) reaction occurs. In the case of severe reaction, lidocaine use will be discontinued.

Research Procedure Name: Muscle Biopsy
Research Procedure Description: A small incision will be made in the skin and fascia of the vastus lateralis. A 5-mm Bergstrom biopsy needle will pass through these incisions with manual suction while the participant is under local anesthesia (1% lidocaine) to collect muscle samples.
Research-related Risks: Percutaneous needle muscle biopsies have been established as a non-routine, but safe research procedure. Similar to blood draws, there is a risk that volunteers will feel faint or may faint, and in very rare instances experience a seizure during or right after a muscle biopsy. If the participant has had problems with fainting during blood draws or muscle biopsies in the past, they may be more prone to them during future procedures. There is some risk of post-biopsy infection, which can be minimized by employing correct sterile procedures and carefully instructing volunteers on care of the wound. Nerve damage is possible. Moderate stiffness, hematoma and swelling around the biopsy site may occur following the procedure, but this usually resolves itself within several days. Some minimal scarring will accompany healing of the incision and formation of a hypertrophic scar or keloid is possible. Although this is a rare event in fair-skinned persons, the incidence of hypertrophic scarring or keloid formation associated with healing of a primarily closed skin biopsy site (i.e., one which was closed with sutures immediately afterward) is 5-10% in dark-skinned persons. Complications of bleeding can be reduced by applying direct pressure to the wound following the biopsy. If symptoms should occur, they usually do not interfere with normal walking or heavier exercise. Volunteers with evidence of bleeding diatheses or with local skin infection or irritation, or recent anticoagulant medication (including aspirin), will not be used.
Measures to Minimize Risks of Harm: (Precautions, safeguards): Volunteers will be instructed about precautions against hematoma and infection. They will be given a handout outlining instructions for proper care of the incision site (**Appendix I**). Dr. Margolis will perform the procedure with sterile technique according the USARIEM Muscle Biopsy SOP and record notes for each biopsy (**Appendix J**). The medical staff at USARIEM will follow-up with participants within 3 days post-biopsy to monitor for any sign of infection, bleeding, or hematoma. To minimize the likelihood of hypertrophic scarring or keloid formation, biopsy wounds will be closed as promptly as feasible.

Research Procedure Name: Exercise
Research Procedure Description: All exercise testing in this investigation will occur on a cycle ergometer, cycling at various levels of intensity based on exercise protocol.
Research-related Risks: Exercise is generally considered safe and beneficial for individuals without cardiovascular disease. The U.S. prevalence of fatal events is approximately 1:100,000 to 1:300,000 in competitive high school athletes and increases to 1:15,000 to 1:50,000 in athletes over the age of 35.

Current civilian and military guidelines state that individuals less than 40 years of age who have no symptoms of or known presence of heart disease or major coronary risk factors have a low risk for cardiac complications during vigorous exercise. All volunteers in this study fall into this low risk category. Local muscle discomfort and fatigue may occur in active muscles during and shortly after exercise. Muscle soreness, ranging in intensity from mild to severe, may persist for 1 to 7 days.
Measures to Minimize Risks of Harm: (Precautions, safeguards): Studies have confirmed the safety of maximal exercise testing, particularly among apparently healthy persons without significant cardiovascular risk factors. As a precaution, there will be at least one spotter during all cycle exercise sessions, and heart rate will be monitored in real time during testing. In addition, exercise monitors and test administrators will be CPR-certified.

C6.2 Incidental or Unexpected Findings

Health problems identified during the screening process will be documented and a copy provided to the volunteer. The volunteer will be encouraged to make an appointment with their primary care provider for a full evaluation of the problem. Volunteers with evidence of any physical, mental, and/or medical conditions that would make the proposed studies relatively more hazardous will be excluded.

C6.3 Potential Benefits

There is no direct health or other benefits related to participation in this study

C7. DATA AND SAFETY MONITORING

C7.1 Monitoring

The PI will, with the assistance of Associate Investigators, continuously evaluate recruitment, the informed consent process, adverse events, and protocol adherence and deviations in order to identify unanticipated problems or risks to the volunteers associated with the research. The PI will ensure that the number of volunteers recruited for this study complies with the protocol. The PI or AI will submit a monthly summary of all adverse events to the Research Monitor to determine whether the number of adverse events is excessive for the risks outlined in the research protocol. The PI (or acting PI) and onsite physician or PA will discuss “discontinuation criteria” for individual volunteers as the study progresses, based on their observations of the volunteer during testing or non-testing periods. Every morning, volunteers will be asked the following questions to evaluate their readiness to test.

- How have you been feeling well since the last test in our laboratory (below average, average, above average)?
- Do you have any pain or symptoms to report that may affect our testing today (e.g., sinus congestion, fatigue, muscle soreness, fever, tooth pain)?
- Have you reported all food and beverages consumed in the last 24 h that were not provided to you by study staff?
- What time did you fall asleep last night and awake this morning?
- Did you perform any exercise or physical activities outside of study activities in the last 24 h?

C7.2 Research Monitor (as applicable)

The research monitor for this study is CPT Steven Yoo. This individual is an appropriate subject matter expert not associated with the protocol. The research monitor shall, at a minimum, review all unanticipated problems involving risk to subjects or others, serious adverse events and all subject deaths associated with the protocol and provide an unbiased written report of the event. Other responsibilities may be assigned by the MRMC IRB as needed.

C8. REPORTABLE EVENTS

C8.1 Expected adverse events

An adverse event is defined as any untoward or unfavorable medical occurrence in a human research participant, including any abnormal sign (e.g., abnormal physical exam or laboratory finding), symptom, or disease, temporally associated with the individual's participation in the research, whether or not considered related to the individual's participation in the research.

A Serious Adverse Event is defined as any adverse event temporally associated with the subject's participation in research that is fatal, life-threatening, permanently disabling, requires inpatient hospitalization, or results in congenital anomalies/birth defect, overdose or cancer, or based on appropriate medical judgment, may jeopardize the participant, or may require medical or surgical intervention to prevent one of the above outcomes.

All medical events that the USARIEM Office of Medical Support and Oversight (OMSO) evaluates will be reported to the ORQC. The PI will report all adverse events to the Research Monitor, if one was appointed for the study.

Expected adverse events which are not serious are reported to the IRB at the time of continuing review of the protocol.

C8.2 Unexpected adverse events and unanticipated problems

All unanticipated problems involving risk to subjects or others, and serious adverse events that are unexpected and determined to be at least possibly or definitely related to study participation, will be promptly reported within one working day by phone (508-233-6306/4811) or email (usarmy.natick.medcom-usariem.mbx.usariem-rqc@mail.mil) to the USARIEM ORQC and the Commander. These events will also be reported to the HQ USAMRMC Institutional Review Board within one working day by phone (301-619-6240), or by e-mail (usarmy.detrick.medcom-usamrmc.other.irb-office@mail.mil).

The research monitor is required to review all unanticipated problems involving risk to volunteers or others, serious adverse events and all volunteer deaths associated with the protocol and provide an unbiased written report of the event. At a minimum, the research monitor should comment on the outcomes of the event or problem, and in the case of a serious adverse event or death, comment on the relationship to participation in the study. The research monitor should also indicate whether he or she concurs with the details of the report provided by the study investigator. Reports for events determined by either the investigator or research monitor to be possibly or definitely related to participation and reports of events resulting in death should be promptly forwarded to the MRMC IRB.

C8.3 Adverse device effects: N/A

C8.4 FDA-regulated research under IND and IDE: N/A

SECTION D: REFERENCES

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SECTION E: ABBREVIATIONS AND ACRONYMS

PGC-1α; peroxisome proliferator-activated receptor gamma coactivator 1-alpha, SIRT1; sirtuin 1, ACC; acetyl-CoA carboxylase 1, AMPK; 5' adenosine monophosphate-activated protein kinase, PDK4; pyruvate dehydrogenase kinase 4, IRS1; insulin receptor substrate 1, GLUT4; glucose transporter 4, CHO; carbohydrate, CON; control, AKT; protein kinase B, ERR; estrogen related receptor, PPAR; peroxisome proliferator-activated receptors, DEXA; dual energy x-ray absorptiometry

SECTION F: DoD PRIVACY RULE AND PROTECTED HEALTH INFORMATION (HIPAA)

☒ NA – institution is not a covered entity

☐ NA – will not use or disclose protected health information

☐ HIPAA authorization will be obtained

☐ An application for waiver/alteration of HIPAA authorization will be submitted