Vascular Dysfunction During Physical Inactivity

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"Understanding Inactivity: Investigating the Impact of Reduced Activity on Vascular and Skeletal Muscle Function"

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BACKGROUND AND INTRODUCTION

Prolonged periods of reduced activity are associated with decreased vascular function and muscle atrophy[1-3]. Physical inactivity due to acute hospitalization is also associated with impaired recovery, hospital readmission, and increased mortality.[4] Older adults are a particularly vulnerable population as functional (vascular and skeletal muscle mitochondrial dysfunction) and structural deficits (loss in muscle mass leading to a reduction in strength) are a consequence of the aging process.[5-8] The combination of inactivity and aging poses an added health threat to these individuals by accelerating the negative impact on vascular and skeletal muscle function and dysfunction. The underlying factors leading to vascular and skeletal muscle dysfunction are unknown, but have been linked to increases in oxidative stress.[9] Additionally, there is a lack of understanding of how vascular function is impacted by inactivity in humans and how these changes are related to skeletal muscle function. It is our goal to investigate the mechanisms that contribute to disuse muscle atrophy and vascular dysfunction in order to diminish their negative impact, and preserve vascular and skeletal muscle function across all the lifespan.

The CDC recently stated the average length of a hospital stay is between 4-5 days, with older individuals (>65 yrs) staying marginally longer, 5-6 days[12]. Injuries or illnesses not connected with hospitalization may still lead to periods of inactivity during the recovery process. As such, it is possible for an individual to experience multiple periods of inactivity throughout life. Studies investigating the negative impact of physical inactivity have utilized bed rest, limb immobilization, reduced activity and prolonged sitting[2, 3, 10-12]. Our objective is to utilize these models to understand and prevent the associated decrements in vascular and skeletal muscle function and preserve physiological function.

Bed rest studies have been used to simulate anti-gravity effects during space flight and bed rest during hospitalization[13, 14]. Bed rest studies have shown muscle atrophy and strength loss, but are time intensive and expensive to administer. A common alternative to the bed rest model has become a single leg immobilization model, with either a knee brace or leg cast used to immobilize one leg. Recent studies have shown that short periods of limb immobilization (5-7 days) have caused muscle atrophy with an accompanied loss of strength[2, 10]. Reducing activity by closely monitoring and decreasing daily step counts by 75% have also shown small decreases in muscle mass, along with impairments in insulin sensitivity and increased inflammatory markers[12]. Furthermore, investigators have shown that sitting while being inactive for 3-6 hours can impair vascular function in the lower limb[11]. Taken together these collective studies show the negative impact of physical inactivity.

A proposed mechanistic link between periods of inactivity and the ensuing decrements in skeletal muscle and vascular function, is an increase of oxidative stress. Oxidative stress is also linked with an age related decrease of vascular function. Non-specific antioxidant supplementations have been shown to restore vascular function to levels that are indistinguishable from healthy young adults[15-18]. Animal studies utilizing prolonged skeletal muscle disuse models have shown oxidative stress increases in the inactive muscle, with mitochondria-targeted antioxidants restoring function with a reduction of oxidative[19-21]. We propose that oxidative stress is one mechanism that is being triggered by inactivity and may, in part, be responsible for the negative vascular and skeletal muscle function due to the insult of physical inactivity. One pathway that is responsible for combating oxidative stress in the body is the activation of NF-E2-related factor 2 (Nrf2). Activation of Nrf2 occurs when it is released from Keap1in the cytosol and translocates to the nucleus. In the nucleus, Nrf2 promotes transcription of antioxidant enzymes. Protandim and PB125 are Nrf2 agonist supplements which likely activate Nrf2. Protandim has been shown to activate Nrf2 and increase antioxidant enzyme expression during an oxidative challenge in human coronary aortic endothelial cells[22]. We propose the use of non-specific antioxidants, mitochondria-

targeted antioxidants and a Nrf2 stimulator to determine the impact of oxidative stress on physical inactivity.

Exercise training and rehabilitation can be used as an alternative approach to combat the destructive effects of oxidative stress on aging and disease. An effective exercise training intervention can improve arterial compliance and vascular endothelial function[23, 24]. Exercise training has been shown to alter the pro- and antioxidant balance resulting in improved endogenous antioxidant defense mechanisms[15, 25]. Moreover, exercise training concomitantly improves musculoskeletal strength and function, cardiovascular function, body composition, blood chemistry (decreased triglyceride and cholesterol levels), and overall well-being[26-28]. NASA scientists developed an integrated resistance and aerobic exercise program during a strict 14 day bedrest study. With less than one hour per day performing exercise, participants preserved muscle size, strength and aerobic capacity[29]. We aim to uncover the effect of exercise training and rehabilitation during physical inactivity.

We propose that physical inactivity induces a loss of vascular and skeletal muscle function and structure, which may be mechanistically linked to increased oxidative stress. Through the models and pathways described previously, our aim is to utilize non-specific antioxidants, mitochondrial-targeted antioxidants, Nrf2 promoter supplements and exercise training and rehabilitation in order to understand and mitigate the negative impact of physical inactivity.

OBJECTIVES: An experimental protocol has been designed to specifically evaluate the role of inactivity on functional (vascular and mitochondrial) and structural (muscular) outcomes in young and old individuals.

Specific aim 1: Determine the impact of pre-training on functional (vascular and mitochondrial) and structural (muscular) outcomes before and after inactivity. Hypothesis: Pre-training (or prehabilitation) will increase the reserve of participants as evidenced by functional (endothelial and mitochondrial) and structural (muscular strength and size) improvements prior to inactivity. Following inactivity, a higher level of function will be present in those individuals that performed pre-training. It is expected that improved function will be associated with improved redox balance as a result of pre-training-induced increases in endogenous antioxidant capacity.

Specific aim 2: Determine the impact of exercise during a stint of inactivity on functional (vascular and mitochondrial) and structural (muscular) outcomes. Hypothesis: One to twenty days of exercise during a period of inactivity will minimize the decrements in functional (endothelial and mitochondrial function) and structural (muscular strength and size) outcomes evoked by inactivity. It is expected that the attenuated decline in function will be associated with improved redox balance as a result of exercise induced increases in endogenous antioxidant capacity.

Specific aim 3: Determine the impact of post-training on the recovery of functional (vascular and mitochondrial) and structural (muscular) outcomes following a period of inactivity. Hypothesis: Four to twelve weeks of post-training (or rehabilitation) following a period of inactivity will recover functional (endothelial and mitochondrial function) and structural (muscular strength and size) measures lost during inactivity. It is expected that the augmented recovery compared to baseline will be associated with improved redox balance as a result of exercise induced increases in endogenous antioxidant capacity.

Specific aim 4: Determine the contribution of oxidative stress in inactivity-induced losses in functional (vascular and mitochondrial) and structural (muscular) outcomes. Hypothesis: Administration of antioxidants such as an oral antioxidant cocktail (vitamins C, E, alpha-lipoic acid), mitochondrial-targeted antioxidant (MitoQ), or a NrF2 agonist (Protandim and/or PB125) during inactivity will minimize the decrements in functional (endothelial and mitochondrial function) and structural (muscular strength and size) outcomes evoked by inactivity. It is expected that the attenuated decline in function will be associated with improved redox balance as a result of free radical scavenging and/or increased endogenous antioxidant capacity.

PARTICIPANT SELECTION CRITERIA:

Inclusion Criteria:

- Healthy individuals 18 years of age or older
- Individuals who have recently undergone surgery or injury requiring inactivity (limb immobilization or bed rest)

Exclusion Criteria:

- Age of less than 18 years
- Individuals unwilling or unable to give consent
- Cardiac abnormalities considered exclusionary by the study physician (e.g., CHF, CAD, right-to-left shunt)
- Uncontrolled endocrine or metabolic disease (e.g., hypo/hyperthyroidism, diabetes [HbA1c > 6.5%])
- GFR <45 mL/min/1.73m^2 or evidence of kidney disease or failure
- Vascular disease or risk factors of peripheral atherosclerosis. (e.g., uncontrolled hypertension, obesity, diabetes, hypercholesterolemia > 250 mg/dl, claudication or evidence of venous or arterial insufficiency upon palpitation of femoral, popliteal and pedal arteries)
- Risk of DVT including family history of thrombophilia, DVT, pulmonary emboli, myeloproliferative diseases including polycythemia (Hb>18 g/dL) or thrombocytosis (platelets>400x10^3/mL), and connective tissue diseases (positive lupus anticoagulant), hyperhomocystinemia, deficiencies of factor V Leiden, proteins S and C, and antithrombine III
- Use of anticoagulant therapy (e.g., Coumadin, heparin)
- Elevated systolic pressure > 180 or a diastolic blood pressure > 110
- Implanted electronic devices (e.g., pacemakers, electronic infusion pumps, stimulators)
- Cancer or history of successfully treated cancer (less than 1 year) other than basal cell carcinoma
- Currently on a weight-loss diet or body mass index > 35 kg/m^2
- Inability to abstain from smoking for duration of study
- A history of > 20 pack per year smoking
- HIV or hepatitis B or C*-
- Recent anabolic or corticosteroids use (within 3 months)
- Subjects with hemoglobin or hematocrit lower than accepted lab values
- Agitation/aggression disorder (by psychiatric history and exam)
- History of stroke with motor disability
- A recent history (<12 months) of GI bleed
- Depression [>5 on the 15 items Geriatric Depression Scale (GDS)]
- Alcohol or drug abuse

- Liver disease (AST/ALT 2 times above the normal limit, hyperbilirubinemia)
- Respiratory disease (acute upper respiratory infection, history of chronic lung disease with resting oxygen saturation <97% on room air)
- Women who are pregnant
- Any other condition or event considered exclusionary by the PI and faculty physician

DESIGN: The proposed work falls within the scope of a single protocol. Specifically, this study is designed to investigate the impact of inactivity (in the form of bed rest, limb immobilization or reduced activity) and oxidative stress on functional and structural outcomes. All procedures for the proposed protocol are strictly research-related.

Pregnancy testing will be performed on all women of child bearing age and potential. Any pregnant participants will be excluded from the study.

STUDY PROCEDURES

An overview of each visit to the laboratory, including chronology of the protocol, is listed on the following pages. This is followed by separate headings which provide details for participants, instrumentation, and the techniques and measurements identified in the protocol.

PRELIMINARY SCREENING AND BASELINE DATA COLLECTION (all participants, 1-2 hours/day)

After obtaining informed consent, participants will report to the laboratory (VA Bldg 2, Room 1D21) for orientation, completion of medical history and activity questionnaires, and may have a medical examination performed by one of the study physicians. A screening questionnaire will be filled out that will include questions concerning risks such as: age, family health history, smoking, hypertension, hypercholesterolemia, and physical activity and allergies (iodine, latex, drugs, etc). A venous blood sample (4 ml) may be drawn from which a Metabolic Panel, Complete Blood Count (CBC), and a Lipid Panel will be assessed.

Next, eligible participants may be asked to complete one *or* more of the following exercise tests: a graded exercise test to volitional exhaustion (bike or treadmill) with or without a mouthpiece to determine oxygen consumption, maximal and sub-maximal handgrip test, maximal and sub-maximal leg extension test, and maximal and sub-maximal plantar flexion test. Sufficient recovery will be allowed between tests, and ECG will be assessed at all times. The hand grip, leg extension, and plantar flexion graded exercise tests are considered low cardiovascular risk tests due to the recruitment of an isolated small muscle mass that results in minor cardiovascular stress. The graded whole-body exercise tests to volitional exhaustion (bike, treadmill), when performed in participants over the age of 45 will be completed under physician supervision, and the participant will be monitored by an ECG throughout the exercise test. Any abnormal ECG responses during the exercise test (overt arrhythmia, severe [3 mm] ST segment depression, elevated [1mm] ST segment in non-Q wave lead, frequent extra-ventricular systoles) or adverse responses such as chest pain, leg pain, or unsteadiness will serve as criteria to stop the test, in accordance with American Heart Association guidelines. Questionable findings will be referred to a cardiologist for inspection/diagnosis prior to further involvement in the protocol. Additionally cognitive assessment such as measuring the participant's ability to follow basic instruction regarding exercise training and physical assessments such as a 6-minute walk test may be performed. Additionally, participants may be asked to wear an accelerometer to measure activity levels.

If the participant is unable to complete the testing listed above additional visits may be scheduled to complete the testing.

Following the exercise testing, participants may be given instruction and equipment for physical activity monitoring.

Due to the nature of the testing performed under this protocol, we will not enroll pregnant women. Additionally, women of child-bearing age will be asked to undergo a urine pregnancy test provided by the research site to confirm that these female participants are not pregnant. Additionally, these participants will be asked not to become pregnant while participating in this protocol. If a female participant becomes pregnant she will be asked to inform the PI or other designee immediately and will be withdrawn from any part of this protocol.

EXPERIMENTAL PROTOCOL:

Prior to reporting to the laboratory participants will be enrolled in one of the following modes of inactivity:

- Bed Rest (1 - 10 days) performed at the University of Utah Center for Clinical and Translational Science (CCTS)
- Limb Immobilization (1 - 21 days)
- Reduced Activity (3 hours - 14 days)

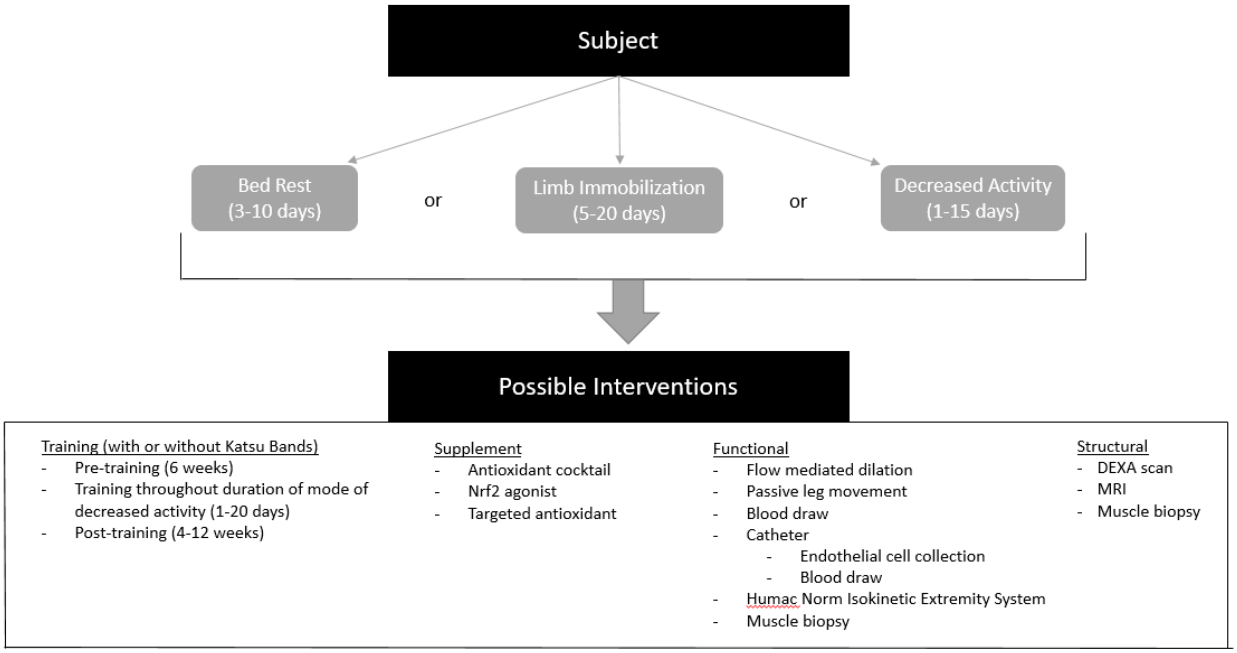
* Detail of each inactivity protocol is described below

Participants may perform exercise training before (1-6 weeks), during, or after (4-12 weeks) inactivity (see Participant **Instrumentation** for more detailed description of exercise protocols). Participants may undergo a period of dietary supplementation (1-20 days) by consuming Protandim (PRO, 675mg/day), PB125 (100-200mg/day) an antioxidant cocktail consisting of vitamins C (500mg), E (200IU) and alpha lipoic acid (300mg), or the mitochondrial targeted antioxidant Mito-Q (40-80mg) before, during, or after inactivity for 1-20 days.

Participants may report to the following laboratories (Utah Vascular Research Lab (UVRL) located at the VA Medical Center Bldg. 2, the CCTS located in Research Park at 421 Wakara Way, Rm 360 or the University of Utah Hospital in the Neuro Acute Care Center, the Utah Center for Advanced Imaging Research (UCAIR) located at 729 Arapeen Dr., or the Skeletal Muscle Exercise Research Facility (SMERF) located in the Department of Physical Therapy at 520 Wakara Way) in a fasted state before, during, and after the inactivity protocols listed above. During these the following tests/evaluations may be performed:

- Blood collection from an antecubital vein
- Muscle biopsy from the gastrocnemius, soleus, or vastus lateralis.
- Flow mediated dilation (FMD) to assess vascular function
- Passive leg movement (PLM) to assess vascular function
- Oral Glucose Tolerance Test (OGTT) and continuous glucose monitoring
- Exercise testing on a Humac Norm Isokinetic Extremity System.
- Body composition assessed by dual x-ray absorptiometry (DEXA) scanner, magnetic resonance imaging (MRI), or D3 creatine.
- Assessment of oxygenated blood using Near Infrared Spectroscopy (NIRS)

- Catheters may be placed in the leg (common femoral artery, dorsalis pedis artery, posterior tibial artery, or femoral vein) or arm (brachial artery, radial artery, or antecubital vein) by a licensed physician using standard sterile technique.
    - Following the catheterization process endothelial cells may be collected using a sterile J-wire (see Participant **Instrumentation** for more detailed description of this procedure) by an approved member of the investigative team.
    - Resting measurements of arterial blood pressure, heart rate, and limb blood flow (ultrasound Doppler) may be taken, and resting blood samples (arterial and venous) may be collected.
- KE, plantar flexion, or handgrip exercise may be performed at several exercise intensities including, but not limited to, 25, 50, and 75% maximal work rate (WR_{max}) or maximal voluntary contraction (MVC) as determined during preliminary testing. Each exercise intensity may be continued for up to 5 min to achieve steady-state pulmonary oxygen consumption. During each work bout the following sequence of events may be conducted:
    1) sampling of femoral arterial and venous blood for the direct measurement of free radicals and blood gases,
    2) continuous measurement of central hemodynamics (heart rate, stroke volume, cardiac output) and arterial and venous pressures (indwelling catheter pressure), and
    3) leg or arm blood flow via ultrasound Doppler.



Inactivity Protocols:

1) **Bed Rest**: After completion of pre-inactivity assessments, participants will adhere to bed rest lasting from 1 to 10 consecutive days. This bed rest model was designed to mimic a traditional inpatient hospital stay and reflect the level of muscle unloading, rest and recuperation that occurs following an acute illness, injury or infection.[30, 31] During bed rest participants will spend a majority of their time in bed and will be allowed to adjust the hospital bed head height for reading, eating

and watching television but otherwise will be instructed to lay the bed flat for sleeping. Bathing and hygiene activities will be performed at the sink in a wheel chair. Subjects will also access the bathroom using a wheel chair. Adherence to bed rest will be monitored by nursing staff 24 hours a day.
2) **Limb Immobilization:** After completion of the pre-inactivity assessments, participants will adhere to unilateral leg immobilization which will be accomplished by a removable knee brace set at 60º flexion (for 1 - 21 days). Crutch assisted locomotion will be utilized to minimize weight bearing of the immobilized leg. Unilateral limb immobilization for 14 days in humans provides an effective and feasible model to evoke significant structural and functional losses in response to unloading resulting in a ~20% reduction in muscle strength, 10% reduction in exercise capacity, 5-6% loss of leg lean masses, and 5-10% decrease in fiber cross sectional area [32-35].

3) **Reduced Activity:** After completion of the pre-inactivity assessments, subjects will adhere to either short term (3 to 6 hours of sitting) or extended (1-14-days) of reduced physical activity at their home/work as has been conducted before.[12, 36] During the short term protocol subjects will remain in the seated protocol with minimal to no movement of the lower extremities as has been described previously.[3, 11, 37, 38] During reduced activity, the goal will be for the participant to drop their physical activity levels to 75% of their normal activity levels as determined by a monitor that the subject will be able to see and record. We will inform the participant that their normal diet should remain the same. A second step monitor will be taped to their leg (waterproof) for the investigators to assess the participants' compliance to the assigned step/day guidelines. For the extended protocols lean mass, strength, power, and physical function measurements will be re-assessed on a later day following the reduced activity study.

PARTICIPANT INSTRUMENTATION (alphabetical order)

Antioxidant and blood measures: Ascorbic acid will be measured in the plasma by the vitamin C assay kit (CosmoBio, Carlsbad, CA). **Ferric Reducing Ability of Plasma** will be determined according the spectrophotometric technique previously described [39]. The FRAP method uses the ability of antioxidants to reduce a FE^{3+}-TPTZ complex to its blue colored Fe^{2+} form. **GSH, GSSG and Total Glutathione** will be measured using BioVision Glutathione Assay Kit (#K264-100) (San Francisco, CA). **Lipid hydroperoxides** will be assessed by the lipid hydroperoxide (LPO) assay kit (Caymen, Ann Arbor, MI). **Nitrite/Nitrate** levels will be measured in the plasma using a standard fluorometric assay kit (Caymen, AnnArbor, MI). **8-isoprostane**, a marker of lipid perodixation and oxidative stress, will be analyzed in the plasma using Cell Biolabs 8-iso-PGF2α kit (#STA-337) (San Diego, CA).

Antioxidant Cocktail: An antioxidant cocktail consisting of vitamins C (500mg), E (200IU) and alpha lipoic acid (300mg) will be consumed daily for 1-20 days. The PI's and Co-I's have previously utilized antioxidant cocktail supplementation in studies of both aging and disease when testing the ability of antioxidant supplementation to prevent free radical-mediated damage [40-45].

Blood Draw: Blood draws will be performed by Dr. Trinity or one of the other trained investigators in phlebotomy technique in our group or by CCTS nursing staff. In the rare case that this procedure is particularly difficult, as this is a standard procedure performed in our laboratory, the participant will be escorted to the VA Medical Center's blood laboratory in an adjacent building where full-time phlebotomists will perform the blood draw.

Body Composition: Dual X-ray Absorptiometry (DEXA) will be used to measure body composition (% body fat, % Lean Tissue, Bone Density) non-invasively. This test will be

performed while lying on a padded bed while the low-level x-ray probe passes over the participant's body (0.0004 cGy radiation exposure associated with the DEXA). Body composition, specifically total body muscle mass, may be determined by the creatine (methyl-d3) dilution method [46]. This methods involves consumption of 30mg enriched creatine, provided in a pill capsule, and subsequent urine analysis of deuterated and unlabeled creatine by liquid chromatography mass spectrometry.

Catheterization: At each catheter insertion site, local anesthesia with lidocaine will be applied (1% lidocaine; 1.33mg/kg assuming 75 kg body weight, with an upper limit of 10 ml). Sterile technique will be applied to minimize risk of infection. 1.0 mg of atropine will be available in case of sudden vagal reactions. The leg (femoral artery and vein, 18G Cook Catheter or equivalent) will be cannulated. A qualified and privileged physician will be responsible for the placement and removal of these catheters. Following catheter removal, wounds will be dressed and the participants educated as to proper care. Participants will not be released until hemostasis is clearly present. Follow-up contact with the participant will continue over the following two days. It should be noted that the Drs. Trinity, Richardson, Supiano and Wray have significant experience with catheter-related studies [47-56], having coordinated the use and insertion of these exact catheters hundreds of times in the last twenty years without incidence of bleeding, infection or tissue damage. In fact, several participants have performed multiple studies with our group, indicating the benign nature and positive outcome of the procedures.

Central hemodynamics: Heart rate, stroke volume, cardiac output, and mean arterial pressure will be determined non-invasively with a finometer (Finapres Medical Systems, Amsterdam, The Netherlands) positioned at heart level. The finometer uses beat by beat arterial pressure waveform analysis as assessed by photoplethysmography using the Modelflow method (Beatscope, version 1.1; Finapres Medical Systems), which in combination with heart rate has been documented to accurately estimate cardiac output during a variety of experimental protocols [57, 58]. This technique requires a blood pressure cuff be placed on the upper arm (biceps) and on the finger of the participant. Mean arterial blood pressure will also be measured by an automatic sphygmometer (Tango+, Suntech, North Carolina) at rest and during exercise.

Continuous Glucose Monitoring: A commercially available continuous glucose monitor will be placed on the participant by CCTS nursing staff or team physician. This device will record 24 hour glucose responses (including in response to meal intake) over the inactivity period.

Diet Stabilization during Bed Rest: Metabolic variability will be reduced by standardizing the diet throughout the hospital stay. Meals will be prepared by the University of Utah CCTS Metabolic Kitchen (dietician) using the Harris-Benedict equation to estimate daily energy requirements, including adjustments for inactivity, and based on the information from a dietician-patient dietary interview, and the nutrient distribution recommended by the American Dietetic Association. The absolute protein content of each meal will be calculated using the pre-bed rest body weight. Macronutrient intake will be evenly distributed between three meals, and uneaten food items will be recorded. Dietary data will be analyzed using The Food Processor software program (Salem, Oregon).

Humac Norm Isokinetic Extremity System: Isokinetic and isometric strength will be assessed in both legs through maximal voluntary contraction effort developed by the knee extensors/flexor (quadriceps/hamstrings) and plantar/dorsi flexor muscles on a CSMI Humac Norm dynamometer.

Electron paramagnetic resonance spectroscopy (EPRS) Ex-vivo spin-trapping with PBN (190 mM/L in 0.9% NaCl) will be utilized for the downstream detection of lipid-derived radical species in blood, as previously described [59-61]. We will use the spin-trap concentration of 3:1 ratio of whole

blood-trap that has proved optimal in our previous studies. We will also use a series of ferrous iron-dithiocarbamate ligand spin-trap complexes to directly assess NO concentration in blood. Briefly, spin-trap adducts will be extracted from serum (or solute in the case of tissue) into toluene, vacuum degassed for 2 cycles and analyzed at 21°C using an EMX X-band EPR spectrometer fitted with an ER TM110 cavity (Bruker, Karlsruhe, Germany). Spectrometer conditions will be as follows: 20mW experimentally validated non-saturating incident microwave power, 0.5 G modulation, 1 x 105 receiver gain, 82 msec time constant, 3450G magnetic field centre and ± 50G scan width, for 15 incremental scans. EPR spectral parameters will be obtained using commercially available software (Bruker Win EPR System, Version 2.11) and filtered identically. The average spectral peak-to-trough line height will be considered a measure.

Endothelial Cell (EC) collection and protein expression: The procedures for the collection of EC and measurement of endothelial protein expression have been previously described [62]. Briefly, following the placement of an arterial (placed by a physician) or venous (placed by an approved member of the investigative team) 1 to 5 sterile J-wires are advanced through the catheter into the artery and/or vein (~4 cm beyond the tip of the catheter) and retracted through an 18-gauge catheter by an approved member of the investigative team, then transferred to a dissociation buffer solution, where ECs are recovered by washing and centrifugation. Collection of ECs may be performed both before and after an acute intervention of exercise, inactivity, or dietary supplementation. EC protein expression will be determined according the method described by Donato et al 2007 [62]. Following EC collection, collected cells will be fixed with 3.7% formaldehyde and plated on poly-L-lysine coated slides (Sigma Chemical, St Louis, Mo). For immunofluorescence staining ECs will be rehydrated with PBS and nonspecific binding sites blocked with 5% donkey serum (Jackson Immunoresearch, West Grove, Pa). Afterward cells will be incubated with monoclonal antibodies for one of the following: nitrotyrosine (Abcam; Cambridge, Mass), xanthine oxidase (US Biological; Swampscott, Mass), NAD(P)H oxidase-p47phox (Abcam; Cambridge, Mass), CuZn SOD (Upstate; Lake Placid, NY), Mn SOD (Research Diagnostics; Concord, Mass), catalase (Abcam; Cambridge, Mass) or NF-KB p65 (Novus; Littleton, Colo). Cells will next be incubated with CY3-conjugated secondary antibodies (Research Diagnostics; Concord, Mass). For analysis, slides will be viewed using a fluorescence microscope (Eclipse 600, Nikon, Melville, NY) and EC images will be digitally captured by a Photometrics CoolSNAPfx digital camera (Roper Scientific, Inc., Tucson, Ariz). ECs will be documented by cell staining of von Willebrand factor and nuclear integrity will be confirmed using DAPI (4',6'-diamidino-2-phenylindole hydrochloride) staining. Once endothelial cells with intact nuclei are identified, they will be analyzed using Metamorph Software (Universal Imaging Corp, Downingtown, Pa) to quantify the intensity of CY3 staining (i.e. average pixel intensity). Values will be reported as ratios of EC protein expression/human umbilical vein EC (HUVEC); this minimizes the possible confounding effects of differences in intensity of staining among different staining sessions.

Exercise Prescription during Limb Immobilization: Total time for each exercise session includes allotted time for warm-up and cool-down before and after resistance and aerobic interval exercise training sessions.

	EXERCISE		
	Resistance	**Aerobic Continuous**	**Aerobic Interval**
Day-1	2-4 sets x 12 repetitions of squats, leg press, heel raise,leg curl, knee extension. Final set to exhaustion *Total Time = 30-60 min*	15-30 min @ 75-80% VO2max *Total time = 15-30 min*	
Day-2			3-6 X 2 min stages @ 70, 80, 90, 100, 90, 80% VO2max.2 min active recovery between intervals *Total Time = 16-32 min*
Day-3	2-4 sets x 12 repetitions of squats, leg press, heel raise,leg curl, knee extension. Final set to exhaustion *Total Time = 30-60 min*	15-30 min @ 75-80% VO2max *Total time = 15-30 min*	
Day-4			3-6 X 30 sec maximal efforts with 4 min active recovery between maximal efforts *Total Time = 16.5-33 min*
Day-5	2-4 sets x 12 repetitions of squats, leg press, heel raise,leg curl, knee extension. Final set to exhaustion *Total Time = 30-60 min*	15-30 min @ 75-80% VO2max *Total time = 15-30 min*	
Day-6			2-4 X 4 min @ 90% VO2max 3 min active recovery *Total Time = 17.5-35 min*
Day-7			
Day-8	2-4 sets x 12 repetitions of squats, leg press, heel raise,leg curl, knee extension. Final set to exhaustion *Total Time = 30-60 min*	15-30 min @ 75-80% VO2max *Total time = 15-30 min*	
Day-9			3-6 X 2 min stages @ 70, 80, 90, 100, 90, 80% VO2max.2 min active recovery between intervals *Total Time = 16-32 min*
Day-10	2-4 sets x 12 repetitions of squats, leg press, heel raise,leg curl, knee extension. Final set to exhaustion *Total Time = 30-60 min*	15-30 min @ 75-80% VO2max *Total time = 15-30 min*	
Day-11			3-6 X 30 sec maximal efforts with 4 min active recovery between maximal efforts *Total Time = 16.5-33 min*
Day-12	2-4 sets x 12 repetitions of squats, leg press, heel raise,leg curl, knee extension. Final set to exhaustion *Total Time = 30-60 min*	15-30 min @ 75-80% VO2max *Total time = 15-30 min*	
Day-13			2-4 X 4 min @ 90% VO2max 3 min active recovery *Total Time = 17.5-35 min*
Day-14			

Pre and post inactivity exercise training (i.e.; prehabilitation and rehabilitation): The prehabilitation and rehabilitation interventions mimic the cardiac rehabilitation program currently used by the VA SLC and University of Utah Cardiac Rehabilitation centers with modifications developed in conjunction with Dr. Paul LaStayo (Co-Investigator, Physical Therapy) to optimize improvements in aerobic capacity and skeletal muscle strength. The prehabilitation and rehabilitation interventions will include the following:

A. Initial Orientation:

1) Preliminary questionnaires to assess diet, current physical activity, and psychosocial health (SF-12-Health Survey, PHQ-9 -Patient Health Questionnaire to assess depression, DASI-Duke Activity Status Index Score, and DRA-Dietary Risk Assessment)
2) Interview with a registered nurse to review medications and past medical history.
3) Cardiopulmonary physical therapy examination to assess readiness for exercise.
4) Anthropometric measurements – waist circumference, height, weight, body mass index calculation (BMI)

5) Cycle ergometry test to assess exercise capacity and 10 repetition maximum testing to determine appropriate workloads for resistance exercises.
6) Initial exercise prescription will be based on results of the cycle ergometry and 10 repetition maximum tests.The personalized PREHAB exercise prescription plan is described below (***See 3. Exercise Prescription, Performance, and Progression***).

B. Pre-exercise assessment:

1) Weight is obtained from subject.
2) Resting blood pressure and heart rate are obtained.
3) Subjects' telemetry is evaluated before, during, and after exercise.
4) Proper warm-up exercises are taught, demonstrated, and performed.

C. Exercise Prescription, Performance, and Progression:

1) Subjects will perform PREHAB or REHAB 3 times per week for the duration of the intervention.
2) The prescribed exercise is based on the individual's baseline aerobic capacity and strength.
3) Evaluations of aerobic capacity and strength will be assessed at 2 week increments in order to adjust exercise intensities and maintain an adequate training stimulus. The

	Exercise Prescription			
Day 1	***Sets***	***Reps / Time***	***Intensity ****	***Rest***
Aerobic High Intensity Intervals	4	4min	80 - 85% of VO2max	4 min
Resistance Exercise				
Reverse Lunge	2-3	10/side	70 - 80% 1 RM	1 min
Pushups	2	10	70 - 80% 1 RM	1 min
Hamstring Curls	2-3	10	70 - 80% 1 RM	1 min
Heel Taps	2	8/side	70 - 80% 1 RM	1 min
Day 2	***Sets***	***Reps / Time***	***Intensity ****	***Rest***
Aerobic Continuous Exercise	1	30 min	65 - 70% VO2max	NA
Resistance Exercise				
Deadlift	2-3	10	70 - 80% 1 RM	1 min
Low Incline 1 Arm DB Bench Press	2	10/side	70 - 80% 1 RM	1 min
Step Up	2-3	10/side	70 - 80% 1 RM	1 min
Day 3	***Sets***	***Reps / Time***	***Intensity ****	***Rest***
Aerobic High Intensity Intervals	10	1 min	90 - 95% VO2 max	1 min
Resistance Exercise				
Box Squat	2-3	10	70 - 80% 1 RM	1 min
1 Arm Cable Row	2	10	70 - 80% 1 RM	1 min
Unilateral Knee Extension	2-3	10/side	70 - 80% 1 RM	1 min

exercise intensity for aerobic exercise will be set at predetermined work rates (watts) known to elicit a given % of VO_2max. The work rate verse VO_2 relationship will be determined during baseline VO_2max and ventilatory threshold testing. As aerobic capacity increases absolute work rates will be progressively increased. The intensity for resistance exercise will be based on 10 repetition maximum. During the final set of each resistance exercise subjects will be instructed to continue until volitional exhaustion. If the subject is able to perform more than the prescribed number of repetitions, the load will be increased accordingly for the next session. If the subject is not able to complete the prescribed number of repetitions the load will be maintained or decreased accordingly for the next session. Abbreviations: DB (dumbbell).

D. Post-Exercise Assessment:

1) Proper stretching exercises are taught, demonstrated, and performed.
2) Subject rests for five minutes and post-exercise blood pressure and heart rate are obtained.

E. Documentation of Exercise Sessions:

1) All data collected during an exercise session is written on an intake sheet.
2) All data is then entered into EPIC.

F. Final Evaluation:

At the completion of the prehabilitation and rehabilitation program, the subject will receive an exit interview to review the following:

1) Preliminary and final tests are given to subject to evaluate outcomes.
2) Subjects' current exercise prescription
3) Subjects' anthropometric measurements
4) Summary of the outcomes obtained by the subject through the program.
5) Note is forwarded to referring physician and Medical Director (Dr. Supiano).

Exercise Testing: Passive leg movement will be performed by a member of the research team moving the participant's leg through a normal range of motion (90°- 180°).

Single contraction exercise protocols will be performed with either the upper (handgrip) or lower (knee extension) extremity. A single maximal voluntary contraction (MVC) will be established for each participant using a handgrip or knee extension dynamometer with an analog output. This MVC value will be used to calculate 10%, 20%, 30%, 40% and 50% of MVC for the single contraction. Following a 2 min baseline period the participant will perform the rapid (< 1 sec) single contraction and central and peripheral hemodynamics will be assessed for 1 min post contraction.

Active leg exercise will be performed using a custom-built knee-extensor ergometer with the participant moving the leg between 90°- 180° at a rate of approximately 60 rpm.

Plantar flexion exercises will be performed using an ergometer specifically designed to isolate the gastrocnemius and the soleus complex at a rate of approximately 60 rpm.

Handgrip exercise will be performed with the participant laying supine on the laboratory bed with handgrip dynamometer in their hand. The arm will be extended perpendicular to the participant's torso and supported by a height-adjustable table. Doppler ultrasound will be performed by a trained sonographer in order to obtain brachial artery blood flow measurements. Exercise will be performed for 4 stages each lasting 3 - 5 minutes and separated by at least 1 min of rest. Intensity will range from 10 to 60% of the participant's maximal handgrip strength. During exercise ECG and blood pressure will be continuously monitored. Arm exercise will involve rhythmic handgrip using a computer-interfaced dynamometer.

The graded exercise test will be performed on a cycle ergometer or treadmill using a conventional ramp test to exhaustion. Work rate/exercise intensity will be increased by an appropriately tailored level from 2.5-25 watts in each step, depending upon capabilities. Pulmonary ventilation and gas exchange will be determined continuously using a low-resistance two-way breathing valve (Hans-Rudolph 2700, dead space 90 ml) and a pneumotachometer (Fleisch no. 3) on the expiratory side. Signals will be assimilated by a PC and O_2 and CO_2 analysis will be performed in real time (Parvo Medics, UT). All of this equipment is currently in place and has been used routinely in our laboratory with both healthy volunteers and patients. Throughout all our exercise testing, safety

is ensured through careful echocardiogram (ECG) monitoring, availability of a defibrillator, an oxygen source, emergency drugs, and attendance of qualified personnel.

Flow Mediated Dilation (FMD): After 15-20 minutes in the supine position a blood pressure cuff will be placed on the leg or forearm. Ultrasound images of the artery and Doppler waveforms will be obtained prior to cuff inflation (baseline). The cuff will then be inflated to 250mmHg for 5 minutes. Following the cuff release, ultrasound images of the popliteal artery and Doppler waveforms will be recorded continuously for 2 minutes. During this time, arterial images during diastole will be captured by image analysis software (Medical Imaging Applications). All recorded ultrasound images and velocity spectra segments will be saved to the GE Logiq 7 hard drive for off-line image and waveform analysis. The time averaged, and intensity-weighted mean blood velocity (Vmean) will be averaged across 5 second intervals for the entire 2 minute clip following cuff release. In addition, edge detecting software will be utilized to measure arterial diameter frame by frame, which will also be averaged into 5 second intervals. Summed shear rate throughout the 2 minutes will be calculated based on vessel diameter and blood velocity. The maximum change in vessel diameter, relative to baseline, will be determined and expressed as %FMD and %FMD/summed shear rate. A greater FMD response is an indicator of healthier vessels. A typical response for a young healthy participants would be 8 - 12% or greater dilation, whereas a poor response would be <5%. In addition, it is also important to be able to express the change in diameter relative to shear stress, as the shear stress is the stimulus for the NO-induced dilation. Again, greater FMD/shear values are indicative of healthier vessels.

Limb blood flow: Blood flow will be measured with an ultrasound Doppler (Logiq 7, GE) equipped with linear array mechanical sector transducers operating at an imaging frequency of 10-14 MHz. Vessel diameter will be determined at a perpendicular angle along the central axis of the scanned area, where the best spatial resolution can be achieved. The blood velocity profile will be obtained using the same transducers with Doppler frequency of 4.0-5.0 MHz, operated at high-pulsed repetition frequency mode (2-25 kHz) with a depth of 1.5-3.5cm. Special care will be taken to avoid aliasing, to ensure that probe position is stable, the insonation angle does not vary, and that the sample volume is positioned in the center of the vessel and adjusted to cover the width of the diameter and the blood velocity distribution. Blood velocity measurements will be obtained with the probe at an appropriate angle to maintain an insonation angle of 60º or less and the sample volume centered. Using arterial diameter and mean velocity (Vmean), blood flow will be calculated as: *Blood Flow (mL/min) = Vmean · π · (Vessel Diameter/2)2 · 60.*

MitoQ: A mitochondrial targeted antioxidant supplement containing the antioxidant quinone moiety covalently attached to a lipophilic triphenylphosphonium cation (TPP). The TPP moiety on MitoQ enables its accumulation within mitochondria driven by the negative membrane potential. Once within mitochondria, nearly all the accumulated MitoQ is adsorbed to the matrix surface of the inner membrane where it is continually recycled to the active quinol antioxidant form by complex II in the respiratory chain.[63] Participants will consume 40-80mg/day for 1-20 days as previously described.[64, 65]

Muscle biopsies: All biopsies will be taken from either the gastrocnemius approximately 3.5 cm deep, anteromedial aspect of gastrocnemius muscle belly 10 cm distal to the tibial tuberosity or the vastus lateralis approximately 3.5 cm deep, 15 cm proximal to the knee and slightly distal to the ventral mid-line of the muscle. The 5 mm diameter biopsy needle (Bergstrom) is attached to sterile tubing and a syringe to apply a negative pressure to assist in the muscle sample collection [66]. Lidocaine (2%) will be used as local anesthetic. The Principal Investigator has been involved in over 100 of these procedures with no negative outcomes. Additionally, in support of this technique, a series of subjects recently underwent 5 biopsies each over a period of time, illustrating that the biopsy experience is acceptable to the subjects. The muscle samples from

each biopsy will be immediately frozen in liquid nitrogen and stored at -80°C for future microscopy, EPR spectroscopy, molecular and biochemical assays or immersion-fixed in gluteraldehyde for EM analysis. With our current technique and experience, utilizing 2-3 passes of the needle and double cut, we are able to attain as much as 300-400 mg of muscle which will permit the performance of all proposed analyses.

Oral Glucose Tolerance Tests (OGTT): This is a standard clinical procedure to determine the blood glucose and insulin responses to a standardized glucose challenge. This takes approximately 2 hours. Glucola, a sugary beverage containing 75g of glucose, will be administered orally over 2 to 5 minutes. Blood samples from an intravenous catheter will be determined before and 30, 60, 90, and 120 minutes after Glucola consumption. Approximately 5 mls of blood will be collected during the OGTT.

Passive Leg Movement (PLM): Participants are moved into an upright sitting position for ~10 min before the start of the data collection and remain in this position throughout the entire protocol. The protocol consists of 60-s of resting baseline data acquisition followed by a 2-min bout of passive leg extension. PLM is achieved by a member of the research team moving the participant's lower leg through a range of motion, defined by 90° and 180° knee joint angles, at a rate of 1 Hz. Throughout the protocol, the non-moving leg remains fully extended and supported. Real-time feedback to the investigator is provided by a metronome to maintain the cadence. Before commencing, and throughout the protocol, participants are encouraged to remain passive and resist any urge to assist with leg movement. To avoid a startle reflex and active resistance to the PLM, participants are made aware that the assessment will start in the next minute, but to minimize the chance of an anticipatory response, they are not informed of exactly when movement will begin 122. There are no known risks of this procedure.

PB125: The product PB125 (Pathways Bioscience, Aurora, CO) to be used in this study contains extracts derived from three botanical sources: *Rosmarinus officinalis*, standardized to carnosol content of 6%, 68mg of extract; *Withania somnifera* (aka Ashwagandha), standardized to withaferin A content of 1%, 23mg of extract; and *Sophora Japonica*, standardized to luteolin content of 98%, 9mg of extract, to create a total daily dosage of 100mg of combined extracts administered in a single capsule administered up to twice daily (i.e. 100-200mg/day). Other inactive ingredients consist of rice flour and hydroxypropyl cellulose (vegetarian capsule). PB125 meets or exceed GMP quality standards. The product does not contain gelatin, dairy, yeast, wheat, or gluten.

Physical Activity Assessment: Physical activity of the participant may be assessed by accelerometery (i.e. activity monitor) and/or physical activity questionnaire. With accelerometry, participants will be asked to wear a small activity tracker attached to a belt fastened around their arm, leg, or waist for approximately 7 days. These activity trackers (Actigraph, Actilife, USA) simply record the number of steps a person takes each day, and the amount of movement a person makes each day. Participants may also be asked to complete a physical activity questionnaire (International Physical Activity Questionnaire, IPAQ) or IPAQ for Elderly123. These short questionnaires simply assess how many minutes per day an individual performs physical activity.

Protandim: A nutritional supplement comprised of five plant extracts (milk thistle, bacopa, ashwagandha root, turmeric, green tea) that activates the Nuclear factor (erythroid-derived 2)-like 2, (called Nrf2) transcription factor pathway that is integral to several antioxidant enzymes, including γ-glutamyl cysteine synthase (an enzyme that catalyzes the committed step in glutathione synthesis).[67] Nuclear factor (erythroid-derived 2)-like 2 is a basic leucine zipper protein transcription factor that regulates the expression of antioxidant proteins that protect against

oxidative damage triggered by injury and inflammation. Participants will consume 675mg/day for 1-20 days as previously described.[68]

DATA SAFETY & MONITORING

In the interest of participant safety and to ensure compliance with IRB and FDA regulations, we propose the implementation of a data safety and monitoring group. Four Utah Vascular Research Laboratory faculty members (Drs. Trinity, Richardson, Wray, and Amann), an internal sub-investigator (Mark Supiano, M.D.), four members external to the UVRL: Dr. Alan Light and Micah Drummond, Ph.D., Dorothea Rosenberger, M.D., Jacob Jessop, M.D. and Lisette Perez and Shelby Love, coordinators for the DSMB will make up the monitoring group, all of whom are affiliated with the University and the SLC VA Geriatric Research Education and Clinical Center. These members will meet on a quarterly basis, and additionally will convene in response to any adverse events or unanticipated problems associated with the proposed studies.

All participants participate on a strictly voluntary basis, and thus any protocol may be stopped at any time and for any reason at the participant's request. Additional criteria to discontinue a protocol will be any evidence that the participant's health is at risk, as identified by a member of the investigative team. Participant safety is at the forefront of all studies, and for this reason all investigators are trained to identify overt (i.e. abnormal ECG patterns, acute change in blood pressure, etc.) and more subtle (i.e. skin pallor or redness, changes in participant demeanor, etc.) signs that a participant may be adversely affected by the experimental interventions. Adverse events and unanticipated problems are broadly defined as any instance of a medical complication arising from catheter insertion (i.e. excessive bleeding and swelling at insertion site) or drug infusion (i.e. acute hypertension, bradycardia, etc.) as listed under the "potential risks" section below. In the instance of an adverse event, the protocol will be discontinued and the study will be temporarily suspended while DS&M members will review the cause of the event. When appropriate, unanticipated adverse events will be reported within 10 working days, in accordance with the University of Utah IRB Reporting Policy and to any other pertinent regulatory body(ies).

POTENTIAL RISKS

Due to the nature of the testing performed under this protocol, we will not enroll pregnant women. Additionally, women of child-bearing age will be asked to undergo a urine pregnancy test provided by the research site to confirm that these female participants are not pregnant. Additionally, these participants will be asked not to become pregnant while participating in this protocol. If a female participant becomes pregnant she will be asked to inform the PI or other designee immediately and will be withdrawn from any part of this protocol.

Bed Rest Risks: Bed rest will be performed at the University of Utah CCTS with around-the-clock medical supervision by trained medical staff. To ensure safety and comfort during the 5 days of bed rest we will follow our established guidelines presented below:

- A slight bed-back elevation will be permitted and subjects may raise their shoulders with two pillows.
- Subjects will be encouraged to change position periodically to alleviate positional discomfort and to eat.
- Serial compression devices and compression stockings will be worn to decrease DVT risk.
- Physical therapy will be provided daily to reflect standard of care for bedridden individuals. It will include passive range of motion to lower extremity major joints in all planes (~10 repetitions/plane)

- Bathing and hygiene activities will be performed during bed rest.
- A bedside wheelchair will be provided to transport to the bathroom for urine excretion and bowel movements.
- Daily safety blood draws (D-dimer, complete blood count with platelet count and auto-differential, partial thromboplastin time, prothombin time).
- 24h nursing supervision and care and medical oversight by team physician (Co-I: Mark Supiano, MD)

Following the bed rest period, participants will be immediately evaluated by a physical therapist and CCTS nursing staff to deem if the patient is safe to be discharged from the hospital research unit (CCTS). We have observed that after 5-days of bed rest, healthy older adults experience losses in muscle mass and strength but do not exhibit functional or cognitive impairments and are well enough to return home without inpatient exercise rehabilitation. Patients may return to the CCTS the following day for a MRI scan to determine the effects of bed rest on leg lean mass and afterwards visit the SMERF laboratory for knee extensor leg strength assessment as outlined in the experimental timeline.

Limb Immobilization Risks: Limb immobilization is an established model of inactivity resulting in significant muscle atrophy (~4-5%) and losses in muscle strength (~20%) and exercise capacity (~10%). During limb immobilization participants will remain ambulatory by use of crutches. Participants will be instructed on proper use of crutches by licensed Physical Therapist in an effort to minimize risks of falling and injury. During limb immobilization there is an increased risk of deep vein thrombosis. To minimize this risk periodic blood draws (every 3rd day) during limb immobilization will be performed and assessed for D-dimer, complete blood count with platelet count and auto-differential, partial thromboplastin time, prothombin time. Additionally, participants will be instructed to perform unweighted hip, knee, and ankle contractions at least twice daily to activate the muscle pump and further minimize the risk of DVT. Approximately 10 contractions at each joint will be performed. If advised by the team physician compression stockings may also be worn during limb immobilization.

Myocardial ischemia, infarction, arrhythmia, and death during exercise. These risks will be minimized by the initial pre-screening and non-invasive testing of each participant. If problems are found the participant will be excluded and referred to his/her physician. The responses to absolute and relative indications for the termination of an exercise test are variable depending upon the finding. However, an incident of ischemia presented as chest pain, shortness of breath, acute hypotension (<80/40 mmHg), or abnormal ECG findings (>2-3 mm ST depression or appearance of S_3 or S_4 heart sounds) would result in the immediate termination of exercise. The participant would be placed in a supine position, administered oxygen and assessed by the on-site physician and research staff. If the condition did not improve or worsened 911 would be called. In all cases this occurrence would be documented and the participant would ultimately be referred to their physician. Cardiac function will be monitored during all exercise studies with an ECG monitor.

Biopsy risks: Using sterile technique and a minimal skin incision (about 1/8”) over the anterolateral mid-thigh, the standard needle aspiration method for muscle biopsy has not produced bleeding nor infectious complications to date. There is the possibility of a small scar the size of the incision (1/8”). No subject is released until hemostasis has been secured, and the skin site dressed. Subjects are always carefully instructed in the routine prevention of infection and bleeding of biopsy sites**.** As lidocaine is used as the anesthetic there is the risk of toxicity, however the low concentration 0.5% and the use of less than 10-15 ml greatly reduces this concern. Though multiple biopsy procedures create additional sites which may be prone to infection, as outlined

above the technique employed minimizes this risk. Nonetheless, on protocols where multiple biopsies are performed, special care will be taken with the sterile dressing and bandaging to further minimize risk of infection. Further, the small quantity required for each sample (300-400 mg) is such that attaining multiple biopsies in one visit is not viewed as presenting a compound risk in terms of muscle damage and healing.

Additionally, nerve injury including paresthesia or motor deficits could occur that could be permanent, and chronic muscle atrophy or possibly permanent pain could occur at the biopsy site. If an infection occurs post biopsy, antibiotics or even surgery to repair infection damage may be necessary.

Catheter-related risks. There is also the possibility of fainting, dizziness, and possible pain and bruising as a result of catheter insertion. There is a risk of infection, swelling, and discomfort at the insertion sites, and a risk that bleeding may occur after catheters removal. A clot or excessive bleeding at the puncture site could result in a partial blockage of the blood flow to the leg, which in extreme cases could lead to loss of the limb. Based on the fact that two arteries supply the hand versus only one artery supplies the leg, femoral arterial catheterization bears a greater risk to the participants' health. Accordingly, the participants will be allowed to select the site of the arterial catheterization, the choices are either femoral or radial artery. These risks are minimized by careful patient selection, catheter insertion by a physician with experience using proper equipment and sterile methods, a continuous infusion of fluids thorough the catheters, adequate pressure for >30 minutes following catheter removal, and appropriate patient education and follow-up. The PI and several CO-I's named herein have significant cumulative experience in this areas, having been involved in several hundred successful catheter-based studies with no instances of bleeding, infection, femoral vein thrombosis or soft tissue damage as a result of the procedures proposed.

Pregnancy risks: Due to the fact that little is known about how these procedures will affect a fetus, we cannot allow pregnant women to participate. If you are a female of child-bearing age, you will be asked to undergo a urine pregnancy test provided by the research site prior to participating in any of the actual study days. Acceptable methods of birth control include oral/topical/injected contraceptives, intra-uterine devices, and barrier methods. If you become pregnant while taking part in the study, you must immediately tell your research doctor and you will be withdrawn from the study. We will follow the outcome of your pregnancy and we will continue to follow you according to the study plan.

Exercise Risks: There is elevated risk for injury or cardiovascular events during exercise. However, the laboratory's significant amount of work with exercise in healthy, diseased, and aged populations over the past 20 years has revealed that this is an issue of caution and when approached as such, is not a problem. Exercise PREHAB and REHAB will be performed under strict supervision in a controlled environment (please refer to following section pertaining to protection against risk during exercise protocols).

Radiation Risks DEXA Scan: This research study involves up to 3 DEXA scans. These scans are not standard of care and you are receiving them only because you are enrolled in this research study. These procedures will expose you to radiation. The risk from this radiation exposure is considered to be small and comparable to other every day risks. To give you an idea of how much radiation you will receive, we will compare this radiation to the radiation that you receive from natural sources. Everyone receives a small amount of unavoidable radiation every day. Some of this radiation comes from space while some comes from radiation that is naturally occurring in water, soil, rocks and minerals found in plants and animals. The excess radiation that you will be

exposed to in this research study is equivalent to about 2 days of natural background radiation. This amount does not include any radiation exposures that you may receive from other types of tests

RISK MANAGEMENT PROCEDURES

By far the most important issue is prevention of the above risks by proper participant pre-screening, by high quality technique of catheter and by careful monitoring during exercise (see methods section), and by ensuring post-study patient education and follow-up of catheter site care. For all protocols, ECG activity and arterial blood pressure are monitored by an observer who is not involved in any other aspects of the study. Both this observer and the participant have absolute authority to terminate a study at any time. Participant confidentiality is ensured by the coding of all personal information and the maintenance of all records and samples within a locked structure accessible only to key personnel involved in the research study. Furthermore, during the exercise, RPEs will be obtained from the participants at the end of each stage. This includes evaluation of a) overall feeling, b) leg discomfort, and c) dyspnea. The laboratory is equipped with a fully stocked resuscitation cart that includes bag/mask ventilation equipment, intubation equipment, suction equipment, electrical defibrillator an O_2 source and resuscitation drugs including naloxone. In the event of symptoms or signs of an adverse cardiopulmonary event – anginal symptoms, extreme dyspnea, dizziness, pre-syncope or frank syncope, severe respiratory distress, decrease in S_pO_2 below 90%, tachydysrythmia – exercise will be ceased immediately, with institution of evaluation and management according to ACLS protocol (maintenance of airway, supplemental O_2, ventilatory assistance if necessary, electrical cardioversion or defibrillation if necessary). If the participant requires cardiopulmonary resuscitation or further medical therapy, they will be immediately transported to the Emergency Department.

STATISTICAL METHODS, DATA ANALYSIS AND INTERPRETATION:

A total of 150 participants will be recruited for the protocols outlined above. The experimental design and statistical analyses have been developed in collaboration with the Study Design & Biostatistics Center at the University of Utah. We focus on well-powered testing of the primary hypotheses while minimizing subject burden and study costs. Descriptive summaries will be provided for each outcome prior to initiating formal statistical analyses. Transformations may be sought for outcomes exhibiting substantial skewness to better approximate normality. If approximate normality cannot be achieved following transformation, rank-based non-parametric analyses (such as Wilcoxon rank-sum test) will be substituted for parametric analyses.

Comparisons between conditions (ie; inactivity verse activity, placebo verse intervention, trained verse untrained) may be made prior to, during, and after the inactivity intervention. No baseline group differences are expected while significant improvements in vascular and mitochondrial function are expected prior to inactivity in those participants undergoing exercise training. During inactivity, measures of vascular and skeletal muscle function are expected to decline. Rates of decline are expected to be attenuated when an intervention such as exercise training or antioxidant supplementation (MitoQ, Protandim, PB125, Oral Antioxidant) is administered. A rich set of measures will be obtained from biopsied muscle, blood samples, and measures of strength, lean mass, and aerobic capacity before and after the intervention for each group. These comparisons with different outcome measures will be considered secondary or supportive analyses. For the formal test of the hypotheses we will use analysis of covariance (as implemented SAS PROC GLM) with the final vascular and mitochondrial function assessments

as the dependent variable and baseline as independent variables as suggested by Vickers and Altman [69]. We will also include age and gender as independent variables in the model. Similar models will be constructed for secondary or supportive analyses listed above.

Clinical Records: Patient demographics (DOB, gender, height, weight), past medical history, medication use, and results of cardiovascular tests (echocardiogram, catheterization, stress test) will be collected.

The following procedures are in place in the Vascular Laboratory to assure safety of research participants:

1. Properly trained research personnel will monitor all exercises to ensure they are performed correctly.
2. Individuals performing blood draws are familiar with all disease transmission safeguards and emergency procedures, should a problem arise.
3. Blood pressure cuffs will only be inflated to obtain the necessary measurements, and will be deflated immediately afterwards.
4. Immediate emergency – call 911, as typically employed at the Salt Lake City VA Medical Center that are not covered by the code team from the hospital.
5. A crash cart and defibrillator are maintained in the laboratory.

The following procedures are in place in the Vascular Laboratory to protect sensitive participant information:

1. The laboratory is kept locked when there are no lab personnel present.
2. The Logic 7 is turned off when not in use for vascular analyses. The computer is password protected and only the investigators related to this project know the password.
3. Hard copies of participant information will be kept in locked file cabinet and locked in offices. Any electronic copies will be kept on a password secured computer.
4. Participants are given copies of their measurements on request.
5. Each participant will be identified by code numbers. These numbers will be used in a statistical spreadsheet while all statistical analyses are performed. The maximal length of storing this information is 5 years, upon which it will be destroyed.

ADMINISTRATIVE RESPONSIBILITIES:

Facilities and Equipment

Utah Vascular Research Laboratory (UVRL)*:* The PI, Dr. Joel Trinity, is a member of the UVRL, a consortium that is affiliated with the University of Utah (Department of Internal Medicine) and housed at the Salt Lake City Veterans Administration Medical Center Geriatrics, Research, Education and Clinical Center (SLC VAMC GRECC). At present, the UVRL includes 5 full-time faculty members, 8 postdoctoral fellows, 8 graduate students, 2 full-time laboratory specialists, and 2 clinical research study coordinators. The full-time laboratory specialists include a microbiologist and a biochemist that conduct and assist trainees in the performance of various assays including the biochemical analysis of free radicals and their footprints. Additionally, two full-time research study coordinators are responsible for scheduling and recruiting subjects and maintaining compliance with the University and VA Institutional Review Boards.

Research performed in the UVRL revolves around the link between vascular and skeletal muscle function. Specifically, faculty of the UVRL, are interested in the mechanisms that appear to limit

skeletal muscle function as the result of healthy aging and age-related disease including heart failure, hypertension, and chronic obstructive pulmonary disease. One of the many potential candidates upon which we are currently focusing considerable attention is the role of oxidative stress in the regulation/dysregulation of skeletal muscle metabolism and vascular control. The UVRL consists of 4 human-based laboratories, each approximately 800 sq. ft., and 3 wetlabs ranging from 250 to 800 sq. ft. All laboratories are located at the Salt Lake City VA Medical Center, Building 2. The proposed vascular function assessments will take place in the human based laboratories while chemical analysis including electron paramagnetic resonance spectroscopy, molecular biology, and biochemistry will be performed in 1 of the 3 wetlabs.

Skeletal Muscle Exercise Research Facility (SMERF): Drs. Paul LaStayo and Micah Drummond (Sub-investigator's) Co-Direct the SMERF, a clinical research training environment focused on the interrelationship of impaired muscle strength/power and activation; and mobility/gait disorders and falls. Current research performed at the SMERF is focused on aging muscle and how it impacts movement and human performance; specifically how aging muscles adapt and how these changes help or hinder human function in daily life and following injury or disease. The SMERF, a compilation of 5000 ft^2 of clinical-lab space, is located on the 1^{st} floor in the College of Health's Department of Physical Therapy and Athletic Training. Previous and current clinical trials designed to evaluate muscle countermeasures and commensurate muscle, mobility and quality of life endpoints have been successfully performed in the SMERF across a variety of older individuals and clinical populations. The SMERF is located on the University of Utah campus yet away from busy day-to-day traffic. Therefore, it is easily accessed by car, bus or via the free ParaTrans system; there is ample free parking and an accessible entrance to both facilities for those who are mobility impaired. The SMERF houses standard muscle and mobility training equipment as well as measurement lab stations for assessing muscle structure/function and analyses of mobility/activity performance. The proposed assessments of muscle strength will take place at the SMERF.

Center for Clinical and Translational Science (CCTS): The proposed project will utilize the several services provided by the University of Utah CCTS. Briefly, the CCTS will provide 24 hour care of subjects during the 1-10 days of bed rest. Moreover, assessment of body composition via Magnetic Resonance Imaging (MRI), muscle biopsies, and venous blood collections will be performed by CCTS nursing staff. Members of the investigative team (Trinity, Drummond, LaStayo, Supiano) have successfully performed and are currently performing several bed rest studies at the CCTS.

University Center for Advanced Imaging Research (UCAIR): For studies involving NMR and MRI, we will collaborate with UCAIR. This center consists of a 7000 sq. ft. of office, class room and laboratory space located in the Center for Advanced Medical Technologies (CAMT) at Arapeen Drive in Research Park. Linux and SUN workstations, computer terminals, PCs and MacIntosh computers are available throughout the lab. The lab contains an electronics shop fully equipped with oscilloscopes, signal generators, and other essential test and measurement equipment with bench space and various equipment for electronic fabrication and testing, a small machine shop, and wet-lab space with fume hood in addition to a full stock of electronic parts, manuals, and data books. UCAIR also has a coil lab, equipped with an HP 8752A network analyzer, a HP 4193A vector impedance meter, and a full size test bore/RF shield to simulate the MRI electrical environment. The School of Medicine machine shop is located in the CAMT building and is fully equipped and available for major machining projects. There is approximately 800 sq. ft. of shielded small bore magnet space, and about 200 sq. ft. of preparation area adjacent to the human imaging magnets. The CAMT building also houses two Siemens Trio 3T MR scanners. The scanners have actively shielded water cooled Sonata gradient systems (40 mT/m amplitude,

200 T/m/s slew rate), 32 high speed RF receiver channels and RF body coil, head coil and several coils of variable numbers of elements designed for specific body parts. Custom coils for unique geometries may be designed and fabricated in the coil development lab (above). The systems are also equipped with pulse sequences and appropriate software for cardiac imaging, spectroscopy, functional MRI, diffusion tensor MRI and other high end imaging capabilities. A highly flexible pulse sequence development platform (IDEA) is also included to allow research and development in novel techniques. We have a research agreement and research support from Siemens and collaborate with them.

Personnel

All investigators of this study have extensive experience with each of the techniques described in the proposal.

Recruitment & Participant Compensation

Participants will be recruited by staff at the Utah Vascular Research Laboratory. Recruitment strategies will include recruitment fliers (VAMC SLC and University of Utah), VA and University patient databases, newspaper advertisements, clinic visits for recruitment with the approval of the candidate's MD, word of mouth. A formal analysis of electronic medical records using appropriate ICD-9 codes and drug indices may be used to identify eligible participants. Electronic medical records from both the VAMC SLC and University of Utah are currently available to the investigative team and will be used in this analysis. Following identification of eligible participants a direct mail campaign will take place in which all eligible participants will receive a letter from the research team stating the purpose of the study, the requirements of the study, study compensation, contact information, and information to opt-out from further contact and participation. Eligible participants that expressed interest in the study or chose not to opt-out from further communication will be called by a member of the research recruitment team.

Participant compensation:

- Preliminary testing and screening: Participants will receive $20/hour compensation per visit.
- Bed rest protocol: $150 per/day of bed rest
- Limb immobilization protocol: $60 per day of limb immobilization
- Reduced activity Protocol: $50 per day of reduced activity
- Exercise training session performed before, during or after inactivity: $20 per session.

Communication Plans for Multi-Center Studies (i.e. multiple sites around the nation): N/A.

Participating Sites outside the University of Utah (i.e. multiple sites around the city or state): N/A.

Recordkeeping:

Per 21 CFR 312.57, Investigator records shall be maintained for a period of 2 years following the date a marketing application is approved; or, if no application is filed or the application is not approved, until 2 years after the investigation is discontinued and the FDA is notified.

VA Records Retention:

The VA does not have included in their record control schedule a timeframe for the destruction of research records, as a result, we cannot destroy anything right now and need to ask if it is indicated in the application or supporting documents that records, including identifiers, will be destroyed within a specific timeframe that this timeframe be changed to "in accordance with the VA record control schedule."

Protocol Deviation:

A protocol deviation (or violation) is any departure from the defined procedures and treatment plans as outlined in the protocol version submitted and previously approved by the IRB. Protocol deviations have the potential to place participants at risk and can also undermine the scientific integrity of the study thus jeopardizing the justification for the research. Protocol deviations are unplanned and unintentional events.

Because some protocol deviations pose no conceivable threat to participant safety or scientific integrity, reporting is left to the discretion of the PI within the context of the guidelines below. The IRB requires the **prompt reporting** of protocol deviations which are:

- Exceptions to eligibility criteria.
- Intended to eliminate apparent immediate hazard to a research participant or
- Harmful (caused harm to participants or others, or place them at increased risk of harm - including physical, psychological, economic, or social harm), or
- Possible serious or continued noncompliance

Protocol violations/deviations will be summarized in the FDA Annual IND Report or if safety issues arise they may be reported earlier.

Prompt reporting for VA research is 5 business days upon becoming aware of a reportable problem, event, or deviation. Unanticipated Problems (including Adverse Events)

- The VA does not use the OHRP term of an unanticipated problem involving risks to participants or others. However, the VA uses criteria for reporting problems and events that are nearly exact when compared to the criteria for an unanticipated problem involving risks to participants or others, which is used by the University of Utah IRB. Thus, investigators conducting VA research should follow the standard Unanticipated Problem criteria for evaluating and reporting problems and events.

- The investigator must notify the IRB same-day by email upon becoming aware of any local research-related deaths that are unanticipated. Send notification, including the IRB number, to: irb@hsc.utah.edu. The investigator must then submit a Report Form within 5 business days.

- Investigators must take care in documenting appropriate evaluation of unanticipated problems. Unfounded classification of problems as unrelated or anticipated must be reviewed by the IRB for possible serious non-compliance.

Protocol Amendments:

Any amendments or administrative changes in the research protocol during the period, for which the IRB approval has already been given, will not be initiated without submission of an amendment for IRB review and approval. All amendments that significantly affects the patient safety will be submitted to the FDA for review per 21 CFR 312.30. These requirements for approval will in no way prevent any immediate action from being taken by the investigator in the interests of preserving the safety of all patients included in the trial.

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