PROTOCOL TITLE: Impact of erythropoietin on hematological adaptations and physical performance

SECTION A: RESEARCH TEAM AND LOCATIONS

A1. RESEARCH TEAM

Study Role	Institution/Company and Contact Information
Sponsor	N/A
Principal Investigator	*Name, Rank, and Degree:* Lee M. Margolis, PhD *Title:* Nutritional Physiologist *Institution:* MND, USARIEM *Address:* 10 General Greene Ave Bldg. 42, Natick, MA 01760 *Phone Number:* 508-206-2335 *Email:* lee.m.margolis.civ@health.mil
Associate Investigator(s)	*Name and Degree:* Stefan M Pasiakos, PhD *Title:* Division Chief *Institution:* MPD, USARIEM *Address:* 10 General Greene Ave Bldg. 42, Natick, MA 01760 *Phone Number:* 508-206-2250 *Email:* stefan.m.pasiakos.civ@health.mil

Name and Degree: James P McClung, PhD
Title: Division Chief
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Address: 10 General Greene Ave Bldg. 42, Natick, MA 01760
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Name and Degree: Jess A Gwin, PhD
Title: Nutrition Physiologist
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Name and Degree: Benjamin J. Ryan, PhD
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Project Coordinator

Name and Degree: Julie Coleman, MS, RD, LDN
Title: ORISE Research Fellow
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Ombudsman

Name, Rank, and Degree: Katelyn Guerriere Aaron, MS
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Name, Rank, and Degree: Dina M. Pitsas, MS
Title: ORISE Research Fellow
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A2. ROLES AND RESPONSIBILITIES

A2.1 Principal Investigator

Name: Lee M. Margolis
Study Responsibilities: Dr. Margolis is responsible for the safe and scientifically sound conduct of the study. He will oversee all aspects of the study, ensure safety and ethical treatment of volunteers; 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, muscle biopsies, performing phlebotomy, and exercise testing. Dr. Margolis will be responsible for the conduct of the study in accordance with the protocol. Maintenance of a list of appropriately qualified persons to whom significant study-related responsibilities have been delegated.

A2.2 Associate Investigator(s)

Name(s): Stefan M. Pasiakos, James P. McClung, Jessica A. Gwin, Emily E. Howard, Benjamin J. Ryan
Study Responsibilities: Protocol development; formulation of protocol questions, hypotheses, experimental approach, and design. Assist PI with volunteer briefing, obtaining informed consent, data collection, and sample processing. Associate investigators will also ensure safety and ethical treatment of participants and will contribute to the analysis, interpretation, and publication of the data.

A2.3 Project Coordinator

Name: Julie L. Coleman
Study Responsibilities: Primary responsibilities include assisting with volunteer briefing, obtaining informed consent, data collection, preparing and administering study diets, preparing and maintaining data collection forms, and biological sample processing. She may conduct procedure for which she is qualified and credentialed as outlined in the DOA log.

A2.4 Ombudsman

Name(s): Katelyn Guerriere Aaron and Dina Pitas
Study Responsibilities: The ombudsman shall protect the interests of the research volunteers. The ombudsman will do the following: read the consent form, observe the recruitment briefing and the signing of consent documents, make sure supervisory and command staff are not present, make sure the description of the study and all risks and discomforts are correct, complete, and understandable to the audience, and make sure that the volunteers are not threatened or pressured to participate. She will observe briefings for military participants in the Human Research Volunteer (HRV) program.

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. All testing procedures will take place at USARIEM.

Metabolic Solutions, Nashua, NH: Metabolic Solutions is a state-of-the art stable isotope analytical laboratory that has the basic laboratory facilities and equipment to analyze stable blood. Equipment onsite includes an Agilent 6110 LC-Tandem Mass Spectrometer (LC-MS/MS) Triple Quad with Agilent 1100 LC, Thermo Finnigan Delta XP Isotope Ratio Mass Spectrometer (IRMS) with GC Isolink for ^{13}C, ^{15}N and ^{2}H GC-IRMS and Conflow IV interface, and a Thermo Finnigan Delta V Advantage IRMS with trace GC-Combustion III unit for ^{13}C, ^{15}N and D GC-C-IRMS and Conflow IV interface and elemental Analyzer EA112HT ^{13}C, ^{15}N and D sample combustion unit. Metabolic Solutions has been an industry leader in developing new stable isotope tracer applications. Metabolic Solutions will analyze samples on a fee for service basis.

A4. MULTISITE RESEARCH

***Lead Site**:* N/A

***Performance Site**:* N/A

SECTION B: RESEARCH METHODOLOGY

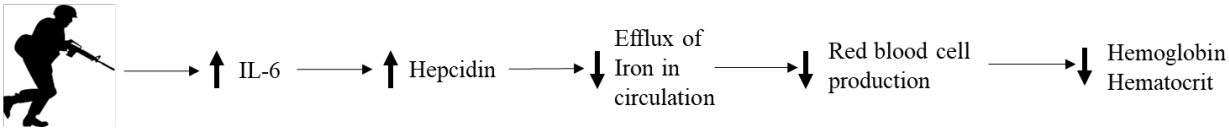
B1. ABSTRACT

Negative hematological adaptations due to prolonged periods of strenuous physical activity may, in part, contribute to declines in physical performance during military operations. Exogenous erythropoietin (EPO) is a potential intervention that may be used to maintain hemoglobin (hgb), hematocrit (Hct), and physical performance during periods of high physical activity. The objective of the current study is to determine the ability of EPO to maintain hgb, Hct, and physical performance compared to baseline measures. Additionally, EPO may result in non-hematological adaptations which increase mitochondria biogenesis and alter substrate oxidation. As such, this study will also assess the influence of EPO on whole-body and skeletal muscle substrate oxidation.. Eight healthy physically active individuals will be recruited to participate in this longitudinal trial. After exercise practice sessions, volunteers will complete baseline physical performance (time trial) and substrate oxidation testing. Participants will then receive EPO injections 3 times per week for 4 weeks. Diet and exercise will be controlled during the injection period. Participants will undergo four weeks of an intense physical training exercise program. Every seventh day during the injection period a safety blood sample, assessing hematocrit, will be drawn, and participants will complete a 5 km time trial to determine the time course of changes in physical performance can be detected. After the 4 weeks of EPO injections volunteers will complete the same physical performance and substrate oxidation testing. Substrate oxidation will be assessed during 90-min steady-state load carriage (30% body mass) exercise on a treadmill at 55 ± 5% of VO_2peak. 6-6-[$^{2}H_2$] glucose tracer technique and indirect calorimetry will be used measure substrate oxidation. Muscle biopsies will be performed to measure muscle glycogen, enzyme activity, and molecular markers of metabolism and inflammation before, and immediately and 3-hrs post exercise. Multiple blood samples will be collected throughout the study to determine alterations in hemoglobin, hematocrit, and markers of substrate metabolism, and inflammation. All study procedures will occur at USARIEM. The primary risks associated with this study include those associated with EPO injection, exercise, blood draws, and muscle biopsies.

B2. BACKGROUND AND SIGNIFICANCE

Overtraining and strenuous military operations result in negative hematological adaptations, with hemoglobin (hgb) concentrations and hematocrit (Hct) values significantly decreased (1-4)(**Figure 1**). These negative effects have been linked to increased circulating concentrations of the pro-inflammatory cytokine IL-6 (5). Increased circulating IL-6 stimulates increased concentrations of the iron-regulatory hormone hepcidin (6, 7). Hepcidin reduces the efflux of iron into circulation by binding to and subsequently degrading the iron transporter protein ferroportin (8). This results in reduced absorption of dietary iron and release of iron from macrophages, inhibiting distribution of iron to tissues in the body (9). Prolonged or multiple bouts of exercise have been shown to result in greater increases of circulating IL-6 (10, 11). Additionally, elevated concentrations of hepcidin can persist for 24 hours post exercise (12). Without adequate recovery, these elevations in IL-6 and hepcidin in response to sustained performance of arduous physical activity results in negative hematological adaptations and potential declines in physical performance. Our laboratory has reported multi-day strenuous military trainings results in increased concentrations of circulating IL-6 and hepcidin (3, 4) and decreased hgb concentrations (3). These negative hematological adaptations to military training has been associated with declines in physical performance (1, 13).

Figure 1
Overtraining



The negative hematological adaptations accompanying strenuous physical training can be partially alleviated by consuming daily iron supplements (13, 14), but hgb and Hct do not fully recover to the levels observed when consuming an iron supplement without performing physical training (14). This blunted response to iron supplementation with training is likely the result of increased hepcidin concentrations inhibiting absorption of dietary iron. Our laboratory recently found following a 3 day simulated military operation, where participants' exercise-induced energy expenditure was ~2000 kcal/d, elevations in IL-6 and hepcidin resulted in a ~50% reduction in iron absorption (15). These physiological consequences to sustained strenuous exercise led to reductions in hgb concentrations and declines in physical performance, despite participants consuming 22 mg/d iron, well above the recommended dietary allowance (RDA) of 8 mg/d for men. Results from this study suggest more robust interventions are required to mitigate associated negative hematological adaptations and declines in physical performance associated with extended periods of strenuous physical activity.

Recognizing the need for more aggressive interventions to the Army has developed the Biomedical Performance Enhancement (BPE) program. One of the goals of this program is to identify pharmaceutical interventions to enhance physical performance. Exogenous erythropoietin (EPO) is one potential intervention that fits within the BPE program to eliminate physiological decrements during military operations. EPO is a hormone produced in the kidney, that stimulates erythropoiesis, production of red blood cells, in bone marrow (16). Stimulation of red blood cell production corrects negative hematological adaptations, increasing hgb concentration and Hct values (16). Exogenous EPO results in higher levels of hgb and Hct compared to placebo (17-19). It is important to note, exogenous EPO requires iron to induce erythropoiesis. Interestingly, administration of exogenous EPO with low systemic iron availability has been reported to reduce circulating hepcidin concentrations by an unknown mechanism, resulting in increased hgb and Hct (20). Furthermore, our laboratory has recently demonstrate that secondary elevations in circulating EPO following exogenous testosterone administration resulted in lower hepcidin and IL-6, and higher hgb and HCT values following 21 days of exercise-induced (~1400 kcal/d) energy deficits compared to placebo while consuming 18 mg/d dietary iron (21);**Table 1**).These finding may suggest that despite reduced iron efflux with overtraining, EPO may still be an effective intervention to maintain hgb, Hct, and physical performance. However, whether elevated IL-6 and hepcidin concentrations with overtraining suppress the effectiveness of exogenous EPO is unknown.

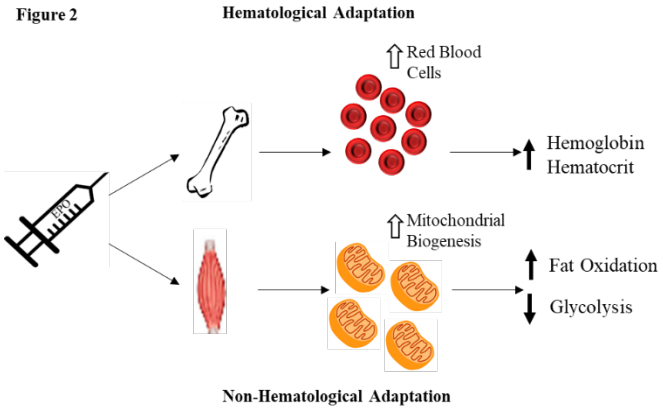
Table 1: Hematological adaptations following 21 days of energy deficit

	Placebo		Testosterone	
	Energy Balance	**Energy Deficit**	**Energy Balance**	**Energy Deficit**
EPO (mIU/mL)	8.8 (7.6, 9.9)[a]	8.5 (7.1, 9.8)[a]	8.4 (7.2, 9.6)[a]	11.3 (9.9, 12.8)[b]
Hgb (g/L)	144 (142, 147)[a]	138 (136, 141)[b]	145 (143, 148)[a]	145 (142, 148)[a*]
HCT (%)	42.8 (42.0, 43.6)[a]	41.5 (40.7, 42.3)[a]	42.9 (42.1, 43.7)[a]	43.6 (42.7, 44.4)[a*]
Hepcidin (ng/mL)	8.1 (6.6, 9.8)[ab]	10.1 (8.5, 11.6)[a]	8.6 (7.0, 10.3)[a]	5.1 (3.4, 6.7)[b*]
IL-6 (pg/mL)	8.5 (6.2, 10.8)[a]	10.2 (7.9, 12.5)[a]	9.2 (6.9, 11.6)[a]	8.5 (6.1, 10.9)[a]

Values mean (95% confidence interval). Data not sharing the same letters are different; $P < 0.05$.
*Different than placebo; $P < 0.05$. Daily exercise induced energy expenditure ~ 1400 kcal/d.

Beyond those well characterized hematological adaptations, exogenous EPO has recently been discovered to result in non-hematological adaptations (22); **Figure 2**). Annaheim et al. (22) reported that changes in physical performance with exogenous EPO were independent of increases in VO_2max and red blood cell volume. Non-hematological adaptations resulting in improved performance may be the result of molecular alterations within skeletal muscle. Exogenous EPO has been shown to increase markers of mitochondrial biogenesis (23) and improve mitochondrial respiratory capacity, increasing oxidative phosphorylation and electron transport capacity (24). However, while some investigations have reported changes in skeletal muscle molecular biology in response to exogenous EPO, there are others that have reported no effect on skeletal muscle (25-27). Controversy regarding the impact of EPO on skeletal muscle revolves around debate of whether an EPO receptor (EPO-R) is present at the skeletal muscle level (28, 29). The absence of EPO-R with skeletal muscle would indicate that there is no direct effect of EPO on skeletal muscle. While it is possible that EPO may not have a direct effect on skeletal muscle morphology, there is potential that EPO has indirect effects that result in alterations in molecular pathways regulating substrate oxidation within skeletal muscle. Exogenous EPO increase fat and decreases carbohydrate oxidation during exercise (19). Alterations in whole-body substrate oxidation during exercise are the result of changes in molecular regulation within skeletal muscle (30). Shifts in substrate oxidation would suggest that alterations do occur within skeletal muscle, if not directly, then potentially due to changes in hormone concentrations of O_2 delivery to skeletal muscle. The influence of exogenous EPO on pathways regulating substrate oxidation within skeletal muscle has not been thoroughly assessed. Additionally, increased capacity to oxidize fatty acids and decreased reliance of carbohydrate for fuel during exercise with exogenous EPO likely results in sparing of muscle glycogen stores. Maintaining high muscle glycogen content has been well-established to improve physical performance (31). Beyond performance improvement, higher glycogen stores may also have additional benefit in reducing the inflammatory response to exercise (5). Muscle glycogen depletion following aerobic exercise results in increased IL-6 gene expression in skeletal muscle, as well as increased circulating IL-6 concentrations compared to higher glycogen content (32). Potential for exogenous EPO to spare muscle glycogen and reduce post exercise inflammation may contribute to lower hepcidin concentrations observed with exogenous EPO administration described above (20).Though some work (19, 24) has been done to show alteration in carbohydrate and fat oxidation following exogenous EPO use, no in-depth analysis has been performed assessing changes in substrate oxidation at the whole body and skeletal muscle level. Specifically, no studies has examined the influence of EPO on glucose kinetics, glycogen storage, or the intramuscular molecular pathways that regulate skeletal muscle oxidation and inflammation. Furthermore, whether non-hematological adaptations with exogenous EPO compensates for potential blunted hematological

Figure 2



adaptations due to reduced iron efflux during overtraining to aid in the maintenance of physical performance has not been explored.

B3. MILITARY RELEVANCE

This study supports the Military Operational Medicine Research Program's Physiological Health and Performance Program Area under the BPE Work Unit. The BPE Work Unit is intent is to execute basic and applied research aimed at identifying and develop biomedical strategies and solutions that safely and ethically enhance Warfighter physical, cognitive, sensory and behavioral performance in training and operational environments, enabling lethality and overmatch. The proposed work aligns to the Physiological Health and Performance Strategic Plan Technical Challenge 1.1.1.1: Identify pharmacological enhancement protocols improving specific task performance under operational stress.

B4. OBJECTIVES/SPECIFIC AIMS/RESEARCH QUESTIONS

Objective

1. Determine the effects of EPO on hematological adaptions compared to baseline during 4 weeks of overtraining.

2. Determine the time course change in physical performance (time trial) with EPO compared to baseline.

3. Assess the influence of EPO non-hematological adaptation (whole-body and skeletal muscle substrate oxidation and glucose turnover) following 4 weeks of overtraining compared to baseline.

Hypothesis

1. Hemoglobin and hematocrit will be maintained with EPO during 4 weeks of overtraining.

2. Physical performance will be maintained with EPO during 4 weeks of overtraining.

3. EPO will increase whole body and skeletal muscle fat oxidation, and reduce reliance on endogenous glucose store following 4 weeks of physical training.

B5. RESEARCH PLAN

B5.1 Research Design

This study will be a longitudinal trial.

B5.2 Research Subjects/Population(s)

B5.2.1 Subject Population(s)

Subject population will be military or civilians 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

A total of 8 volunteers will be required to achieve sufficient statistical power. To complete testing on 8 volunteers, we estimate we will need to enroll 60 individuals. All screening will stop once complete data has been collected on 8 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. These individuals may be recruited from the HRV population, civilians, and active duty military personnel (on the installation).

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 (minimum 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 while on study diets
- Supervisor approval 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, hypertension etc.)
- Personnel or family history of blood clots
- 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 similar)) to be utilized in the study
- History of inflammatory bowel disease
- History of seizures
- 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
- History of malignancy
- Use of oral contraceptives or hormone replacement therapy due to increased risk of clotting
- Musculoskeletal injuries that compromise the ability to exercise
- Blood donation within 8 weeks of beginning the study
- Are unwilling or unable to eat study diets and foods provided and/or follow exercise prescriptions
- Pregnancy, post-partum status, or breastfeeding

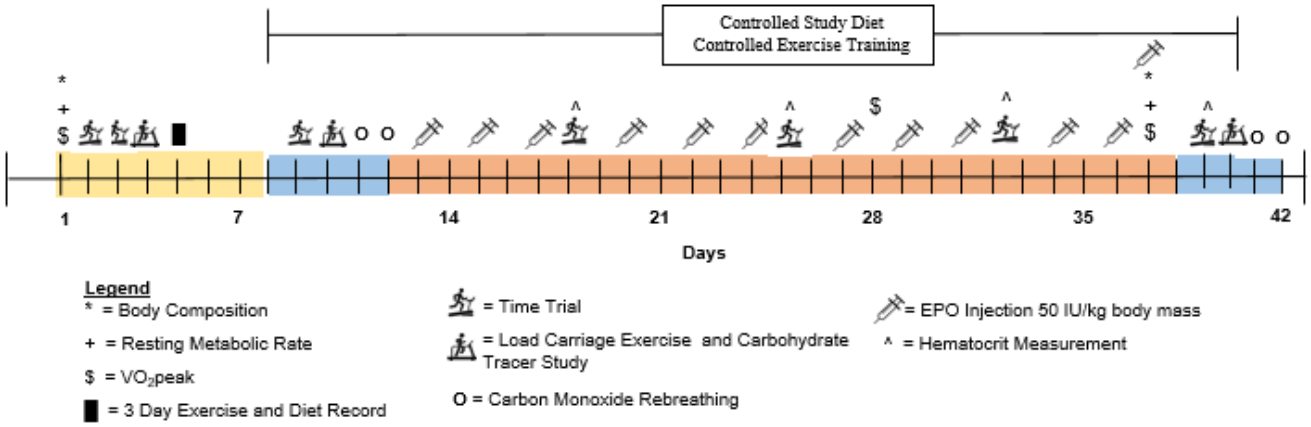
B5.3 Research Procedures

Research procedures conducted by investigators and support personnel will be in accordance with the Delegation of Authority (DOA) Log for Key Research Personnel.

COVID-19 risk mitigation: Study staff and participants will comply with all COVID-19 risk mitigation procedures in place at USARIEM during the time of data collection. As such, participants may be asked to wear face masks and use hand sanitizer during data collection activities (in accord with prevailing recommendations at the time of data collection) and may be asked to wear gloves (i.e., nitrile gloves) during data collection activities.

Study Design Overview: Healthy physically active individuals will be recruited to participate in this longitudinaltrial. After baseline assessments of body composition, resting metabolic rate (RMR), VO_2peak and exercise familiarization; volunteers will complete physical performance and substrate oxidation testing (**Figure 3**). Physical performance will be assessed using time trial. Substrate oxidation will be assessed by volunteers performing 90-min of steady-state load carriage (30% body mass) exercise on a treadmill at 55 ± 5% of their VO_2peak. 6-6-[$^2H_2$] glucose will be used as a tracer to assess glucose turnover. Indirect calorimetry will be used to determine carbohydrate and fat oxidation. Muscle biopsies will be performed to measure muscle glycogen, enzyme activity, and molecular markers of substrate metabolism before, immediately after, and 3-hrs post exercise. Multiple blood samples will be collected on substrate oxidation protocol days. To minimize carry over effects of muscle biopsies on subsequent exercise performance, volunteers will not exercise for 3 days. During the first two days of this period, volunteers will undergo carbon monoxide (CO) rebreathing to measure Hgb mass and blood volume. Volunteers will then receive EPO injections 3 times a week for 4 weeks. During the injection period all volunteers will have safety blood draws to assess Hct once a week. Volunteers will also complete a physical performance test once a week to determine time course change in performance with EPO compared to baseline. Exercise training will be controlled during the injection phase, consisting of a combination of endurance- and resistance-type exercise. During the final week of the injection phase body composition, resting metabolic rate (RMR) and VO_2peak will be reassessed. At the end of the 4-week injection phase volunteers will complete a final physical performance test and substrate oxidation protocol, followed by two CO rebreathing tests on subsequent days. All food and beverages (except water) will be provided to volunteers beginning at the pre injection physical performance and substrate oxidation through the duration of the study. All data collection will occur at USARIEM.

Figure 3: Study Timeline



Erythropoietin Injections *(Credentialed procedure):* A dose (50 IU/kg body mass) of recombinant human EPO (PROCRIT, EPOETIN ALFA, Janssen Products, LP, Titusville, NJ, USA) will be injected subcutaneously 3 times per week for 4 week. Injections will be performed by credentialed study staff at USARIEM. The Office of Medical Support and Oversight (OMSO) will be the prescriber of prescription drugs used in this protocol, to include EPO. The dosing amount and frequency is on-label dosing equivalent to pediatric dosing. This dose and duration has previously been shown to be sufficient to result in hematological adaptions and increase physical performance (18, 19, 33). Hematocrit and hemoglobin will be checked weekly to ensure values remain < 50%. If Hct increases above 50%, EPO injections will be stopped and Hct will be reassessed within 3 days. If hemoglobin increases more than 1 g/dL over a 1 week period EPO injections will be stopped and hemoglobin will be reassessed within 3 days. Reticulocyte count will also be assessed on the first injection day, then again during week 2 and 4 of the injection phase. Additionally, before starting prescribed exercise, volunteers will complete a short clinically relevant health questionnaire and have vital signs checked (blood pressure, heart rate, and SpO_2) which will take about 5 minutes by study staff. Copies of all health related information will be provided to the OMSO daily for review and tracking. After the final injection volunteers will continue to be

monitored for 2 weeks by participating in weekly blood draws and daily monitoring of vital signs and health questionnaire which will continue to be reviewed by OMSO. Study personnel conducting this procedure will be credentialed and listed on the DOA log with code “F.”

Body Composition *(credential procedure):* 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% (34). 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. Measurements of body composition will be performed before and during the last week of the injection phase to characterize changes in fat mass and at-free mass. Female volunteers will complete a pregnancy test prior to body composition testing. Female staff members will oversee pregnancy tests. Study personnel conducting body composition scans will be credentialed and listed on the DOA log with code “U.”

Height and Weights *(non-credentialed procedure)***:** Height will be measured to the nearest 0.1 cm using a stadiometer at baseline. Body mass will be measured 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, once a week during the injection period.

Diet and Exercise Records *(non-credentialed procedure)***:** Volunteers will complete a 3-d diet record (**Appendix B**) and a 3-d activity log (**Appendix C**) according to instructions provided by study team dietitians (35). 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. These forms will be completed at baseline to characterize volunteers’ habitual diet and exercise habits.

$\dot{V}O_2$***peak*** *(credentialed procedure)***:** Following an overnight fast (10 hrs), volunteers will complete a maximal aerobic exercise sessions to determine peak oxygen uptake ($\dot{V}O_2$peak) on a treadmill. $\dot{V}O_2$peak will be determined using an indirect, open circuit respirator system (Parvomedics). 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%). Volunteers will be given adequate time to become familiar with the testing procedures and allowed a 3-min self-paced warm-up on the treadmill. At the start of testing, the volunteer will put on a nose clip and a mouthpiece or face mask connected to a 2-way respiratory valve, which is attached to a head piece to hold it in place. The volunteers will begin by running for 4 min at a pace predetermined as comfortable at a 0% grade. At 4-min, the grade will be increased to 4% followed by an additional 2% every 2 min thereafter until volitional exhaustion. Assessment of $\dot{V}O_2$peak will be done before and during the last week of the injection phase. Changes in $\dot{V}O_2$peak after injections will be taken into account to adjust the speed and grade of the treadmill during steady-state load carriage exercise.

Heart rate will be monitored throughout exercise using a heart-rate monitor (Polar Electro Inc, Oulu, Finland), and record during the last 30 seconds of each workload during all testing. 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. Study personnel conducting maximal aerobic testing will be credentialed and listed on the DOA log with code “J.”

Resting Metabolic Rate *(non-credentialed procedure)***:** Following a 10 hr overnight fast, RMR will be measured using open circuit indirect calorimetry (True Max 2400, Parvomedics, Sandy, Utah, USA) at baseline. Volunteers will rest in the supine position for approximately 30-min before measurement in a quiet and dim, temperature regulated room. To minimize error, volunteers will be instructed to minimize

movement once the hood is placed over their heads to collect expired air. The test will be discontinued when 20-min of steady state oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) are recorded. This measurement will be repeated during the last week of the injection phase to assess for changes in RMR. Baseline assessment of RMR, a factor of daily living, and Harris Benedict equation will be used to determine energy needs for study diets.

Study Diets *(non-credentialed procedure)***:** Registered Dietitians will develop individualized daily menus using Food Processor SQL (ESHA Research, Salem, OR, Version 10.14). The diets will be derived primarily from components of the US military Meals Ready-to-Eat (MRE) rations to achieve appropriate macronutrient proportions. Energy content of diets during glycogen normalization will match volunteers estimated energy needs to maintain body weight. Volunteers will be consuming a diet providing approximately 55% carbohydrate, 15% protein, and 30% fat. Dietary iron will be controlled for providing a minimum of 18mg/day to ensure recommended dietary allowance (RDA) for both males (8mg/day) and females (18mg/day) is met.

Time Trial *(credentialed procedure)***:** Following a 10 hr overnight fast, volunteers will complete a 5 kilometer time trial. The treadmill will be set at a constant 1% grade (36) for the entire test. Following a warm-up period, volunteers will blindly modulate treadmill speed in order to complete the distance as quickly as possible. The only feedback given will be distance covered at half mile increments. At half mile increments volunteers' rate of perceived exertion will be determined using the Borg Scale. Heart rate will be monitored throughout the time trial. Heart rate will be recorded at half mile increments. No motivation will be provided during the time trial. Participants may consume water *ad libitum* during the time trial. Following completion of the test, a self-selected cool-down will occur. Volunteers will complete a minimum of two practice exercise sessions to ensure they are familiar with the performance test. The practice sessions also will establish the coefficient of variation. Study personnel conducting maximal aerobic testing will be credentialed and listed on the DOA log with code "J."

Study Exercise *(non-credentialed procedure)***:** During the injection phase volunteers will conduct prescribed exercise training 4 days and 1 day of physical performance testing (time trial) per week. Volunteers will perform both weighted (treadmill or outdoor; walking, running, and load carriage) and unweighted (cycle ergometry) endurance-type exercise, and resistance-type exercise throughout the protocol. Exercise will be metabolically matched between groups, with exercise-induced energy expenditure being 1000-1500 kcal per day. Prescribed endurance-type exercise will be low-to-high intensity (30-85% $\dot{V}O_2$peak) using the ACSM metabolic equations for steady-state exercise (37) and the compendium of metabolic equivalents for physical activities. This level of training is similar to that conducted during arduous military training in garrison (38). VO_2peak will be assessed after 2 weeks into the injection phase. Exercise intensity will be adjusted based on alterations in VO_2peak. Study personnel conducting exercise will be listed on the DOA log with code "V."

Load Carriage Exercise *(non-credentialed procedure)***:** Volunteers will complete 90-min of weighted steady-state exercise on a treadmill at 55 ± 5% of their VO_2peak, with 30% of volunteer's body mass. The speed and grade of the treadmill will be determined using the ACSM metabolic equation for walking based on desired $\dot{V}O_2$ (39). Prior to the protocol day, participants will perform a practice session of the treadmill exercise to confirm the prescribed speed and grade are appropriate to induce target $\dot{V}O_2$. The treadmill speed and grade will be maintained for both arms of the study to match the absolute workload of the exercise across treatments. Study personnel conducting treadmill load carriage will be listed on the DOA log with code "V."

Carbohydrate Tracer Study *(credentialed procedure)***:** Following an overnight (10 hour) fast, two catheters will be placed into the lower arm (one in each arm). One arm will be used for infusion of 6,6-[$^2H_2$] glucose tracer and the other will be used for blood sampling. Following an initial blood sample collection to determine background enrichments, a primed, continuous infusion of 6,6-[$^2H_2$] glucose will begin (prime, 82.2 μmol·kg^{-1}; continuous rate, 0.78 μmol·kg^{-1}·min^{-1}, **Figure 4**). The 6,6-[$^2H_2$] glucose will

be infused for 100 min under resting fasted conditions to ensure isotopic steady-state is achieved prior to initiating exercise. Volunteers will then perform 90 min of steady-state (~55 ± 5 % of VO_2peak) load carriage (~30% body mass) exercise. During exercise $\dot{V}O_2$, $\dot{V}CO_2$, and HR will be measured 6 times at approximately 0, 20, 45, 60, 75, and 85 min. A total of 12 $\dot{V}O_2$, $\dot{V}CO_2$, and HR during the two carbohydrate tracer studies Credentialed personnel performing venous catheterization will be listed on the DOA log with code "T."

Figure 4: Carbohydrate Tracer Study

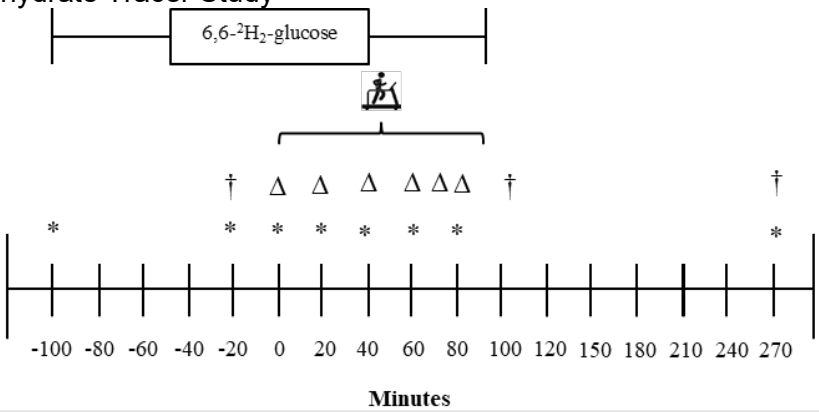


Figure Legend:

= Steady-State (55 ± 5% VO_2peak) † = Muscle biopsy

* = Blood Sampling Δ = VO_2/VCO_2 and Heart Rate

Percutaneous Muscle Biopsy of the Vastus Lateralis *(credentialed procedure)***:** Percutaneous muscle biopsies will be obtained from the vastus lateralis using a 5 mm Bergstrom needle with manual suction while the volunteer is under local anesthesia (1% lidocaine or similar) according to the approved USARIEM SOP (40, 41). Muscle biopsies will be performed immediately before, after, and post load carriage exercise. Each volunteer will have a total of 6 muscle biopsies during the entire study. Credentialed personnel performing muscle biopsies will be listed on the DOA log with code "R."

Blood Sampling *(credentialed procedure)***:** Blood samples will occur after an overnight (10 hour) fast. Blood collection for safety blood draws and CO rebreathing will be conducting using venipuncture. Safety blood draws will occur once a week during the injection (4 times) and follow-up period (2 times) of the protocol. Blood will be collected using an indwelling catheter kept patent using IV saline at approximately -100, -20, 0, 20, 40, 60, 80, and 270 min during carbohydrate tracer studies or venipuncture for weekly monitoring of Hct by a USARIEM credentialed phlebotomist. Blood draws will occur once per CO rebreathing measure. Additionally a finger stick will be used to collect tract amounts of blood during these measures. A total of 26 venipuncture and IV blood draws (Appendix F) will be completed during this study taking ~360 mL of blood sampled from each volunteer over the protocol days (**Table 2**). Blood samples will be used for isotope analysis assessment of nutrient status, hormone responses, and inflammation (**Table 2**).

For glucose kinetic analysis, the tracer/trace ratio (6,6-[$^2H_2$] glucose/glucose) will be measured on the pentaacetate derivative by gas-chromatography-mass spectrometry (Metabolic Solutions, Inc., Nashua, NH). Credentialed personnel performing venipuncture and/or venous catheterization will be listed on the DOA log with code "S and T."

Carbon Monoxide Rebreathing *(credentialed procedure)*: The optimized CO-rebreathing technique (42, 43) will be used to assess total hemoglobin mass (Hbmass, the total mass of hemoglobin in the circulation) and blood volume. Volunteers will sit for 20 minutes for postural control and then a venous blood sample will be collected to assess Hb and Hct. Baseline carboxyhemoglobin (%HbCO) will be

assessed from an arterialized capillary blood sample (finger stick) using a hemoximeter (Radiometer, OSM3, Denmark, or similar). The volunteer will then breathe into a CO detector (Draeger Pac 7000, Draeger, Lubeck, Germany, or similar) to assess end-tidal CO. Next, the volunteer will be administered a bolus of CO (99.99%; men: 1.0 ml/kg body mass; women: 0.8 ml/kg body mass; if body mass index is > 30 kg/m^2 convert body mass to BMI of 25 kg/m^2) into a custom built, closed-circuit spirometer (Spico-CO Respiration-Applikator, Blood Tec, Germany or similar) followed with 100% oxygen from a 3L bag and rebreathe for two minutes. Once the 2 minutes are complete, the volunteer will return to breathing room air. Four minutes after the initial CO inhalation, post-rebreathing end-tidal CO concentration will be assessed using a CO detector. Seven minutes after the initial CO inhalation, an arterialized capillary sample will be collected to determine the post-rebreathing increase in %HbCO. Female volunteers will complete a pregnancy test prior to CO rebreathing measurements. Female staff members will oversee pregnancy tests. Credentialed personnel performing CO rebreathing will be listed on the DOA log with code “W.”

B5.4 Data Collection

Data Element/Variable	Source	Operational Specification
Anthropometric data	Direct measurement	Height, weight, body composition
Dietary intake	Log and direct measurement	Habitual and controlled energy, protein, carbohydrate, and fat intake
Physical activity	Log and direct measurement	Normal and controlled physical activity habits including type of exercise and duration
Substrate oxidation	Blood and breath	Glucose turnover, carbohydrate, and fat oxidation
Physical performance	Direct measurement	TTE
Metabolic response	Blood and muscle	Blood analytes for metabolism, inflammation and iron status, mRNA, protein signaling, microRNA, activity assays, cross-sectional area, and fiber type

Table 2: Carbohydrate tracer study blood analytes

	Time (min)							
	Carbohydrate Tracer Study							
	Resting		Load Carriage Exercise					Recovery
Analyte	**-100**	**-20**	**0**	**20**	**40**	**60**	**80**	**270**
Glucose[1]	x	x	x	x	X	x	x	
6,6-[$^2H_2$] glucose[1]	x	x	x	x	X	x	x	
Insulin[2]		x	x	x	X	x	x	
Free fatty acids[2]		x	x	x	X	x	x	
Lactate[2]		x	x	x	X	x	x	
TNFα[2]		x					x	x
IL6[2]		x					x	x
Hepcidin[2]		x					x	x
C-microRNA[2]		x					x	x
Erythropoietin[2]	x							
Archive	x	x	x	x	X	x	x	x

Table 3: Carbon Monoxide Rebreathing blood analytes

Analyte	Pre	Post
HCT[2]	x	x
HgB[2]	x	x
Hepcidin[2]	x	x
Ferritin[2]	x	x
Soluble transferrin receptor[2]	x	x
Erythroferrone[2]	x	x
Erythropoietin[2]	x	x
Archive	x	x

Samples will be analyzed at [1]Metabolic Solutions (Nashua, NH) and [2]USARIEM. All blood samples will be separated into plasma and serum through centrifugation, aliquoted, and stored at –80˚C until analysis or shipment. Study samples will be collected and aliquoted at USARIEM and specific samples will be shipped on dry ice to Metabolic Solution. Blood sent out to other laboratories for analysis will not have any remaining samples after analysis is completed. USARIEM will retain all archive samples.

Plasma Glucose Turnover Calculations

For calculation of plasma glucose turnover the Steele equation with modifications for non-steady state will be used (44). Enrichment (E) will be expressed as mole percent excess (MPE); calculated as (TTR)/(1 + TTR), where TTR is the tracer to tracee ratio. Appropriate corrections for skewed abundance distribution and overlapping spectra will be made for the TTR of the glucose tracer, 6,6-[$^2H_2$] glucose (44).

$$\text{Total glucose } R_a \text{ (Total } R_a) = (F - ((pV \times ((C_2 + C_1)) / 2) \times ((E_2 - E_1) / (t_2 - t_1)))) / ((E_2 + E_1) / 2)$$
$$\text{Glucose } R_d = \text{Total } R_a - (pV\ (C_2 - C_1) / (t_2 - t_1))$$
$$\text{Metabolic Clearance Rate (MCR)} = \text{Glucose } R_d / ((C_2 + C_1) / 2)$$

Where F represents the infusion rate of 6,6-[$^2H_2$] glucose; *p*V is the effective volume of distribution for glucose, C_1 and C_2 are plasma glucose concentrations at t_1 and t_2, respectively, E_1 and E_2 are plasma enrichments of 6,6-[$^2H_2$] glucose at t_1 and t_2, respectively.

Calculations of Carbohydrate and Fat Oxidation

Carbohydrate, fat, and protein oxidation rates will be calculated from $\dot{V}O_2$ (L/min) and $\dot{V}CO_2$ (L/min) during the 90-min exercise bout as described by Jeukendrup and Wallis (45):

Fat oxidation (g/min) = 1.695 × $\dot{V}O_2$ (L/min) − 1.701 × $\dot{V}CO_2$ (L/min)
Total carbohydrate oxidation (g/min) = 4.585 × $\dot{V}CO_2$ (L/min) − 3.226 × $\dot{V}O_2$ (L/min).

Muscle Glycogen

Glycogen concentration will be determined using ~3 mg (dry weight) freeze dried muscle homogenized in water using a TissueLyser II with a 5-mm steel bead (Qiagen, Valencia, CA, USA). Homogenates will be boiled at 100°C for 5 min and centrifuged at 13,000×g for 5 min at room temperature. Supernatant will be removed and muscle glycogen concentrations will be assessed using an endpoint colorimetric assay (Cat# MAK016; Sigma-Aldrich, St. Louis, MO, USA).

Immunohistochemistry

Resting fasted muscle samples before and after EPO dosing will be frozen for cross-section analysis to include but not limited to fiber typing. Muscle sections will be stained with antibodies against either myosin heavy chain I or myosin heavy chain IIA. Sections will then be incubated with appropriate conjugated secondary antibodies for one hour at 37 °C (Alexa Fluor-568, Molecular Probes, Life Technologies). Fluorescent-conjugated (AlexaFluor488) Wheat Germ Agglutinin (WGA) will be used to stain the extracellular matrix for the calculation of fiber cross-sectional area using Image J software.

mRNA, microRNA, and c-microRNA Expression

Total RNA will be isolated using ethanol precipitation from approximately 20 mg of muscle. Quantity and quality of RNA will be assessed using a Nanodrop ND-1000 spectrophotometer (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 or equivalent) and a TaqMan® microRNA RT kit (Applied Biosystems, Foster City, CA, USA) or equivalent. Individual Taqman® probes (Applied Biosystems) or microarray (Qiagen) will be used to determine the mRNA expression of intramuscular molecular regulation to include but not limited to, PGC-1α, PPARs, SIRT1, p53, CPT1a, FABP, FAT, TNF-α, TNF-αR, IL-6, IL-6R, TWEAK, and TWEAK-R. microRNA analysis will be conducted using individual Taqman® probes (Applied Biosystems) or microarray (Qiagen), assessing microRNA that may be associated with metabolism and inflammation. This microRNA targets will include, but not be limited to, miR-1, miR-23a/b, miR-26, miR-29, miR-34a, miR-103, miR-107, miR-133a/b, miR-146, miR-206, miR-208a, miR-486, and miR-499a.

Expression of microRNA will also be assessed in circulation using Taqman® probes (Applied Biosystems) or microarray (Qiagen). Circulating miRNA will be extracted from 200 µL serum using miRNeasy Serum/Plasma kit, which allows for extraction and purification of small (< 200 nucleotides) cell-free RNA (Qiagen or equivalent). 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 or equivalent) 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) or equivalent 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) or equivalent for miRNA of interest at 0.05X concentration in 1X TE buffer. All serum miRNA will be normalized to the geometric mean of external (Spike-In Control C. elegans miR-39) and internal controls to allow for both technical and inter-individual normalization (46). Geometric mean of controls will be used to correct for possible outlying values and abundance differences between the different controls (47).

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 or similar model). A StepOnePlus™ real-time PCR system (Applied Biosystems) or similar model will be used to perform all mRNA and miRNA analysis. Fold changes will be calculated using the ΔΔcycle threshold ($\Delta\Delta C_T$) method.

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, any additional gene and protein expression of relevant targets will be assessed.

Western Blotting

Approximately 20 mg of muscle will be homogenized in ice-cold buffer (1:10 w/v) 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 or equivalent). Phosphorylation status and total protein content will be determined by 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 difluoride (PVDF) membranes and incubated with commercially available primary antibodies of intramuscular molecular regulation to include but not limited to AMPKα, p38 MAPK, PGC-1α , SIRT1, CaMK, p53, Akt, IL-6, TNFα, NF-kB, and IKKα/β (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 or equivalent). Blots will be quantified using the ChemiDoc XRS from Bio-Rad and Image Lab software (Bio-Rad) or similar model. To confirm equal protein loading per well a normalizing protein such as HSP90 or GAPDH will be assessed.

Enzyme Activity
Assays will be conducting using homogenate from Western blotting to include, but not limited to Pyruvate dehydrogenase (PDH) and citrate synthase. PDH activity will be assessed in lysates using a kinetic colorimetric assay (Cat# MAK183; Sigma-Aldrich). Citrate synthase activity will be determined by colorimetric assay combining 10 mL diluted (1:10; 0.1 M Tris HCl, pH 8.1) sample to 150 mL reaction master mix (1 mL DNTB [5,5-dithio-bis-(2-nitrobenzoic acid)], 3 mg acetyl-CoA, and 8 mL 0.1MTris HCl, pH 8.1) and 10 mL 10 mM oxaloacetate. Enzyme activity data will be normalized to run time and sample protein content.

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, 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 office, or kept in a computer file with password-protected access restricted to the principal investigator. 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. De-identified samples for isotopic analysis will be shipped on dry ice to (blood) and room temperature (breath) to Metabolic Solutions. All samples will be shipped via FedEx and stored in these laboratories until analyzed. Once samples have been analyzed, there will be no remaining sample for storage other than the archived sample stored at USARIEM. Coded data will be transmitted between the above mentioned laboratories via an encrypted email, a secure file transfer site, or using an approved removable media. Only USARIEM will maintain digital copies of these data.

Only the principal investigator and project coordinator will have access to personal identifiable data. No outside laboratory will have access to identifiable 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 ten years after the study has been completed. The master list will be destroyed upon the protocol's closure.

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

De-identified study samples will be stored in -80ºC freezer at USARIEM in room 322 or 304 for potential future use and maintained indefinitely. Only personnel assigned to the research study by the principal investigator will have access to samples. The de-identified data and samples will remain under the control of the PI and may be shared with outside collaborators for future research. Any use of the samples outside of this defined protocol will be submitted as a protocol amendment or a new protocol.

B5.7 Devices, Drugs, Dietary Supplements, Nutritional Supplements, And Biologics

B5.7.1 Devices

5.7.1.1 FDA-approved device being used in this research according to the approved labeling

DEXA, DPX-IQ, Lunar Corporation, True Max 2400, Parvomedics, Sandy, Utah, USA, Polar Electro Inc, Oulu, Finland

5.7.1.2 FDA-approved device being used in this research in a manner other than its approved labeling: N/A

B5.7.2 Drugs

B5.7.2.1 FDA-approved and used in accordance with the approved labeling

B5.7.2.2 FDA-approved and used in a manner not in accordance with its approved labeling: PROCRIT, EPOETIN ALFA, Janssen Products, LP, Titusville, NJ, USA

B5.7.2.3 Any drug not approved by the FDA: N/A

B5.8 Statistical Analysis

B5.8.1 Sample Size Estimation

Based on results for multiple outcomes across different studies (**Table 4**) a sample size of 8 is required to statistical significance.

Table 4: Previous study effect sizes

Study	Outcome	Delta	Effect Size	Sample Size
Thomson et al. (18)	TTE	10 ± 6	1.40	7
Plenge et al. (24)	Hct	5.1 ± 3	1.56	7
Caillaud et al. (19)	CHO Oxidation	0.7 ± 0.3	1.24	4
Caillaud et al. (19)	Fat Oxidation	0.2 ± 0.05	3.51	2

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. Paired t-tests will be used to assess phase effects (PRE vs. POST injection) for glucose turnover, and substrate oxidation. Repeated measures ANOVA will be used to assess main effects of time for physical performance. Mixed-model repeated measure ANOVA will be used to assess main effects of phase (PRE vs. POST injection), time and their interaction for blood analytes and muscle molecular analysis. If interactions are significant, appropriate post-hoc 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

Volunteers will be recruited from the federally and non-federally employed civilian population, the Natick Human Research Volunteer (HRV) Pool, and NSSC active duty military personnel.

C1.2 Recruitment Process

For Soldiers in the U.S. Army Natick Soldier System Center Human Research Volunteer (HRV) Program, the Principal Investigator will furnish a copy of the consent form to the Human Research Volunteer Program Coordinator or designee. The Coordinator will schedule the consent briefing for the HRV platoon.

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

Civilian volunteers and other active duty personnel will be recruited by “word of mouth”, posted flyers (“off-site recruitment flyer” to be used for any flyers posted outside of NSSC, “recruitment flyer” to be used for postings within NSSC), or electronic distribution of the “off-site” flyer to include a text-only, approved version of the flyer. Recruiting materials will be distributed around NSSC, surrounding community, and on bulletin boards at local universities. The text-based flyer will be posted on various USARIEM social media sites and used in distribution media requiring a text format (e.g., electronic newsletters). Recruiting may also be conducted through briefings presented to college classes, clubs, sports teams, or other organizations. Approvals from the requisite parties will be obtained prior to any recruitment activities.

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. An ombudsman (indicated by code “L” on the DOA Log) will be present for HRV briefings. All civilian and non-HRV Soldier briefings will be done one-on-one.

C1.3 Eligibility

Volunteers will be medically cleared by the OMSO before participation in accordance with USARIEM procedures outlined for screening volunteers for research involving exercise, tracer infusions, muscle biopsies and EPO use at USARIEM. 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 volunteers must be willing to consume only food and beverages provided by study staff during the entire study. Additionally, volunteers must be willing to refrain from any additional exercise during the study.

C1.4 Consent Process

Informed consent documents will be provided to each prospective volunteer. Additionally, an oral presentation will be provided to prospective volunteers by the principal investigator or his designee. In case of COVID risk mitigation plans, briefings can be done virtually and consent forms can be emailed and digitally signed. 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. Potential volunteers will have a minimum of an hour, but can take longer, to review the consent form. Interested volunteers will sign the informed consent form prior to undergoing initial screening. If they meet all the medical selection criteria after completing the screening health assessment they will be scheduled to begin data collection. 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

C2. COMPENSATION FOR PARTICIPATION

Military and civilian personnel will receive $50 for each successful blood draw. There are 26 blood draws during the entire study. Volunteers completing all draws will receive $1300. If a volunteer does not complete the entire study, they will be compensated for the number of successful blood draws they did complete. If USARIEM staff fail to get a blood draw and the volunteer completes the study they will be compensated in full. Volunteers will not be eligible for any other form of compensation during this study.

Note: Participants who receive more than $600 in a calendar year will have this income reported to the Internal Revenue Service.

C3. WITHDRAWAL FROM RESEARCH PARTICIPATION

Volunteers will be allowed to withdraw at any time without penalty or loss of benefits to which they would otherwise be entitled. They will do so by informing the PI, AI or a staff member of their intent to withdraw verbally or in writing (electronic or paper/pencil). They will then be asked to verbally discuss their choice to withdraw with the PI so that reasons for withdrawal can be documented. An investigator may stop an individual's participation in the study if the volunteer is unwilling or unable to complete study procedures. An investigator may also withdraw a volunteer if the individual becomes ill or injured or it would not be in the volunteer's best interest to continue. If the participant is withdrawn by the investigator or decides to

voluntarily withdraw him/herself, all further data collection will discontinue. Any samples or data collected prior to withdrawal will be maintained. Participants will be compensated for any blood draws they completed up until that point, and they will be asked to return any remaining food items that were provided, in addition to any wrappers and diet/activity logs that they had completed up to the point of withdrawal.

C4. PRIVACY FOR SUBJECTS

To protect the volunteer's privacy, all of their research-related records will be labeled or "coded" with an assigned research volunteer number that will not include their name or any other form of identifiable information. The principal investigator or project coordinator will keep the link between volunteer number and the volunteer's research records in a locked cabinet. Any documents that will require the volunteer's name, such as the consent form, will be kept in a locked cabinet separate from any research documents that contain the volunteer's ID number. The principal investigator and project coordinator are the only people who will be able to match the research volunteer number with any of their personal identifying information.

When the results of the research are published no information will be included that would reveal the volunteer's identity to others. Photographs, videos, or audio-tape recordings of volunteers will only be used, if the volunteer grants permission through the Audio/Visual Image Release form. Each volunteer will also be asked to grant permission for his/her name to be included on his/her photo or video image or in writing connected to his/her image. If a volunteer does not grant permission through the Audio/Visual Image Release, then no photos or other visual recordings will be taken of him/her. In the event that it is discovered that an individual has been inadvertently photographed or visually recorded without his/her permission, the materials will be immediately destroyed. Permission through the Audio Visual Image Release form will be confirmed before any photographs or other visual recordings are used.

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

Complete confidentiality cannot be promised to military participant because information bearing on the military participant health may be required to be reported to appropriate medical or command authorities.

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 office, or kept in a computer file with password-protected access restricted to the principal investigator and project manager. The master list linking subjects with their data will be destroyed when the study is closed. When the results of the research are published or discussed in conferences, no information will be included that would reveal identity. Study samples will be processed on site at USARIEM. All samples will be stored using the subject identification number. Samples will be shipped for analysis to 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. De-identified samples will be maintained indefinitely.

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 ten 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: Dual energy X-ray absorptiometry (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: Exposure to less than 0.6 mrem X-irradiation in a slow-speed (up to 20 minutes) whole-body scan is possible. This dose is equivalent to approximately 1/500 of normal annual background radiation (300 mrem/year), 1/6 of the radiation received in a transatlantic flight (3.825 mrem), or 1/3 of the radiation received in a chest X-ray (2 mrem). The American College of Obstetricians stated that exposure to less than 5 rem (50 mSV) has not been associated with an increase in fetal anomalies or pregnancy loss.
Measures to Minimize Risks of Harm: 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. Volunteers must be advised that the health effects of very low level exposures to ionizing radiation, if any, are unknown (thought to be minimal). Pregnant women may not undergo this procedure. Women of child bearing age must have a documented negative pregnancy test within the 48 hours preceding the scan. Volunteers will be cautioned against becoming pregnant while participating in the study. Study personnel conducting body composition will be credentialed for such procedures and listed on the DOA log with code "U."

Research Procedure Name: Indwelling Catheters
Research Procedure Description: A 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. 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. This will require another needle to be inserted into your arm. In addition, the catheter can cause irritation, bruising, swelling, infection, or an allergic reaction.
Measures to Minimize Risks of Harm: 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. Subjects will be briefed on the signs and symptoms of potential complications and given information on who to contact for medical assessment and/or treatment. Volunteers should not donate blood for eight weeks before or after this study. Credentialed personnel performing venous catheterization will be listed on the DOA log with code "T."

Research Procedure Name*:* 6,6-[$^2H_2$] glucose infusions
Research Procedure Description: 6,6-[$^2H_2$] glucose will be infused using a indwelling catheter.
Research-related Risks: The primary risks associated with tracer studies are those related to venous catheterization. The catheter can cause irritation, bruising, or infection. There are no known risks or reported side effects associated with administration of 6,6-[$^2H_2$] glucose infusions to humans during clinical or experimental studies. The risks associated with the infusion include volume overload, infection, and allergic reaction to the infused substance. There have been no occurrences of volume overload, no occurrences of infection or allergic reaction attributable to iodine used prior to venipuncture in any of the 200 infusion protocols that the investigators have been involved. To minimize the likelihood of these events occurring, infusion rate will be closely monitored and maintained at less than 30 ml/hr throughout the entire infusion protocol day.
Measures to Minimize Risks of Harm: All staff who directly participate in the infusion studies will be properly trained how to safely monitor (i.e., infusion pumps and IV lines) infusion studies from Dr.

Margolis, who has extensive experience with infusion studies. In addition, infusates will be prepared sterile, pyrogen-free, and in the proper dosages by a licensed pharmacist. If sign of allergic reaction, rash or redness, infusion will be stopped immediately. Credentialed personnel performing venous catheterization will be listed on the DOA log with code "T."

Research Procedure Name: Venipuncture
Research Procedure Description: A 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, and syncope. 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: Participant monitoring, aseptic technique, including sterile disposable blood collection apparatus and adherence to standard medical precautions reduce risk. Trained technicians will perform all venipuncture. Subjects will be briefed on the signs and symptoms of potential complications and given information on who to contact for medical assessment and/or treatment. Credentialed personnel performing venipuncture will be listed on the DOA log with code "S."

Research Procedure Name: Exogenous EPO
Research Procedure Description: A needle and syringe will be used to inject prescribed EPO amounts subcutaneously in the abdomen 3 times a week for 4 weeks.
Research-related Risks: Side effects with EPO injection are redness and pain at the site of injection, increase blood pressure, blood clots, joint pain, nausea, dizziness, headaches, trouble sleeping, and weight loss. Rare but possible side effects can include myocardial infarction and stroke
Measures to Minimize Risks of Harm: To minimize side effects the study will use a low dose EPO injection equivalent to a pediatric dose. Before participants begin the prescribed exercise they will complete a short clinically relevant health questionnaire (**Appendix A**) and have their vital signs (blood pressure, heart rate, and SpO_2) assessed by study staff. Copies of all health related information will be provided to OMSO daily for review and tracking. Hemoglobin and Hct values will be measured weekly during EPO injections. To minimize risk of blood clots Hct values will be maintained < 50%. If Hct values increase > 50% EPO injections will be stopped. Volunteer will return to for another Hct check within 3 days. If hemoglobin increases more than 1 g/dL over a 1 week period EPO injections will be stopped and hemoglobin will be reassessed within 3 days. EPO injections will only be administered by credentialed staff at USARIEM. These individuals will follow administration procedures provided in the PROCRIT® prescriber information guide. Volunteers will continued to be monitored for 2 weeks after the injections are complete to ensure there are no complications from the EPO injections. The OMSO will be responsible for observing and monitoring adverse events of participants in this study.

Research Procedure Name: Exercise
Research Procedure Description: Exercise testing will occur on a cycle ergometer or treadmill. Exercise will be at various levels of intensity based on exercise protocol.
Research-related Risks: Exercise per se rarely provokes cardiovascular events in healthy individuals with normal cardiovascular systems. The prevalence of fatal events in the U.S. is approximately 1:133,000 to 1: 185,000 for men and 1:769,000 to 1:1,500,000 for women who are competitive high school and college athletes. For middle-aged and older adults the relative risk rises to 1:15,000 to 1:18,000 for individuals without a prior history of cardiovascular events. 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 exercise sessions, and heart rate will be monitored in real time during testing. In addition, exercise monitors and test administrators will be CPR-certified. Study personnel conducting maximal aerobic testing and treadmill load carriage will similarly be credentialed for such procedures and listed on the DOA log with code "V."

Research Procedure Name: Percutaneous Skeletal 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 applied to collect muscle samples, while the volunteer is under local anesthesia (1% lidocaine or similar local anesthetic analogue in dosages and composition approved by OMSO).
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 right after a muscle biopsy. If the volunteer has had problems with fainting during blood draws or muscle biopsies in the past, they may be more prone to them during future procedures. The most common risks associated with muscle biopsies are pain (~1.27%), erythema (~1.27%), and ecchymosis (1.27%) (48, 49). Panic episode, bleeding, and edema have also been reported (0.21%, 0.42%, and 0.84%, respectively) (48). Denervation, numbness, and atrophy may occur but have not been verified in the literature. 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.
Measures to Minimize Risks of Harm: Lidocaine (or similar anesthetic) will be used to minimize pain or discomfort. Potential risk for complications of bleeding will be reduced by applying direct pressure to the wound following the biopsy. If soreness symptoms should occur, they usually do not interfere with normal walking or heavier exercise. Volunteers with evidence of bleeding diathesis should be excluded during medical clearance; those with local skin infection or irritation or recent use of anticoagulant medication not identified during initial medical screening (including aspirin) will be withdrawn by the PI in consultation with OMSO. Volunteers will be instructed about precautions against hematoma and infection. Volunteers will refrain from non-steroidal anti-inflammatory medications for 10 days before and 5 days after the muscle biopsies. They will be given a handout outlining instructions for proper care of the incision site (Appendix C). Muscle biopsies will be performed using sterile procedures and equipment by study staff members who are credentialed to perform skeletal muscle biopsies and who will abide by USARIEM's Percutaneous Skeletal Muscle Biopsy SOP (OMSO-approved USARIEM SOP for Invasive Procedures, Chapter 10) of 11 July 2017 in all regards. Either Dr. Margolis, other credentialed individual who performed the muscle biopsy and OMSO will follow-up with volunteers within 3 d post-biopsy to monitor for any sign of infection, bleeding, or hematoma. Study personnel conducting muscle biopsies will be credentialed for such procedures and listed on the DOA log with code "R."

Research Procedure Name: Lidocaine (or similar) Injection
Research Procedure Description: Approximately 8-10 mL of 1% lidocaine (or similar local anesthetic analogue in dosages and composition approved by OMSO) 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 (or similar). Unlikely, but possible side

effects could include: dizziness, confusion, shakiness, visual changes, nausea, and unusually slow heartbeat.
Measures to Minimize Risks of Harm: Volunteers will be screened for all allergies during the initial general medical clearance. Volunteers will be instructed to notify a Study Investigator or any staff member immediately if an allergic (i.e., swelling, itching, rash, hives, difficulty swallowing, or difficulty breathing) reaction occurs. In the case of severe reaction, OMSO will be notified immediately and lidocaine (or similar) use will be discontinued and if indicated emergency medical staff will be contacted. In the event of an extremely severe reaction, an Epinephrine Auto-Injector will be available onsite. Only credentialed PIs will administer the lidocaine (or similar), and medical staff will be on duty.

***Research Procedure Name*:** Optimized CO-rebreathing
***Research Procedure Description*:** CO-rebreathing has been safely used to assess Hbmass for over 100 years. In this procedure the volunteer will inhale a bolus of CO (99.99%,; men: 1.0 ml/kg body mass; women: 0.8 ml/kg body mass; if body mass index is > 30 kg/m^2 convert body mass to BMI of 25 kg/m^2) followed with 2 minute rebreathing 100% oxygen (from a 3L bag) using a closed circuit spirometer (Spico-CO Respiration-Applikator, Blood Tec, Germany or similar). Arterialized capillary blood (finger-tip) will be collected at the beginning of the rebreathing procedure and after seven minutes of the initial CO inhalation to assess blood %HbCO. Sodalime will be used to prevent accumulation of CO_2.
Research-related Risks: Minor risk of performing the optimized CO-rebreathing procedure include headache (extremely rare). Additionally, finger-sticks may result in pain, dizziness, fainting, infection, bruising, tenderness, and/or edema at puncture site, skin irritation, nausea and vomiting. It is unknown what potential risks might be present for pregnant women, so those who are pregnant will be excluded from participation.
Measures to Minimize Risk of Harm (Precautions, safeguards): This method uses a small, quantified volume of CO aimed at increasing the %HbCO by 4-7% above baseline. CO-rebreathing will not be performed if baseline %HbCO is >3%, thus reducing the likelihood of headache. Additionally, only one CO-rebreathing procedure will be completed per day. Study staff will also monitor volunteers. Finger capillary sticks will be performed by credentialed and proficient research personnel who will use aseptic technique. Risk of fainting or dizziness will be minimized by having subjects seated during blood draws/fingersticks. Female volunteers will self-administer a pregnancy test prior to undergoing CO rebreathing tests.

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. Information gathered from this research may benefit other people in the future.

C7. DATA AND SAFETY MONITORING

C7.1 Monitoring

The Principal Investigator will be responsible for monitoring the data collected. The Principal Investigator, with the assistance of the Study Coordinator, will ensure that the data is being collected according to the methods described in the protocol. Electronically collected data will be downloaded daily and checked for

quality. This also includes continuous evaluation of th/e following: recruitment, the informed consent process, adverse events, protocol adherence, and protocol deviations. This will occur continuously in order to identify unanticipated problems or risks to the volunteers associated with the research. The Principal Investigator will ensure that the number of volunteers recruited for this study complies with the protocol. If adverse events occur, the Principal Investigator will submit a monthly summary of the related adverse events to OMSO to determine whether the number of adverse events is excessive for the risks outlined in the research protocol. The Principal Investigator and Study Coordinator will be responsible for ensuring that the appropriate regulatory and IRB documentation is on file and up to date.

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.

Expected adverse events are local bruising and soreness from blood draws and at the site of EPO injections, and muscle soreness or pain from exercise. Expected adverse events for muscle biopsies are local soreness, redness, or bruising. Expected adverse events which are not serious are reported to the IRB at the time of continuing review of the protocol.

All medical events that the USARIEM Office of Medical Support and Oversight (OMSO) evaluates will be reported to the ORQC.

C8.2 Unexpected adverse events and unanticipated problems

A serious adverse event is 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 will be reported to USARIEM's Office of Medical Support and Oversight (OMSO). OMSO staff will retain a copy of the report in the subject's OMSO medical file as a means of tracking and analyzing trends in medical events.

All unanticipated problems involving risk to subjects or others, 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 (508-206-2371/2200) or email (usarmy.natick.medcom-usariem.mbx.usariem-rqc-protocol@health.mil) to the USARIEM ORQC and the Commander. These events will also be reported to the HQ USAMRDC IRB within one working day by phone (301-619-6240), or by e-mail (usarmy.detrick.medcom-usamrdc.other.irb-office@mail.mil)

Adverse events assessed by the PI as not serious and serious adverse events that are deemed to be unrelated to participation in the study will be reported to the IRB at the time of continuing review of the protocol.

In the event of a medical emergency at facilities on the Natick Soldier Center, the local Emergency Medical Services (EMS) will be contacted immediately by dialing 5911. The installation security personnel will direct the ambulance to the proper location on the installation. While awaiting their arrival,

Basic Life Support will be rendered by study personnel or on-site medical coverage. EMS response time to USARIEM is approximately 5 minutes. Transport time to definitive care is approximately 8 minutes.

C8.3 Adverse device effects

N/A

C8.4 FDA-regulated research under IND and IDE

The investigation is not intended to be reported to the FDA as a well-controlled study in support of a new indication of use or any other significant labeling or advertising for the drug. The investigation will be conducted in compliance with the requirements for institutional review per 21 CFR Part 56 and the requirements for informed consent per 21 CFR Part 50. The drug will not promoted as safe or effective and the study will be conducted in compliance with 21 CFR Part 312.7.

SECTION D: REFERENCES

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SECTION E: ABBREVIATIONS AND ACRONYMS

See the "Guide for Investigators"

Erythropietin; EPO, Hemoglobin; Hgb, Biomedical Performance Enhancement; BPE, Hematocrit; Hct, Office of Medical Support and Oversight; OMSO, prothrombin time; PT, partial thromboplastin time; PTT, mole percent excess; MPE, tracer to tracee ratio; TTR, Human Research Volunteer; HRV,

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

Click in the appropriate box See the "Guide for Investigators" for definitions and further information.

☒ 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

Appendix A: Clinical Questionnaire

Have you experienced in the last 24 hours any:

1. Chest pain
2. Shortness of breath
3. Dizziness, lightheadedness, clamminess, diaphoresis (sweating), or fatigue
4. Nausea, vomiting, belching, or indigestion
5. Extremity swelling, pain, warmth, erythema
6. Difficulty speaking, walking or understanding language
7. Paralysis, numbness, or weakness of the face, arm, or leg
8. Vision or hearing changes
9. Headache
10. Alteration in mental status (including, but not limited to confusion, memory loss, disorientation, or loss of alterness)
11. Fever or chills
12. Cough
13. Itching
14. Skin abnormalities to include, but not limited to rash or blistering
15. Depression Symptoms:
    a. Depressed mood
    b. Loss of interest or pleasure
    c. Change in appetite or weight
    d. Sleep disturbance(s)
    e. Psychomotor disturbances (i.e. hand wringing, pacing, or fidgeting; slowness in body movements, thinking or speech)
    f. Feelings of worthlessness or guilt
    g. Thoughts about harming yourself or others

Appendix B: Food Records

Keeping Food Records

GENERAL INSTRUCTIONS:

☑ **Record your food and beverage intake from midnight to midnight (24 hour) on each of the days on which you are asked to record your intake, for a total of three days.**

☑ Record **everything** you eat or drink. Don't forget to record all beverages, alcoholic beverages, snack foods, candy, etc. Record items and amounts as soon as possible after eating to prevent memory error.

☑ Write only **one food or ingredient per line**. Use more than one food record form for a day if necessary.

☑ List all **items added** to food/drink (e.g., jelly, mustard, mayonnaise, margarine, sour cream, creamer, syrup).

☑ Indicate if you **removed** anything from an item (ex. olives from salad, skin from chicken, etc)

☑ Please record if you consumed any **nutritional supplements**. Record the brand name and the nutrition information from the label or provide the product label or container with your record.

FOOD OR BEVERAGE DESCRIPTION, BE SPECIFIC:

☑ Record the **restaurant or source** where you got prepared food (e.g., Pizza Hut, Subway, PX, Dining Facility, cafeteria, vending machine, etc.).

☑ Record the **brand names** of foods and beverages (e.g., *Chips Ahoy* mini chocolate chip cookies, *Stouffer's* Macaroni & Cheese). Please supply the product label if possible.

☑ Record the **type** of food, (ex. white bread, 2% low fat milk, honey-roasted peanuts, fried chicken breast, bottled orange juice vs. orange drink, cake or raised doughnut).

☑ Record how the food was **prepared**, (e.g., raw, baked, broiled, fried, sautéed, steamed, grilled, etc.).

☑ If you ate something like **PIZZA**, specify the *number of slices* eaten as well as the *number of slices in the entire pizza* and the *size* of the entire pizza.
EXAMPLE: Pepperoni pizza from Pizza Hut, 5 slices from a large (16"), 10-slice pizza.

☑ List or describe the **ingredients** in unique dishes or combinations of foods, such as stews, casseroles, and salads.
EXAMPLE: Lasagna (per piece)- lean ground beef (1/4 cup), mozzarella cheese (1 oz), ricotta cheese (1oz), tomato sauce (½ cup), 2 noodles. If food is home cooked, please provide **recipe** information on a separate sheet.

AMOUNT CONSUMED:

☑ Record the **size** of the bag, box, bottle, or restaurant order. Use the package weight on the label or menu information. (e.g., 1-lb bag of *M&M*s; 20 fl. oz. or 2 liter bottle C*oke*, *McDonalds* jumbo fries). Don't confuse fluid ounces (fl. oz.) with weight ounces (oz.).

☑ Specify the **quantity or proportion** of foods/beverages consumed (ex, 3 10-oz *Bud Lite* drafts; 1/2 of a 16-oz bag of *Baked Ruffles*; 15 *Ritz* crackers; half 16-oz porterhouse steak, 1/6 of 9"diameter pie).

☑ **Count** number of pieces (ex., 6 chicken nuggets).

☑ Report amount in **household measures** (cup, fl. oz., Tbsp, tsp). Actually measure if possible or use the tips sheet to visually estimate.

☑ Measure the **dimensions** thickness, length, and width if possible (ex., baked potato—4" x 3").

☑ **Use your hand: Fist = 1 cup, Palm of hand = 3oz meat, Golf ball = 2 Tablespoon.**

Helpful Hint: It's easier, and your food record will be more accurate, if you write everything down as it is happening. **Don't wait until the end of the day to record everything**, because you'll forget the specifics.

Sample Food Record

Approximate Time	Food/Drink Item	Method of Preparation	Description/ Brand Name	Amount
11:00 AM	Eggs Sandwich made with:			
"	Toasted Bagel, Plain, Lender's original			1
"	scrambled eggs, in pan with cooking spray			2 eggs
"	ketchup			1 Tablespoon
11:00 AM	Orange Juice		Tropicana	1.5 Cups
3:00 PM	Chocolate chip cookies		Chips Ahoy	3
4:30 PM	Cheeseburger w/ bun		McDonald's	1
"	Ketchup + Mustard		"	1 packet of each

”	Medium Fries	McDonald’s	1
”	Vanilla shake	McDonald’s	¾ of 1 large
7:00 PM	Vanilla Ice cream	Haagen-Dazs	1 cup

Volunteer ID:_____ Date:_____ **Study Day # _____**

Record 1 day of records per double sided sheet

Time	Meal	Food/Drink Item	Description	Amount	Place
	B-Breakfast **L**-Lunch **D**-Dinner **S**-Snack	One item per line. Skip line between each meal or snack	Be specific. Give details such as brand name, cooking method, etc. Attach recipes or food labels if applicable.	Specify oz., cup, tsp, Tbsp, etc.	Barracks, DFAC, restaurant (give name)

Appendix C: Activity Log

Training Log Instructions:

Guidelines:

I. 3-Day Training Log:

- Record your purposeful exercise each day for 3 days on the Training Log provided.
- Be specific about the type of exercise
    - Cardio…
        - What type of cardio did you do?
        - How long did you work out?
        - How far did you go?
        - Did you do the activity continuously, or did you rest during?
    - Resistance…
        - What muscle groups did you work?
        - How many sets did you do? How many repetitions per set?
        - How long was the rest time between sets?
- Use the Weeklong Training Log Entry Example below as a guide.

II. What/When to Return:

- Return completed log as directed.

Example of Training Log Entry

Day/Date:

Time	Type/Description of Activity	Time/Distance/Reps
7:45 AM	Ran	3.0 miles in 27 min 30 sec.
8:15 AM	Weight Lifted - Chest & Back	45 min total: 4 exercises/muscle group, 8 repetitions/set (exercise), 1 min rest between sets

Helpful Hints:

1. Don't change your exercise habits. This should be an accurate description of your typical physical activity.
2. It's easier, and your activity record will be more accurate, if you write everything down as it is happening. **Don't wait until the end of the day to record everything**, because you'll forget the specifics.
3. Call if you have any questions or concerns

Date:_____

Time	Type/Description of Activity	Time/Distance/Reps

Date:_____

Time	Type/Description of Activity	Time/Distance/Reps

Date:_____

Time	Type/Description of Activity	Time/Distance/Reps

Appendix D: Muscle Biopsy Care

Muscle Biopsy Care
Information for the Participant

You have just had a percutaneous muscle biopsy. The muscle biopsy site is closed with a steri-strip. This is covered with 4 x 4 gauze pad, a transparent dressing to prevent the incision from getting wet, and an elastic ACE bandage.

As the anesthetic wears off, you may feel a dull ache at the biopsy site for ~1-3 days. Many people have no pain at all. Instructions for the care of the biopsy site are as follows:

1. You may perform all your normal activities immediately after the biopsy, including light exercise.

2. Leave the ACE bandage in place for 5 hours after the biopsy. After this time, remove the elastic bandage. The following morning, the biopsy site will be examined by the study principal investigator or the US Army Research Institute of Environmental Medicine (USARIEM) Office of Medical Support and Oversight (OMSO). The principal investigator or OMSO will remove the transparent dressing and gauze pad.

3. An appointment will be scheduled with OMSO within 3 d of your procedure to ensure proper healing.

4. Do not take any aspirin or aspirin-containing products for 5 days after the biopsy unless the OMSO MD/PA approves. Also avoid ‘aspirin-like’ drugs such as Motrin, Indocin, etc. Tylenol is OK.

5. After 2 days, no special precautions are required and the site should be almost completely healed.

6. If any of the signs below occur, please contact the study principal investigator:

    - Fever
    - Bleeding from biopsy site
    - Inflammation at biopsy site (warmth, redness, tenderness, pus formation, clear discharge, opening of incision, lump under incision, or rash around incision)
    - If you are not sure if it is healing normally, alert the study principal investigator who will direct you to on duty medical staff
    - Persistent numbness in the leg
    - Pain at the biopsy site more than one week after the biopsy

Appendix E: Photo Release Form

US ARMY RESEARCH INSTITUTE OF ENVIRONMENTAL MEDICINE

Audio/Visual Image Release*

***Only complete this form if you are willing to be photographed or recorded for some or for all of the below-described purposes.**

We are asking your permission to take photographs, videotape, or digital video recordings of you during your participation in the study titled, Erythropoietin induced hematological adaptations to enhance physical performance.

Please note you may be permitting researchers to use and keep your images despite Consent Form language that your identity would otherwise remain anonymous and that records will be destroyed

For Project Illustration and Presentation at Academic Conferences, I grant permission to be photographed or otherwise visually recorded during this study **even if someone could recognize me**: *Please initial* one Yes _____ No _____

Please note you may be permitting researchers to use and keep your images despite Consent Form language that your identity would otherwise remain anonymous and that records will be destroyed

For Marketing, Posting Online, and Public Affairs, I grant permission to be photographed or otherwise visually recorded during this study,

	Initials	
Even if someone could recognize me.	_____	*If you agree, initial one OR the other (otherwise leave blank)*
OR		
Provided that I cannot be recognized.	_____	

Can we use your name?

I grant permission for my name to be on a photo or video image of me or in writing connected to my image.

	Initials	
Yes	_____	*Initial one OR the other*
OR		
No	_____	

I understand that I will not be paid or otherwise compensated for the use of my image.

1. Participant		
Typed or printed name (Last, first, middle initial)	Signature	Date(YYYYMMDD)
2. Witness		

Typed or printed name (Last, first, middle initial)	Signature	Date(YYYYMMDD)

Appendix F: Study Timeline

	Days																																						Weeks
	SV	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	≈ 2
Study Phase	<----Baseline Phase---->								<----Protocol Phase---->																															Post Injection Follow Up			
														<----Injection Period---->																												
Medical Screening	X																																										
Body Composition		X																																			X						
Height		X																																									
Weight		X																	X							X						X							X					
VO2peak		X																										X									X						
Resting Metabolic Rate		X																																			X						
3-d Exercise & Diet Record						X	X	X																																			
Practice Exercise			X	X	X																																						
Study Diet									X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Study Exercise															X	X	X	X				X	X	X	X				X	X	X	X				X	X	X	X					
Time Trial										X									X							X							X							X				
Load Carriage Exercise											X																														X				
Carbohydrate Tracer Study											X																														X				
Blood Sampling (IV Catheter)											X																														X				
Breath Sampling											X																														X				
Muscle Biopsy											X																														X				
CO Rebreathing												X	X																													X	X		
EPO Injection														X		X		X			X		X		X			X		X		X			X		X		X					
Blood Sampling (Sticks) hgb & Hct												X	X						X							X						X							X		X	X	X	X
Clinical Questionnaire															X	X	X	X	X			X	X	X	X	X	X		X	X	X	X	X			X	X	X	X	X	X	X	X	X
Vital Signs															X	X	X	X	X			X	X	X	X	X	X		X	X	X	X	X			X	X	X	X	X	X	X	X	X