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Title: Nitrite Supplementation to Mitigate Fatigability and Increase Function in Long COVID Patients

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Principal Investigator: Daniel Forman

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Summary

Prevalence of Long COVID is high among survivors of an acute COVID-19 infection¹. Long COVID is associated with progressive declines in function and well-being that commonly lead to disability, diminished quality of life, and high healthcare costs. Typical symptoms of Long COVID include fatigue, shortness of breath, and myalgia, suggesting there are systemic manifestations that extend beyond the therapeutic focus of acute COVID management². Skeletal muscle pathophysiology has been implicated as a significant determinant of COVID symptoms and risks³. In this trial, we will study the benefits of nitrite therapeutics, using nitrate-rich beetroot juice to increase serum nitrite. We hypothesize that increased nitrite will reduce fatigability and improve physical function in Veterans with Long COVID as a result of enhanced skeletal muscle mitochondrial respiration.

Nitric oxide (NO) is a signaling molecule that contributes to numerous physiological functions, including enhanced mitochondrial respiration⁴, which can thereby result in decreased fatigability and increased physical function. NO is commonly produced from the conversion of the amino acid L-arginine to L-citrulline in the presence of oxygen. However, inorganic nitrate obtained from the diet can act as alternate source of substrate for bioactive nitrite and downstream NO, particularly during the ischemic stress of exercise⁵.

In a proof-of-concept SPiRE pilot, we will study 30 Veterans with Long COVID in a randomized, placebo-controlled clinical trial, comparing 15 who consume two weeks of daily nitrate-rich beetroot juice versus matched Veterans who consume a nitrate-depleted placebo. Both groups will be encouraged to undergo simultaneous physical therapy. Primary endpoints center on meaningful clinical changes, including fatigability (i.e., rating of perceived exertion during steady-state walking), cardiorespiratory fitness (i.e., peak oxygen utilization [VO₂] as well as submaximal VO₂ at anaerobic threshold [VAT]). In addition, serology assessments will include nitrate and nitrite levels and skeletal muscle mitochondrial respiration to analyze nitrite-mediated mechanisms underlying functional changes.

List of Abbreviations

ADL	Activities of Daily Living
AE	Adverse event
BP	Blood pressure
COVID	Coronavirus disease
COVID-19	Coronavirus disease 2019
CPET	Cardiopulmonary Exercise Testing
CPOC	Clinical and Population Outcomes Core
CR	Cardiac Rehabilitation
CRF	Case report form
CTC	Clinical Trials Center
DSMB	Data Safety Monitoring Board
GEC	Geriatric and Extended Care
GEM	Geriatric Evaluation and Management Clinical
GRECC	Geriatrics Research Education and Clinical Center
HFpEF	Heart Failure with preserved ejection fraction
HFrEF	Heart Failure with reduced ejection fraction
ICF	Informed consent form
ID	Identifications
IND	Investigational New Drug
IRB	Institutional review board
MetHb	Methemoglobin
MD	Medical Doctor
NIA	National Institute on Aging
NO	Nitric oxide
OXPHOS	Oxidative phosphorylation
PCP	Primary Care Provider
Peak VO ₂	Cardiorespiratory Fitness
PhD	Doctor of Philosophy
PHI	Private health information
PI	Primary Investigator
PT	Physical Therapist
RNA	Ribonucleic acid
ROB	Research Office Building
RPE	Rating of perceived exertion
SAE	Serious adverse event
SBP	Systolic Blood Pressure
SPiRE	Small Projects in Rehabilitation Research
SPPB	Short Physical Performance Battery
SUIT	Substrate-uncoupler-inhibitor titration
VA	Veterans Administration
VAPHS	VA Pittsburgh Healthcare System
VAT	VO ₂ at anaerobic threshold
VHA	Veterans Health Administration
VMI	Vascular Medicine Institute
VO ₂	Peak oxygen utilization or maximum rate of oxygen consumption
400MCW	400-Meter Corridor Walk

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1.0 Study Personnel

- **Qualifications of Investigators**

Daniel Forman, MD., Project Director/Principal Investigator

- Dr. Daniel Forman will oversee all aspects of the project, including oversight of all aspects of participant recruitment, enrollment and retention; beetroot juice acquisition; intervention delivery; functional assessments; data collection, analysis and dissemination. Dr. Forman will also complete skeletal muscle biopsies at VA Pittsburgh Healthcare System (VAPHS). Dr. Forman is responsible for all aspects of participant safety and wellbeing.
- Dr. Forman is a cardiologist and geriatrician. His research has focused predominantly on utility of exercise training to improve physical function in older adults with cardiovascular disease and frailty. He has honed significant expertise in exercise training (including aerobic, strength, balance, and inspiratory muscle) in older, frail populations to enhance clinical outcomes. Likewise, he is a leading investigator and national thought leader in Cardiac Rehabilitation. Dr. Forman is also leading a study of nitrite supplementation (administered as an investigational drug) for adults aged ≥ 70 years who are sedentary and/or who have heart failure. The primary endpoint of that study is focused on changes in skeletal muscle mitochondrial bioenergetics due to nitrite. Fatigability and cardiorespiratory fitness are secondary endpoints. The current study on nitrate therapeutics entails nitrates administered as an easily ingested nitrate-rich beverage for adults with Long COVID. The primary endpoints of this study are fatigability and cardiorespiratory fitness

Erika Hoffman, MD, Co-Investigator

- Co-Director of the VAPHS Long COVID Program
- Dr. Hoffman will work with Dr. Forman and his study team to recruit, monitor, and retain study patients as they proceed through the protocol. She will work with Dr. Forman to ensure safety of study patients. She will also participate in the analysis, interpretation and dissemination of study data.

Steven Handler, MD, PhD, Co-Investigator

- Director of the VAPHS Geriatrics and Extended Care and GRECC
- Dr. Handler will work with Dr. Forman and his study team to ensure successful implementation of this study, including all aspects of logistics, safety, recruitment, and retention of study patients. He will also participate in the analysis, interpretation and dissemination of study data.

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Kala Markel, PT

- Dr. Markel will work with Dr. Forman and his study team to ensure that all participants (both those receiving active dietary supplement and placebo) undergo physical therapy administered safely, effectively and in standardized fashion. She will also participate in the analysis, interpretation and dissemination of study data.

Collaborators/Other Institutions

University of Pittsburgh:

Personnel at the University of Pittsburgh will collaborate and/or provide consultation for this study. They will not be engaged in the research or require IRB approval.

Paul Coen, Ph.D., Consultant

- Dr. Coen is a mitochondrial respirometry expert with an established track record of working with Dr. Forman on his current nitrite supplementation analyses. He will work with Dr. Forman and his study team to analyze skeletal muscle data in relation to functional endpoints. He brings unique perspective of skeletal muscle respiration to the study team, which is not available at the VAPHS. Dr. Coen will also participate in the analysis, interpretation and dissemination of study data. He will have access to only de-identified skeletal muscle, serologic, and functional data as part of our shared analytic efforts.

Nancy Glynn, Ph.D. Consultant, University of Pittsburgh

- Dr. Glynn is a fatigability expert. She will work with Dr. Forman and his study team in maintaining quality control in all functional assessments. Dr. Glynn has an established track record of working with Dr. Forman on his current nitrite supplementation analyses. She will participate in the analysis, interpretation and dissemination of study data. There is no one at VAPHS with expertise that is equivalent to Dr. Glynn. She will have access to only de-identified functional data as part of shared analytic efforts.

Subashan Perera, Ph.D. Consultant, University of Pittsburgh

- Dr. Perera is a biostatistician. He will work with Dr. Forman and the study team to ensure precision of analytic method, as well as in all the analysis, interpretation, and dissemination of all study data. Dr. Perera has an established track record of working with Dr. Forman on his current nitrite supplementation analyses. Similar statistical expertise is not available to Dr. Forman in the VAPHS. He will not have access to study data but will provide oversight and help if needed.

Frank Sciurba, MD, Consultant, University of Pittsburgh

- Dr. Sciurba is a pulmonologist with an established track record of COVID-19 research. He will work with Dr. Forman and the study team in the analysis, interpretation, and dissemination of all study data, and adding particular expertise in respect to pulmonary function tests, cardiopulmonary exercise tests, and clinical impact of COVID-19 on clinical status. There is no one at VAPHS with expertise that is equivalent to Dr. Sciurba. He will have access to only de-identified functional data as part of shared analytic efforts.

- **Non-Financial Conflicts of Interest**

N/A

Package uploads (Section 1.0):

- **Research Staff Form**
- **Research Answering Service Form (for GTM studies)**

2.0 Introduction

- **Scientific Background/Rationale**

Prevalence of Long COVID is surging among Veterans¹³. Veterans Health Administration (VHA) is prioritizing goals to remedy the debilitating effects of this disease¹⁴. This study harmonizes with these VHA goals. For Principal Investigator, Daniel Forman, this study's

focus on Long COVID constitutes a shift in research direction as it diverges from what has been a predominant orientation to cardiac rehabilitation, exercise training, and adjunctive therapies for older adults with heart disease, sedentariness, and frailty. Nonetheless, this study also flows logically from Dr. Forman's expertise in functional restoration for adults afflicted with disease-related disability, as well his insights and infrastructure in nitrite therapeutics⁷.

A burgeoning literature highlights that skeletal muscle mitochondrial dysfunction underlies functional deficits associated with many non-muscle diseases, possibly representing a common pathophysiological effect¹⁵. While mechanisms underlying Long COVID are not fully elucidated, skeletal muscle mitochondrial dysfunction is recognized as a probable component of the disease³. Dr. Forman's lab is currently studying the biological and clinical impact of inorganic nitrite salts to older adults who have heart failure and frailty. Nitrite is administered as dietary supplement capsules in a thrice-daily, 12-week regimen. Safety and adherence to nitrite capsules have been remarkably successful. Moreover, ex vivo skeletal muscle analyses show that nitrite supplementation is associated with increases in mitochondrial state 3 respiration⁶ with associated potential to reduce fatigability and improve daily activity¹⁶.

When ingested, nitrite is decomposed to nitric oxide in the stomach under acidic conditions. Administering nitrite as a capsule has advantages in respect to achieving high serum nitrite levels. However, this advantage is counterbalanced by the difficulties of administering a thrice-daily regimen, with hemodynamic risks as well as the significant regulatory and cost challenges associated with an investigation new drug (IND) medication. Beetroot juice provides an alternate source of nitrites. Beetroot juice itself contains inorganic nitrate salts but once ingested, nitrate accumulates in the salivary glands and is then reduced to nitrite by oral commensal bacteria¹⁷. Achieving increased nitrite through beetroot juice has advantages over capsules in respect to lower cost, greater safety, as well as greater ease of administration. Administration is only once a day, and hemodynamics are less labile. While serum levels of nitrite achieved by beetroot juice are usually not as high as nitrite capsules, they still can be therapeutically robust, especially with the insights that have accrued over time regarding optimal timing and dosing⁴.

In a seminal study, Larsen et al. analyzed oral nitrate benefits in healthy trained adults (age 28±6 years) showing that the nitrates improved submaximal endurance exercise performance¹⁸. The daily nitrate dose corresponded to the amount normally found in 150–250 g of nitrate-rich vegetables such as beetroot. In contrast, a study of older adults (69±7 years) with heart failure with preserved ejection fraction (HFpEF) consumed Beet-It Sport Shot (70 ml or 6.1 mmol of nitrate) for one week. While increases in serum nitrate resulted, there were no increases in serum nitrite, and no functional gains. Investigators concluded that the nitrate dose was too low¹⁹. Nonetheless, a study by Hirai et al which used 12.9 mmol nitrate supplementation in older adults (age 63±5 years) with heart failure with reduced ejection fraction (HFrEF) for 9 days achieved elevated nitrate and nitrite levels, also showed no improvements in exercise capacity²⁰. Investigators in that study concluded that patients with HFrEF have high peripheral oxygen extraction as a manifestation of their heart failure physiology and may not derive as much benefit from nitrate as much as other populations.

Overall, prior studies indicate that dosing strategies and clinical features of recipients are integral to successful beetroot juice therapeutics. Woessner et al. reviewed data from other studies to conclude that efficacy of nitrate supplementation to decrease cost of oxygen exercise and increase power output and time to exhaustion entailed using a minimum of 5.2 mmol nitrate for 6 days²¹. A longer dosing protocol (15 days) demonstrated that

improvements in steady-state VO_2 seen at day 5 were maintained but no further increases at day 15. In another study, Kenjale et al. showed even doses of 18.1 mmol nitrate/day dose were feasible and safe for patients aged 67 ± 13 years with peripheral arterial disease²². These studies inform rationale for a Beet-It nitrate preparation (James White Drinks Ltd., Ipswich, UK) at 140ml (approximately 266mg of nitrate/day) versus a 210 ml of nitrate-depleted placebo in respect to both safety and efficacy, and respect to potential efficacy in a Long-COVID population.

3.0 Objectives

- **Study Objectives**

Aims: We will conduct a pilot randomized controlled trial to study the benefits of nitrate-rich beetroot juice versus placebo in 30 Veterans with a diagnosis of Long COVID. All participants will receive a daily study juice intervention for 2 weeks. All participants will be encouraged to participate in the standard physical therapy program in the Long COVID clinic.

- Aim 1: To study the efficacy of nitrate-rich juice supplementation to reduce fatigability as measured by rating of perceived exertion (RPE) during submaximal steady-state 1.5 mile per hour walking⁸ after two weeks of treatment in patients with Long Covid. Walking efficiency (VO_2 per kg) will also be assessed in association with fatigability.
- Aim 2: To study the benefits of nitrate-rich juice supplementation to increase cardiorespiratory fitness (peak oxygen utilization [VO_2], VO_2 at anaerobic threshold (VAT), 400-meter corridor walk (400MCW), short physical performance battery (SPPB)¹¹ in patients with Long COVID after two weeks of treatment.
- Aim 3: To explore the utility of nitrate-rich juice supplementation to enhance skeletal muscle mitochondrial respiration in patients with Long COVID as measured by ex vivo Oroboros analysis¹².

- **Hypothesis**

We hypothesize that nitrate-rich juice supplementation will reduce fatigability and improve physical function in patients with Long COVID as a result of enhanced skeletal muscle mitochondrial respiration.

- **Relevance to Veterans**

At the VAPHS, Veterans continue to be afflicted by acute COVID-19. Prevalence of Long COVID is high among survivors of an acute COVID-19 infection¹. Long COVID is associated with progressive declines in function and well-being that commonly lead to disability, diminished quality of life, and high healthcare costs. Typical symptoms of Long COVID include fatigue, shortness of breath, and myalgia, suggesting there are systemic manifestations that extend beyond the therapeutic focus of acute COVID management². Skeletal muscle pathophysiology has been implicated as a significant determinant of COVID symptoms and risks³. Escalating prevalence of Long COVID is devastating to many Veterans in terms of their quality of life and progressive disability. This pilot study is extremely significant in applying novel nitrite therapeutics to address this critical challenge.

This study lays a foundation for an anticipated MERIT application for Veterans with Long COVID and potentially to other Veteran patient populations who contend with fatigability and functional declines as part of disease-based disability (e.g. Veterans with heart failure or chronic obstructive lung disease). This study is aligned with Rehabilitation Research and Development's mission to maximize Veterans' functional independence, quality of life and participation in their lives and community.

The value of this research initiative is reinforced by the recent expansion of Dr. Forman's VA leadership role as Director of Translational Research for the Geriatrics Research Education and Clinical Center (GRECC) and Director of Transitional Care of the Geriatric and Extended Care (GEC), with broad endorsement and encouragement to launch such innovative clinical research. Overall, this study aligns with the Veteran's Affairs Office of Research and Development's commitment to research that helps Veterans affected by COVID-19, and it also aligns with the Rehabilitation Research and Development's mission to maximize Veterans' functional independence, quality of life and participation in their lives and community.

4.0 Resources

VAPHS Resources

Dr. Forman is a geriatrician and cardiologist, with a primary faculty appointment in the Pittsburgh VA Healthcare Center (VAPHS) with associated appointments at the Geriatric, Research, Education and Clinical Center (GRECC) and Geriatrics Extended Care (GEC). He is also a Professor of Medicine in the Divisions of Geriatrics and Cardiology at the University of Pittsburgh School of Medicine, with associated appointments in the Pittsburgh Pepper Center and the University of Pittsburgh Aging Institute (AI). Dr. Forman's expansive configuration provides a range of resources.

VA Pittsburgh Healthcare System: VAPHS is a tertiary care institution that provides a broad spectrum of inpatient and outpatient services. The University Drive Division is a 146-bed facility that provides routine medical, neurological, surgical, and critical care, as well as specialized services such as bariatric treatments and liver and kidney transplants. Additionally, there are 78 secure, private, psychiatric treatment beds; and a step-down unit for Veterans who were no longer in need of the intensive care provided in the Intensive Care Unit but greater need than would be provided in a general ward.

VAPHS research programs are housed in a new state-of-the-art Research Office Building (ROB) located at the University Drive division of VAPHS, immediately adjacent to the primary VAPHS clinical building. The VAPHS Institutional Review Board has been continuously accredited since 2002, first by the National Committee for Quality Assurance and by the Association for the Accreditation of Human Research Protection Programs (AAHRPP) since 2007. VAPHS investigators have access to an on-site full-service health sciences library, video and teleconferencing equipment, and other media services, such as scientific poster preparation.

Dr. Forman has an office on the ground floor of the Research Office Building (ROB) immediately adjacent to the clinical building. In the ROB Dr. Forman has extensive office space for study personnel, including infrastructure for data entry, data storage, and study-related participant phone calls. Moreover, the ROB includes a Clinical Studies

Unit. The Clinical Studies Unit also includes a research translational unit Veteran Health Enhancement Activity Research Testing Center (VET-HEART).

- VAPHS Veteran Health Enhancement Activity Research Testing Center (Director, Dr. Forman) is housed within the Veterans Health Foundation Clinical Trials Center (CTC) in the Research Office Building at the VAPHS. The CTC consists of a 1500 sq. feet facility with four fully equipped exam rooms that also contain computers, phlebotomy room, and blood processing and storage with associated staffing and expertise. The VET-HEART center includes its own adjoining 600 sq. foot space for exercise testing, aerobic and strength assessments, exercise training sessions (staffed by an exercise physiologist), and muscle biopsies (staffed by cardiologists, nurses, and research assistants).
- VAPHS Clinical Cardiac Rehabilitation Program: (Director Dr. Forman). The VAPHS Cardiac Rehabilitation (CR) program is a joint program with backing of cardiology, rehabilitation services, and GRECC. It provides not only the option of space for facilitating exercise training and testing but also additional staff who are oriented to research initiatives, and who work synergistically with the VET-HEART staff. This program provides inpatient and outpatient CR with referrals for patients who receive stents, cardiac surgery, cardio defibrillator device implants and/or who are status post heart failure decompensation. The clinical program is a multifaceted program in which we are implementing new models of care, including home-based and hybrid-based (hospital and home-based) options for CR. The dedicated CR space is equipped with exam rooms, telecommunications equipment and exercise space equipped with treadmills, recumbent cross trainers (NuSteps), stationary bicycles, arm ergometers, free weights, telemetry and multiple computers. The program is well staffed with MDs, nurses, physical therapists, and exercise physiologists.
- Geriatric Research, Education and Clinical Center (GRECC): The GRECC mission is to improve the care and services for the increasing number of older Veterans through the advancements in scientific knowledge gained through research, training, and development and evaluation of improved model of care for older veterans. The VAPHS GRECC program is known for its multidisciplinary Geriatric Evaluation and Management clinic (GEM), home based primary care that goes into the home to reach a greater number of patients with travel limitations, Clinical Video Telehealth TeleDEMENTIA clinic, along with a depth of training programs for multi-disciplinary staff. Dr. Forman is a member of the GRECC faculty and he is able to utilize available resources from the GRECC including their advanced education learning system for older adults with education materials geared to older adults.
- VAPHS has an internal network and secured servers that allow staff to access their secured study drives throughout the hospital. All systems are backed up daily to prevent loss. All facilities have computers, printers, email, internet and various software programs. VAPHS provides 24/7 computer support and service.
- Geriatric Extended Care centered at the HJ Heinz Division is home to a 262 bed Community Living Center and offers hospice, rehabilitative, dementia, and outpatient services. This campus also includes a 65-bed Veterans Recovery

Center and 31 psychiatric residential rehabilitation treatment beds. VAPHS also supports Community-Based Outpatient Clinics in 5 surrounding Pennsylvania counties. In addition, VAPHS is home to multiple clinical and research training programs supported by the Office of Academic Affairs, including fellowship programs in women's health, health systems engineering, geriatrics, mental illness, spinal cord injury rehabilitation, patient safety, and clinical simulation.

- Dr. Forman has additional office space at the HJ Heinz campus and anticipates expanding research infrastructure under the evolving plans to integrate research with GEC clinical program.

Veteran Health Enhancement Activity Research Testing Center (VET-HEART) in the Research Office Building (ROB) of the VAPHS University Drive Campus

- *MedGraphics CPET Ultima System*- Ultima Cardio2 combines powerful BREEZESUITE™ Gas Exchange Software and PC-Based CArdio Perfect ECG into one easy-to-use system. This system allows for high resolution color comparisons of complexes, easy detection of arrhythmias, and breath by breath analysis. This state-of-the-art system allow for a quantitative comparison of changes in functional capacity over time.
- *Woodway Bari-Mill Treadmill*- The medical grade, adjustable handrails are customizable to each patient's size. The software in the treadmill is compatible with the MedGraphics CPX Ultima System..
- *Keiser Strength equipment- Leg Press*
The Keiser strength equipment is adjustable and uses an air compressor for resistance rather than weight stacks which allows for the weight to be set in 1lbs increments with the touch of a button.
- Additional equipment located in the VET-HEART Center
 - Electronic Bariatric Exam Table- weight capacity of up to 600lbs
 - Portable force plate
 - Mobile Manual Blood Pressure Cuff- Welch Alyn
 - Digital Stadiometer- Seneca
 - Digital Scale with 450 LB weight limit
 - Grip Strength Dynamometer
 - Bariatric Chair weight capacity of 450lbs
 - Workstations with Computer
 - Well-equipped exam rooms, with sufficient space, lighting, storage for muscle biopsies.

Service Line Impact

- **Long COVID clinic:**

Study screening and recruitment will occur on the Long COVID unit. Participants enrolled in the study will be encouraged to utilize usual care while inpatient, including 2-3 days of physical therapy a week.

Participant Payments

Participants will either be paid using Electronic Fund Transfer or Direct Debit Cards depending on the Veterans' preference. Veterans will be offered a \$25 stipend for their participation in each assessment and a \$100 payment for each biopsy. Based on a planned enrollment of 5 Veterans in Year 1 and 25 Veterans in Year 2 for a total of 30 Veterans receiving 2 assessments each and 2 biopsies each (baseline and 2-week follow-up), we estimate the following assessments costs:

- Year 1: 10 assessments at \$25 each = \$250
10 biopsies at \$100 each = \$1,000
- Year 2: 50 assessments at \$25 each = \$1,250
50 biopsies at \$100 each = \$5,000

Total participant payments at this site for years 1-2: \$7,500

Year 1

Task	# Participants	Amount	Total
Baseline assessment	5	\$25	\$125
Follow-up assessment	5	\$25	\$125
Biopsy #1	5	\$100	\$500
Biopsy #2	5	\$100	\$500
<i>Year 1 total</i>			<i>\$1,250</i>

Year 2

Task	# Participants	Amount	Total
Baseline assessment	25	\$25	\$625
Follow-up assessment	25	\$25	\$625
Biopsy #1	25	\$100	\$2,500
Biopsy #2	25	\$100	\$2,500
<i>Year 2 total</i>			<i>\$6,250</i>

Off-Site Ancillary Activities

Off-site work will entail specialized analysis of coded identification skeletal muscle tissue with techniques (mitochondrial respiration and microscopy techniques) that are not available at the VAPHS. This constitutes only about ~10% of the study work.

Vascular Medicine Institute of the University of Pittsburgh Campus.

Dr. Forman will be sending coded identification muscle samples to the Vascular Medicine Institute (VMI) core lab of mitochondrial metabolism. The VMI currently

has 4 Oroboros machines for oxidative phosphorylation (OXPHOS) analysis. These units combine concepts of bioenergetics and biochemical pathways related to mitochondrial core energy metabolism and provide the basis for the design of substrate-uncoupler-inhibitor titration (SUIT) protocols in O2k-assays. Skeletal muscle respirometry assessments will occur at Jurczak lab.

Package Uploads (Section 4.0):

- **Letters of Support**
- **Agreements**
- **Budget**
- **Financial Letter of Support for Patient Payments (Business Service line, Veterans Health Foundation)**

5.0 Study Procedures

- **5.1 Study Design**

Screening:

Veterans (N=30) who are being treated in the Long COVID clinic and in Primary Care clinics at VAPHS will be enrolled. Inclusion criteria include Veterans with prior COVID-19, with residual symptoms of Long COVID including increased fatigability documented in CPRS with a Long COVID diagnosis. Patients will be screened for eligibility through CPRS by study staff and referred by the clinical staff of the of Primary Care clinics and Long COVID program. If they are eligible, they will be approached only if they express interest in learning about the research.

Exclusion criteria will principally exclude those with end-stage disease or orthopedic limitations which confound assessments, as well as anemia, or hypotension (BP systolic <110 mmHg), which diminish nitrite safety. Veterans using excessive anti-bacterial mouthwash or antacids will also be excluded as these treatments can interrupt nitrate/nitrite/NO biologic pathways which depend on commensal oral bacteria and an acidic gastric environment.

Research data:

All study staff will also be blinded except for those doing the data analysis.

Screening log and informed consent will be collected prior to baseline assessments. Baseline and post intervention assessments will occur over two days. Assessment day 1 will include questionnaires (demographics, PSQI, VR-12, Vaccination form, and modified REAP-S) and physical assessments (SPPB, 400MCW, SSWT, and CPET) along with collecting Medication Inventory, Vital Signs and a Physical Exam. Questionnaires can also be answered over the phone before the first assessment day.

After 48 hours, the participant will return for the lab assessments (blood draw, muscle biopsy). Bio-Collections and Muscle Biopsy Procedures Log will be reported. Once bio-specimen are analyzed, Skeletal Muscle Mitochondrial Respiration and Blood Lab forms will be complete for the corresponding timepoint. Randomization happens on the lab assessment day once all lab assessments are completed.

If beverage adherence is poor, the study staff will alert the PI with a summary of the subject adherence obstacles. The PI will determine an appropriate improvement plan.

The participant will then go into the 14-day intervention where they will consume either the study beverage or placebo. During this period, they will be encouraged to go to physical therapy, and they will receive phone calls to ensure safety of the study beverage, muscle biopsy recover, and physical therapy attendance.

Post intervention assessments will occur 14 days after they start consuming the study beverage. The assessments days will be identical to the baseline assessments, except the only questionnaire being asked is the VR-12. All other assessments will follow the same schedule as listed above.

Lab assessments may occur before the functional assessments during baseline and post-intervention timepoints if the visits are approximately 48 hours apart.

If the participant opts out of doing muscle biopsies, the blood draw can be performed during the functional assessment.

On all enrolled participants, study staff will collect a set of established measures.

Measure	Timeline				
	Screening	Baseline*	Intervention	Follow up ~3, 7, & 11 days during intervention	Post intervention assessments 14 (+7) days
Screening Log	x				
Demographics		x			
Pittsburgh Sleep Quality Index (PSQI)		x			
Veterans RAND-12 (VR-12)		x			x
Modified Rapid Eating Assessment for Participants Shortened Version (modified REAP-S)		x			
Vaccination Form					
Medication Inventory		x			x
Physical Exam		x			x
Vital Signs		x			x
Short Performance Physical Battery (SPPB)		x			x
400 Meter Corridor Walk		x			x
Steady State Walking Test (SSWT)		x			x
Cardiopulmonary Exercise Testing (CPET)		x			x
Muscle Biopsy Procedure Log		x			x
Bio-Collection		x			x
Skeletal Muscle Mitochondrial Respiration		x			x

Blood Labs		x			x
Intervention Check-In Call				x	x
* Randomized after baseline.					

Screening:

- **Screening Log:** Study staff will initially screen patients in the Long COVID clinic and/or Primary Care clinics through electronic medical record review in CPRS to identify patients who are eligible for the study. In-person screening will occur afterwards to confirm Long COVID diagnosis from the Long COVID clinic physician and to confirm other inclusion and exclusion criteria.

Baseline Testing:

- **Demographics** and additional information regarding medication, diet, alcohol use and other factors important for study participation will be evaluated. The PI can withdraw the participant from the study if they report clinically significant alcohol intake or substance abuse.
- **Pittsburgh Sleep Quality Index (PSQI):** is a questionnaire to evaluate sleep habits that could affect fatigue of the participant during the past month.
- **Veterans RAND 12 (VR-12):** is a brief, generic, multi-use, self-administered health survey comprised of 12 items. The instrument is primarily used to measure health related quality of life, to estimate, disease burden and to evaluate disease-specific benchmarks with other populations.
- **Modified Rapid Eating Assessment for Participants (modified REAPS):** *will assess the participants' baseline dietary quality.*
- **Vaccination Form:** is a questionnaire to collect information on if the participant received a vaccination and what kind of vaccine they received.
- **Medication Inventories:** A list of current medications from the participants CPRS charts will be reviewed by the PI to assure safety when consuming the study beverage/placebo and during assessments. Those who are unable to hold warfarin, novel oral anticoagulants (NOACs) or aspirin for 3 days prior to muscle biopsy, or to hold thienopyridine medications for 5 days prior to muscle biopsy or are unwilling to hold Viagra-like drugs (i.e. Viagra, Cialis, Levitra, other phosphodiesterase inhibitors) will be withdrawn from the study.
- **Physical Exam:** will be performed by the study physician to assure the participant's medical stability (cardiovascular, abdominal, neurological, etc.) look normal and that the patient is safe to perform assessments.
- **Physical Function Measures:**
 - **Short Physical Performance Battery (SPPB):** This entails a measure of time to sit-to-stand out of a chair 5 times, walk over a 4-meter course (walking speed), and to stand in different positions to measure their balance

- **400m Corridor Test (400MCW):** This test measures the amount of time it takes the participant to walk a 400-meter course to assess cardiovascular and pulmonary fitness or to predict adverse outcomes such as mobility disability.
- **Cardiopulmonary Exercise Testing (CPET):** a physical exam performed by a study physician or nurse prior to completing all functional assessments. CPET will be used to initially assess oxygen consumption (VO_2) at steady state walking (SSWT) which entails walking at 1.5 mph.

Exercise Intensity then progresses (by increasing incline and speed) to measure peak VO_2 , which is a metric of cardiorespiratory fitness.
- **Vital Signs:** Blood pressure (BP), heart rate, pulse oximetry, methemoglobin %, and respiration will be assessed at the initial study visit, including sitting and orthostatic hemodynamics.
- **Bio-Collections:**
 - **Muscle Biopsy:** Needle muscle biopsies will be completed at baseline and final visit.

Muscle biopsies of the vastus lateralis muscle will be completed in the non-dominant leg. A small piece of muscle (approximately 100 to 200 mg) will be collected for mitochondrial respirometry, Western blots, and histomorphometry. Samples will be stored for RNA-seq.

Participant's current medication inventory will be attained through CPRS or self-reported by the participant and reviewed by the PI. For participants using antiplatelet or antithrombotic medication, primary prescribing physician approval will be required. If the primary physician feels it is safe, medicines will be held 3- to 5-days prior to the procedure (depending on the medication), and then resumed after the muscle biopsy. Thienopyridines will be held for 5 days. Any medication that falls into this category will be recorded in the Medication Inventory form.
 - **Skeletal Muscle Mitochondrial Respiration:** Muscle samples collected during the muscle biopsies are being transported to the Jurczak Lab for analysis. Skeletal muscle will go through mitochondrial respiration which is the set of metabolic reactions and processes requiring oxygen that takes place in mitochondria to convert the energy stored in macronutrients to adenosine triphosphate (ATP). This is completed for the samples collected at baseline and at the final visit.
 - **Blood Labs:** 5 ml of blood will be collected from participants and spun down using a centrifuge to separate the plasma from the rest of the blood. These samples will be sent to the Shiva lab where they will analyze nitrite levels in the blood. Serological sampling (5 mL) is completed at baseline and at final visit: Plasma nitrite and nitrate levels will be measured. Blood samples will be obtained by qualified research study staff who are trained and certified with a valid VA appointment and will follow all clinical and/or research standards for collection,

processing, and analysis. The blood samples will be transported to the Shiva lab at the University of Pittsburgh for the nitrite analysis. A new material transfer agreement (MTA) will be executed prior to any transport of samples outside of VAPHS.

Randomization

- Randomization will occur once all baseline assessments are complete. A high quality pseudo-random deviate generator in SAS® (SAS Institute, Inc., Cary, North Carolina) will be used to randomize participants to nitrate-rich beetroot juice or placebo in a 1:1 ratio. We will employ a blocked randomization scheme using random block sizes chosen from small multiples of 2.

Intervention:

- **Intervention check-in call:** This study will ask questions to ensure the study juice is administered safely. Will be completed by study staff on days 3, 7, and 11 (+/- 1 day). Questions regarding symptoms that correspond to potential nitrite-related side effects will be utilized. These calls will also cover adherence to study supplement/placebo, muscle biopsy healing, physical therapy and adverse events.
- **Physical Therapy Attendance (part of standard care of the Long COVID clinic):** All participants will be encouraged to undergo physical therapy as part of the Long COVID standard practice protocol. If participants are coming from Primary Care Clinics, the study physician will see if the participant is interested in physical therapy. If they are, the study physician will request PT in CPRS and notify the participant's PCP. Physical therapy is scheduled 2-3 times per week based on tolerance for activity and recovery needed after each session. During the intervention check in calls, we will collect participants' self-reported number of physical therapy sessions attended, potential barriers, and frequency of exercise. We will record how many physical therapy sessions the participant attends over the 14-day intervention. Physical therapy will be offered on site or at home (or a hybrid combination). Physical therapy includes:
 - Strength maneuvers
 - Balance maneuvers
 - Inspiratory muscle training
 - Aerobic training

Follow Up Data Measures:

- **VR-12**
- **Medication Inventory**
- **Physical exam**
- **Vital Signs**
- **SPPB**
- **400m Corridor Walk**
- **SSWT**

- **CPET**
- **Bio-Collections**
- **Skeletal Muscle Mitochondrial Respiration**
- **Blood Labs**

Measures that will be collected when/if needed:

- **Serious Adverse Event/Adverse Event Report Forms**
- **Protocol Deviations Log**
- **Progress Note**
- **Study Completion Form**
- **Interval history:** If the participant requests to be seen by a physician outside of a study visit, the physician will examine the participant and address any questions or concerns that the participant may have.

Experimental Dietary Supplement Treatment:

All participants will be randomized (1:1 ratio) into one of two study groups. Study group one will receive 140ml Beet-It nitrate-rich beverage (James White Drinks Ltd., Ipswich, UK) to provide approximately 266mg of nitrate/day, while study group two who will receive nitrate-depleted placebo (i.e., a cranberry beverage). Both groups will be blinded to which supplement they are taking and will be instructed to follow study beverage instructions for 14 days. These groups both will complete the same functional and tissue assessments before and after the intervention.

Both groups will receive 3 phone calls during the 14-day intervention along (for safety surveillance and adherence reinforcement) and will be encouraged to complete the physical therapy that is part of the Long COVID clinical program. The participant will receive a 3-week supply of the study supplement to ensure they have enough supplies if they cannot schedule their muscle biopsy directly after the 2-week intervention.

The Beet-IT™ Sport Shot and placebo will be stored at ambient temperatures in the VAPHS UD-Outpatient Pharmacy. When an enrolled study participant is ready to be randomized the blinded study staff will submit an order to IDS/Pharmacy to dispense the study product. The unblinded data analyst will utilize the randomization list generated by SAS® (SAS Institute, Inc, Cary, North Carolina) to determine the study participant's treatment assignment and notify IDS/Pharmacy. The Investigational Drug Service (IDS) /Pharmacy will affix adhesive labels to the containers of the Beet-It™ Sport Shot and Placebo to obscure the manufacturers' labeling and preserve the blind. The containers will be prepared as kits each containing the appropriate number of containers based on the treatment assignment and will include instructions for the study participant. The containers will be placed in an opaque bag or box when provided to the study staff to prevent inadvertent unblinding of the contents.

Risks and Benefits

Potential Risks:

Long COVID is associated with functional decline, fatigue, and disability. In this trial, inorganic sodium nitrite supplementation administered as nitrate-rich beetroot juice will be tested as a means to increase physical function and diminish fatigability. Serum nitrate and nitrite levels will be assessed at baseline and after the study intervention to evaluate the successful metabolism of nitrate (in beetroot juice) to nitrite. Assessment of key biological effects of nitrite in the skeletal muscle will also be completed.

Nitrite Therapy:

Before initial dosing, we will establish that the subjects' vitals are stable and systolic blood pressure (SBP) is >110/60 mmHg.

We expect beetroot juice will be particularly well-tolerated in the study population. In multiple trials in the literature, safety parameters of this nutraceutical have been excellent.

The development of nitrite therapies extends beyond 50 years ago. Subsequent to 2001, various investigators have continued to explore the use of nitrite/nitrate compounds for potential therapeutic uses in animal and human research. Multiple therapeutic mechanisms are theorized. Animal research examples include evidence of positive benefits on type II muscle fiber of oral nitrate via beetroot juice in Sprague Dawley rats¹ and on pulmonary vasculature in wild type mice.²

Human studies were undertaken and evidence of nitrate efficacy (via high nitrate food derivatives, principally beetroot juice) has accumulated. Vanhatalo and colleagues (2010) were first to report the physiological consequences of dietary nitrate supplementation in humans over a time period beyond 4–6 days.³ Subjects tolerated the therapy and reported no ill effects while showing positive effects on VO₂ and other aerobic performance parameters.

Consideration for therapeutic benefits of nitrates or related compounds in individuals with cardiovascular disease was a logical follow up. Zamani reported increased VO₂ max with a single dose of beetroot juice in subjects with heart failure with preserved ejection fraction (HFpEF).⁴ Borlaug reported on the use of infused sodium nitrite in subjects with HFpEF, noting decreased pulmonary capillary wedge pressure with no adverse events.⁵

Prior work with oral sodium nitrite most applicable to this application is that done by Devan.⁶ These investigators randomized a group of 50-79 year old men and women to 40 or 80 mg oral sodium nitrite or placebo, taken for 10 weeks. Measures of carotid artery elasticity and endothelial function were improved.

Regarding safety, no severe adverse events occurred. Both doses of nitrite were well-tolerated without symptomatic hypotension or clinically-relevant elevations in blood MetHb %. Treatment-emergent adverse events, all of which were expected, were mild and included headache, nausea, fatigue, dizziness/lightheadedness; asymptomatic orthostatic intolerance and dry mouth also were reported.

Our research group completed a pilot study of oral sodium nitrite therapy administered as 20-40 mg capsules tid for one month to 10 sedentary controls, aged ≥70 with minimal adverse effects. We theorized functional benefits inclusive of positive changes on mitochondrial function and muscle fiber as well as others. Only one subject underwent a dose reduction from 40 to 20 mg tid as a preventive measure, i.e., not as a result of any symptomatic hypotension. We demonstrated subject safety via pharmacokinetics at first

dosing via MetHb % levels, SBP and minimal reports of adverse symptoms as seen in Table 1. Pharmacokinetic data for 10 control subjects.

Healthy controls	Systolic blood pressure (mmHg) (mean)	MetHb % (mean)	Symptoms
pK Baseline	132	1.0	
pK at 60 minutes	117	1.7	
Any pK time point			1 subject

During the drug dosing period which ranged from 32-49 days (variable due to scheduling of post-drug assessments), there were no serious adverse events; mild symptoms of dry mouth, occasional lightheadedness, and increased nocturia were noted.

Thus, the previous work referenced here as well as our own pilot data has demonstrated feasibility and safety for older adults with higher dose nitrite therapeutics.

Risk potential with nitrite therapy:

Hemodynamic:

- Common: none
- Frequent: none
- Infrequent: hypotension
- Rare: none

Gastrointestinal

- Common: none
- Frequent: none
- Infrequent: dry mouth
- Rare: nausea, abdominal pain, and vomiting

Hematologic

- Common: none
- Frequent: none
- Rare: methemoglobinemia

Cardiovascular

- Common: none
- Frequent: none
- Rare: flushing, tachycardia

Neurologic

- Common: none
- Frequent: none
- Infrequent: headache, dizziness,
- Rare: seizure, coma

Respiratory

- Common: none
- Frequent: none
- Rare: tachypnea, dyspnea, cyanosis

Genitourinary

- Common: none
- Frequent: none
- Infrequent: increased nocturia
- Rare: none

The most significant risk of nitrates relevant for this protocol is nitrate's blood pressure lowering effects. While in some participants this could be of benefit, it becomes a risk for those with lower blood pressure. Therefore, entry criteria exclude candidates with BP <110/60 mmHg.

Also, candidates for participation will be assessed for orthostatic hypotension. If the participant demonstrates symptomatic orthostatic hypotension, s/he will be assessed by Dr. Forman to ensure safety before continuing. For participants who have asymptomatic hypotension, we will emphasize surveillance during routine follow-up calls to ensure patients remain asymptomatic and stable.

CPET (to assess VO₂ at Steady State and Peak exercise):

The exercise stimulus of maximal (peak) exercise testing is associated with a 1 in 10,000 chance of significant untoward outcome (e.g., myocardial infarction, arrhythmia), including the possibility of death.⁷ However, all those enrolled will have a physical exam immediately before to best ensure they are stable and they will have a qualified staff performing the stress test to be sure they are maximally safe and well-cared for if any problems develop. A current de-identified ECG of the participant from CRPS will be attached to the study files to ensure there are no major changes.

The physician and exercise testing personnel are all BLS trained and a code cart is in the vicinity. A study physician will also examine all study candidates before the initial CPET to ensure their stability.

Muscle biopsies:

Muscle biopsies are also associated with a small chance of infection (less than one in 1000) and pain. Careful steps to use sterile technique and keep the area free of germs will minimize infection risks. Xylocaine numbing medicine is used to reduce any possibility of pain, but sometimes the Xylocaine itself produces a brief burning feeling as it enters through the skin.

The study staff member will make every attempt to contact participants approximately 48 hours after the procedure to ask how the muscle biopsy site is healing and if they have any pain or other symptoms. Participants with excessive pain or reports that the biopsy sites are not healing well will be asked to come in for further evaluation.

If the participant does not answer the call, the study staff member will attempt to leave a general message with contact information and request a call back. If there is no way to leave a message, the study staff member will make at least 3 attempts to call the participant approximately 48 hours after the biopsy.

Peripheral blood sampling:

Risks are minimal and include mild bruising or infection. Careful steps to use sterile technique and keep the area free of germs will minimize infection risks.

Specimen/data access and management:

Standardized case report forms (CRF) will be used for recording all data from each subject. The investigator will ensure that these CRFs are properly completed. Either original de-identified source documents will be used or, in a minimum number of instances, medical data that are extracted from medical records (e.g., recordings of vitals) and CRFs will be audited against the appropriate component of the medical record. The CRFs will be kept current to reflect subject status at each phase during the trial. In all cases, subject will not be identified on CRF by name. Appropriate coded identifications (ID) will be used. Every effort will be made to collect complete data from each study visit. Documents which contain identifiable private health information (PHI) (i.e., informed consent document, contact information, prior medical records) will be separated from the deidentified CRFs at conclusion of subject participation. A participant master list will be maintained to link participant study ID with identifying PHI. The master list will be stored securely on the project-specific VA research drive in a password-protected file with access limited to approved study personnel. Causes of missing data will be fully documented.

All data with identifying information will be stored using a double-lock method i.e. in a locked cabinet in a locked office for hard copies, or password-protected computer files on the study specific research drive behind the VA firewall. Hard copies of completed CRFs will be kept at VAPHS. Physical copies will be stored in the Research Office Building (BLDG 30) which requires a personal identity verification (PIV) to enter. These copies will be located in either rooms GA-127 or G29 within a locked filing cabinet. Electronic files will be secure within a VA portal, behind VA firewalls with limited access to only study staff. It will be located in a designated folder named Forman 1700250, which are only accessible to those who have authorization from the study coordinator or PI. REDCap will also be used to record study data and questionnaires. This information will also be protected and only accessed by study personnel who are assigned to the study by the study coordinator or PI. *Specimens being stored in -80 freezers will be stored in Freezerwork, a password protected, de-identified data storage system or de-identified data will be stored in REDCap until Freezerwork is operational.* Unidentified data collected from muscle assessments at the Jurczak's lab will be entered into Lab Archives; a password protected, unidentified data storage system. Only study personnel will have access to this program. Unidentified data collected from muscle and blood nitrite assessments at the Shiva lab will be entered into Micromolar; a password protected, unidentified data storage system. VA REDCAP Servers are maintained by VIREC <https://vhacdwwweb05.vha.med.va.gov/>.

Data being analyzed will be identified by subject codes and identifying information will be removed. The identity of participants will not be revealed in the presentation or publication of any results from the project. All project staff working on the project will be educated about the importance of strictly respecting participants' rights to confidentiality and will have completed several training courses including proper practice in accordance with HIPAA regulations, protection of human subjects, and computer security. The codes that link the names of participants and their ID numbers will be kept confidential by the PI in a password protected document on a limited access secured network drive. These data will only be accessible to the PIs and staff directly working with the study.

All personnel proposed for this project will have the required human subjects and confidentiality training, which includes information about maintaining data integrity and security. Confidentiality will be guarded using established procedures such as storing data in locked cabinets within locked offices or locked data rooms, coding by study identification numbers rather than any personally identifying information to avoid

revealing the identity of subjects, and aggregating data across participants. Only study personnel will have access to the data sets on protected servers. In order to maintain the highest standard of data entry quality, all data will be double entered, with discrepancies highlighted so that they can be reviewed by the project coordinator. Oversight of all aspects of data management is the responsibility of the PI.

The Investigator will retain the data for the entire period of this study. The Investigator may continue to use and disclose subjects' de-identified information for the purpose of this study for a minimum of seven years after final reporting or publication of the study. If the subject and/or legal representative decide to withdraw or be withdrawn from study participation, they may request that the study data and samples be destroyed.

Questionnaire Tools:

Questions about mood and quality of life entails some potential for individual stress both due to the questions themselves and the sense of being evaluated. Study personnel will take precautions to guide participants patiently, with efforts to minimize stress and any sense of discomfort.

Participation in a research study:

There is a potential risk of stress and anxiety while participating in this study. It is likely that at least mild anxiety will occur while the subject is participating in this study, including the logistic challenge of attending multiple study visits.

Overall subject burden:

Subject burden is a possibility due to the broad spectrum of questionnaires, blood tests, muscle tests, and physical function tests. However, as noted above, precautions have been integrated with the assessments to minimize risk to the subjects.

Benefits:

Participation in the proposed research may or may not provide a direct benefit to participants in this research. This research involves an intervention or procedure that presents greater than minimal risk to the involve participants but holds the potential for direct individual benefit as well. The risk is justified by the extent of potential benefit to the involved adults, which includes possibly of decreasing fatigability, improving physical function, and revealing mechanisms underlying these functional gains. These data are important for improving care and outcomes for the population of Veterans with Long COVID.

Alternatives:

Theoretically, subjects may be able to attain some benefit from beetroot juice consumption they initiate on their own. Furthermore, usual treatment options offered by primary care and specialty physicians, inclusive of medications, physical therapy, and complementary approaches are the available to patients.

Package uploads (Section 5.1):

- **Study Procedures Table (required for GTM studies and minimal risk studies that use SOC)**
- **Patient ID Card (if study involves treatment or intervention)**

- **Screening documents, tests, questionnaires, surveys, or any worksheets participants will complete**
- **Case Report Forms**

5.2 Study Population, Recruitment Methods and Informed Consent Process

The maximum number of subjects we plan to enroll at VAPHS is 30. We will recruit for this study from the VAPHS Long COVID clinic and the Primary Care clinics. The Long COVID clinic has the capacity to see 144 patients in 12 months. A recruitment letter will be sent out to potential participants who have visited the Long COVID clinic or Primary Care clinics in the past. Flyers will also be advertised within both of these clinics. We will also utilize StatCore to pull a query from the corporate data warehouse to extract a list of potentially eligible participants based on exclusion/inclusion criteria. Study staff will pre-screen potential participants from this list and utilize the approved recruitment letter and flier. *Participants may be recruited by displaying flyers/brochures at recruitment/informational tables sanctioned by the VAPHS Research Office as part of National VA Research Week activities or other outreach efforts. Study team members may elect to sit at the recruitment/informational tables to provide additional information and/or enroll eligible participants.* After launch of recruitment, we anticipate recruiting 2-3 people per month, targeting total of 30 patients to be recruited over a 12-month recruitment window. We do not anticipate any participant discontinuation since this is a 3-week study. During the second year of the study, we will begin analysis of data collected on fatigability, cardiorespiratory fitness, and skeletal muscle mitochondrial respiration.

We have a waiver of HIPPA authorization to review the CPRS records of Veterans for the study. The research coordinator (RC) at each site will review records of potentially eligible. Potentially eligible Veterans will be mailed a letter endorsed by the study PI and Long COVID clinic physician along with a brochure describing the study. If (s)he is interested in participating, (s)he will call the study research coordinator (RC), whose name and contact information will be provided in the brochure. In addition, 14 days after we have mailed letters, if we have not been contacted, we will call potential participants to ascertain their interest in participation.

Sample Size Justification

This is an initial small pilot study, and unlike larger definitive studies, sample size and power computations strictly based on rejection of null hypotheses at the 0.05 level are not appropriate. Findings from the proposed study will be used for rigorous sample size and power computations in planning a larger study to establish efficacy. The sample size of 15 per group is also consistent with the generally accepted range in pilot studies of a variety of disciplines in health sciences.^{24,25,26}

Inclusion and Exclusion Criteria

Pre-screening chart review of the electronic medical record will occur by study staff before going to approach the patient. Patients will be approached while they

are receiving care in the Long COVID program and in Primary Care clinics. Patients in the Long COVID program and Primary Care clinics are screened to verify that symptoms are from COVID-19 and not from other etiologies. These common conditions are generally ruled out by the Long Covid Clinic. The study staff approaching candidate patients may ask additional questions about the inclusion and exclusion criteria that may not be sufficiently clear in their medical notes to ensure they are good candidates for this research study.

Inclusion Criteria:

- Veterans with prior COVID-19, with residual symptoms of Long COVID including increased fatigability

Inclusion limited to age 18 and over

Exclusion criteria:

- Blood pressure <110/60 mmHg, either systolic or diastolic value which may affect participant safety during assessments will be at the discretion of the study physician.
- Unable to hold warfarin, novel oral anticoagulants (NOACs) or aspirin for 3 days prior to muscle biopsy, or to hold thienopyridine medications for 5 days prior to muscle biopsy
- Current use of organic nitrates or phosphodiesterase type 5 (PDE5) inhibitors
- Orthopedic or other chronic condition which limits physical activity or functional testing assessments
- End-stage disease
- Dementia or other reason unable to give informed consent
- Anemia (hemoglobin ♂ <11.0 g/dL or ♀ <10.0 g/dL)
- Unstable psychiatric diagnosis
- Clinically significant alcohol intake or substance abuse
- Chronic use of oral corticosteroids or medications that affect muscle function
- Use of anti-bacterial mouthwash or antacids that confound the nitrate/nitrite/NO pathway.
- Suicidal ideations
- Unwilling to hold Viagra-like drugs (i.e. Viagra, Cialis, Levitra, other phosphodiesterase inhibitors)
- Enrollment in another greater than minimal (GTM) risk study
- Other clinically unstable medical condition as determined by the study physician

We will recruit from the population of eligible VA patients with Long COVID. In VAPHS, 685/4360 of acute cases of COVID were identified as Black and 905/4360 of acute cases of COVID were identified as Women. Based on these numbers, we aim to recruit 20% of women and minorities respectively into this study. If necessary, eligible minority and women candidates will also be oversampled to achieve 20% recruitment goals. No children will be recruited in this study of Long COVID in Veterans.

Recruitment

VAPHS provides a robust research environment and Veterans are accustomed to research protocols. Dr. Forman has offices in the Research Office Building as well as the Heinz campus, providing capacity to organize and implement this pilot study flexibly and efficiently. We will recruit for this study from the VAPHS Long COVID clinic. We will also utilize StatCore to pull a query from the corporate data warehouse to extract a list of potentially eligible participants based on exclusion/inclusion criteria. The Long COVID clinic has the capacity to see 144 patients in 12 months.

Following notice of award there will be a startup phase to develop the manual of operations, initiate training and to build the study database. After launch of recruitment, we anticipate recruiting 2-3 people per month, targeting total of 30 patients to be recruited over a 12-month recruitment window. During the second year of the study we will begin analysis of data collected.

	Year 1				Year 2			
Months	3	6	9	12	15	18	21	24
Set up	x	x	x					
Recruitment/Enrollment (2-3) subjects/month)			x	x	x	x	X	
Assessment				x	x	x	X	
Intervention				x	x	x	x	
Data analysis							x	x

Screening

Trained study personnel will pre-screen electronic medical records daily to identify patients in the Long COVID clinic and Primary Care clinics with a qualifying diagnosis and related symptoms of fatigue who may be potentially eligible study candidates. Trained study personnel will also pre-screen electronic medical records of veterans identified through the data query via StatCore to determine eligibility. A waiver of HIPAA is requested for screening purposes as study personnel may access records of potential candidates to ascertain eligibility prior to obtaining informed consent.

After potentially eligible candidates are identified through chart review screening, study personnel will approach the patient's clinical provider in the Long COVID clinic or the study physician will review potentially eligible patients in the Primary Care clinics to confirm that there are no concerns or objections regarding enrollment in the research study. Clinical staff will then obtain permission from the patient to approach. Study staff will only make initial contact with potentially eligible patients-in person; there will be no cold-calls. We may also receive direct referrals from study physicians from Long COVID clinic and Primary Care clinics. Fliers will also be advertised within the clinics to help with referrals. All referred patients will undergo the same eligibility process. Potential participants who are identified through chart screens that are not readily available at an upcoming clinic appointment will be mailed an approved recruitment letter and flier. The letter indicates to call research study staff if they are interested in participating in this research study.

Informed Consent

If no concerns are identified and the clinical care team obtains permission from the potential candidate for research personnel to approach, a trained study staff member who is approved to administer informed consent will approach the candidate to confirm eligibility, ascertain interest and explain the study. If it has been determined that a candidate is interested and eligible to participate, the study staff member will explain the informed consent document in detail, and the candidate will be given time to review the consent document and ask questions. When the subject feels they understand the study, a study physician will talk to the subject about the study and again review risks. If the candidate remains interested, the informed consent document will be signed by the subject and study staff who administered the consent. Female participants under the age of 55 will be asked to take a pregnancy test to assure they are not pregnant during the

first baseline assessment. It will also be made clear to study participants that they have the right to withdraw consent at any point.

Only individuals who provide written informed consent may participate. In accordance with IRB regulations, we will obtain written informed consent from each participant by a physician investigator or study coordinator after a thorough explanation of the nature of the investigation, the types of assessments and treatment involved, the potential risks involved in participation and study procedures. We will give all subjects ample opportunity to ask and receive answers to questions. In addition, participants will receive an explanation of how their information will be handled, including all parties involved, data management, and plans to publish data in group format without identifying information. The study team will obtain Institutional Review Board (IRB) approval of the protocol and consent forms prior to the initiation of recruitment. Participants will also be informed that they are free to withdraw from the study at any time without any consequences. There will be no advertisements used for this study and no cold calling to the Veterans. Participants who consent to the study will be added to a password-protected participant master list stored in the project-specific secured research drive upon completion of the informed consent process. Only approved study personnel will have access to the research drive and the participant master list.

Package Uploads (Section 5.2):

- **Inclusion/Exclusion Checklist (required for GTM studies)**
- **Inclusion of Non-Veterans Worksheet**
- **Recruitment Materials**
- **Documentation of Public Affairs Approval for any Advertisements**
Waiver Requests if applicable: Waiver of Documentation of Informed Consent; Waiver of HIPAA Authorization; Waiver of Informed Consent
- **Consent Documents, HIPAA Authorization Forms and/or Combined Consent and HIPAA Authorization**

5.3 Data Analysis

Sample Size Justification

This is an initial small pilot study, and unlike larger definitive studies, sample size and power computations strictly based on rejection of null hypotheses at the 0.05 level are not appropriate. Findings from the proposed study will be used for rigorous sample size and power computations in planning a larger study to establish efficacy. The sample size of 15 per group is also consistent with the generally accepted range in pilot studies of a variety of disciplines in health sciences^{24,25,26}

Data Analysis

Data analysis will be performed by approved study personnel at VAPHS. Dr. Subashan Perera (Professor of Medicine and Biostatistics, Division of Geriatric Medicine, University of Pittsburgh) will be consulted as needed. If needed, we will execute a material transfer agreement with the University of Pittsburgh that would allow sharing de-identified data with Dr. Perera.

As this is a preliminary pilot study with a limited sample size, we will use appropriate descriptive statistics, magnitudes of model estimates and graphical methods using data at the individual level (such as spaghetti plots) in addition to standard analytic

techniques (described next) to draw conclusions. Such graphical techniques focused on individual-level data have been used in other small studies from a range of disciplines^{25,26,27}

We will first compare the baseline characteristics of the two groups of participants using independent samples *t*-, Wilcoxon rank sum, chi-square and Fisher's exact tests. Any significant differences will be noted and controlled for as covariates in adjusted analyses. Next, to examine between group differences hypothesized in all 3 aims, we will fit a series of analysis of covariance models with each of the baseline to post-treatment change in outcomes as the dependent variable; participant group (nitrate-rich beet juice/placebo) as the main independent factor of interest; baseline value of outcome as a covariate; and any other characteristics found to be significantly different at baseline as additional covariates. We will be mindful of the small sample size and will keep the number of covariates to a bare minimum, if any. Magnitude and statistical significance of the participant group coefficient will serve as preliminary evidence supporting the Aims 1-3 hypotheses.

Package Uploads (Section 5.3):

- **Sponsor Protocol, if applicable**

5.4 Withdrawal of Subjects

Participation in this study is voluntary. Participants do not have to take part in this study, and their refusal to participate will involve no penalty or loss of rights to which they are entitled. They may withdraw from this study at any time without penalty or loss of VA or other benefits to which they are entitled.

Participants can choose to withdraw from the study at any time but will only receive payment for the visits they complete. If they withdraw from the study before all visits are completed, the researchers still may use the data they have already collected. If they withdraw consent, participants will be asked to stop taking the Beet-It nitrate-rich beverage or nitrate-depleted placebo and to continue with the care provided by the Long COVID unit. Theoretically, subjects may be able to attain some benefit from beetroot juice consumption they initiate on their own which they can purchase online. If the participant no longer wants to take the study beverage during the intervention, we will ask if they would like to complete the post intervention assessments.

Participants have the right to withdraw any biospecimen samples which they provided for the study. If a participant withdraws consent for biospecimens, those biospecimen samples will be removed and destroyed to prevent them being used in future studies.

A study participant may be withdrawn from the research without their consent if any of these anticipated circumstances occur:

- Symptomatic Blood pressure drop
-

If subjects report via phone call monitoring or other contact that they are symptomatic for ≥ 24 hours with dizziness, lightheadedness, weakness, or if they report SBP < 100 mmHg or one or more readings from their home blood pressure

monitor, subjects will be asked to come in for an interim visit to assess blood pressure. A study physician will evaluate that subject. Depending on the assessment, the participant may need to be discontinued from the study.

Overall protocol specific stopping rules

The development of any untoward events causing an intolerance of the study procedures that cannot be managed by usual care practices will be assessed by the investigators, and continued participation in the study will be determined on an individual basis. Participants who are unable to tolerate protocol procedures will not be asked to continue participating in the study.

Package Uploads (Section 5.4):

- **Communication scripts (letters sent to participants for follow up purposes), if applicable**

6.0 Data Safety Monitoring

A data and safety monitoring plan (DSMP) will be implemented to ensure that there are no changes in the benefit/risk ratio during the study and that confidentiality of research data is maintained. Investigators, study personnel, and the clinical coordinators involved in the study will meet quarterly to discuss the study progress, modifications, recruitment, retention, and plans for data analysis and confidentiality and address any issues or concerns at the time. These meetings will be overseen by the principal investigator. Any instances of adverse events, protocol deviations, or other problems identified during the meetings will be reported as soon as possible within the required reporting timeframes using the standard forms and/or procedures set forth by the IRB. In addition, clinical coordinators may review study documentation and/or consent forms to ensure that subject's confidentiality is maintained.

Monitoring of safety and data quality in the proposed study will be the responsibility of all personnel on the project, with primary responsibility and supervision by the PI. Institutional oversight is required and provided by the three offices under the umbrella of the Research Conduct and Compliance Office. First, the Institutional Review Board (IRB) will review and approve the protocol prior to recruiting or obtaining consent from any participants. Inclusive of this review will be approval of the Informed Consent Form (ICF) for the study with each participant signing the ICF prior to participating or undergoing any research procedures. IRB will provide oversight of data and safety issues. The study will be reviewed on an annual basis by the Review committee. To ensure participant safety, once participants are enrolled in the study, study staff will he PI will report all SAEs according to the requirements of the IRB for their review and direction.

With regard to monitoring of data quality and protected health information, all study personnel for this project will have the required human subjects and confidentiality

training, which includes information about maintaining data integrity and security. Confidentiality will be guarded using established procedures such as storing data in locked cabinets within locked offices or locked data rooms, coding by study identification numbers rather than any personally identifying information to avoid revealing the identity of subjects, and aggregating data across participants. The key linking names and study identification numbers will be kept separately from the data sets with limited access by study personnel. Only study personnel will have access to the data sets on protected servers. Oversight of all aspects of data management will occur with the PI.

Data will be collected using standardized forms and will only be identified using the participant's ID number (no names or identifying information will be on the forms). The codes that link the names of participants and their ID numbers will be kept confidential by the PI on a password protected document on a limited access network drive. These data will only be accessible to the PIs and staff directly working with the study. Data will be entered in the computer independently by trained data entry staff and data entry discrepancies will be corrected by the site project coordinator, based on available source documents. The quality of the data will be monitored on an ongoing basis. Data quality will be monitored by inspection of the completed forms by a research assistant and any problems detected will be discussed with the PI. In the electronic study database (VA REDCap), the REDCap data query resolution workflow module will be used to monitor data input and address any data quality concerns identified on a regular basis throughout the duration of the study.

If a Data Safety Monitoring Board (DSMB) is requested, Dr. Forman will prepare a detailed written summary of serious, unexpected, and treatment related adverse events, and will compare and contrast the event with prior events. The detailed written summary will be provided to the DSMB and the IRB at annual reporting unless otherwise indicated.

If requested, the DSMB Report addressing the following information will be submitted to the IRB at the time of continuing review annually or more often as required:

- A list of the research personnel who participated in the data and safety monitoring.
- The frequency of monitoring that took place during the renewal intervals and/or the dates that data and safety monitoring was conducted.
- A summary of cumulative data related to unanticipated problems (including adverse events) including a determination of causality and whether the risk to benefit assessment has changed.
- If appropriate, a summary of pertinent scientific literature reports, therapeutic developments, or results of related studies that may have an impact on the safety of study participants or the ethics of the research study.
- A summary of the outcome of reviews conducted to ensure subject privacy and research data confidentiality.
- Final conclusions regarding changes to the anticipated benefit-to-risk assessment of the study participation and final recommendations related to continuing, changing, or terminating the study.

Parameters to be monitored

The following progress will be monitored throughout the course of the research to ensure

the safety of subjects as well as the integrity and confidentiality of their data.

- Reported symptoms, blood pressures and heart rates while taking nitrate-rich beetroot juice or placebo throughout the intervention period
- An evaluation of the progress of the research study, including subject recruitment and retention, and an assessment of the timeliness and quality of the data.
- A review of collected data (including adverse events, unanticipated problems, and subject withdrawals) to determine whether there is a change to the anticipated benefit-to-risk assessment of study participation and whether the study should continue as originally designed, should be changed, or should be terminated.
- An assessment of external factors or relevant information (e.g., pertinent scientific literature reports or therapeutic development, results of related studies) that may have an impact on the safety and study participants or the ethics of the research study.
- A review of study procedures designed to protect the privacy of the research subjects and the confidentiality of their research data.

The severity of adverse changes in physical signs or symptoms will be classified as follows:

- Grade 1 (Mild): asymptomatic or mild symptoms; clinical or diagnostic observation only; intervention not indicated.
- Grade 2 (Moderate): minimal, local or noninvasive intervention indicated; limiting age-appropriate ADL (Activities of Daily Living).
- Grade 3 (Severe): medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self-care ADL.
- Grade 4 (Life-threatening): consequences; urgent intervention indicated.
- Grade 5 (Death): event is a direct cause of death.

Frequency of monitoring

Dr. Forman will review subject safety data as it is generated. Dr. Forman and the research staff will meet at -weekly intervals to re-evaluate study goals and progress, subject recruitment and retention, data quality, documentation and identification of adverse events, complaints and confidentiality of subjects.. Dr. Forman will also review adverse event data to determine whether there is any change to the anticipated benefit-to-risk ratio of study participation and whether the study should continue as originally designed or should it be re- evaluated and changed.

If a DSMB is required, it will also be expected to meet as needed, but not less than, every six months to provide an overall summary status report to the regulatory agencies. An emergency meeting of the DSMB may be called at any time by the Chair should participant safety questions or other unanticipated problems arise.

Reportable adverse events

For this study, a serious, unexpected, and related local (internal) adverse event related to the research. A Serious Adverse Event (SAE) is defined as an adverse event that results in:

- Death
- A life-threatening experience
- -Hospitalization (for a person not already hospitalized)

- -Prolongation of hospitalization (for a patient already hospitalized)
- -Persistent of significant disability or incapacity
- -Congenital anomaly and/or birth defect
- -Requires medical, surgical, behavioral, social, or other intervention to prevent one of the preceding outcomes

If clinically important and unexpected adverse experiences or clinically important study-related adverse experiences occur, they will be recorded on the adverse event case report form and reported according to the VAPHS table of reporting requirements.

Adverse Events (AEs) and Serious Adverse (SAEs) Events:

In the proposed study we will use the VHA definition of adverse events (AE) and SAE as the reporting guideline. In the event that a participant either withdraws from the study or the PI decides to discontinue a participant due to an SAE, the participant will be monitored by study staff until (a) a resolution is reached (e.g., the problem has resolved or stabilized with no further change expected), (b) the SAE is determined to be clearly unrelated to the study intervention, or (c) the SAE results in death. Outcomes of SAEs will be regularly reported to the IRB. A summary of the SAEs that occurred during the previous year will be included in the local IRB progress reports as well as in the annual IRB renewal. Suicidal ideation and AEs will be formally assessed immediately after treatment and referrals for further care will be made as needed.

If a participant expresses suicidal ideation during any questionnaire or any study assessment, we will follow local facility policy on suicidal risk. Any positive response will result in referral to PCP if not already receiving care from a mental health provider. Any identification of suicidality will result in the patient being escorted to the ED or appropriate Mental Health provider. If the participant is presenting suicidal ideations over the phone, study staff will follow the Veterans Crisis Line guidance document to assure the participant receives appropriate help.

The study will be conducted at the VAPHS. Emergency services will be available to anyone who participates in this project. We will have specifically outlined in the Informed Consent Form the availability of emergency services to participants who may seek them during and after normal working hours. In the Informed Consent Form, we will provide specific information about emergency contacts. Participants are instructed to contact Dr. Forman during working hours. After hours, participants will be provided the information on how to contact the study coordinator or Dr. Forman, with triaging determined on an ongoing basis.

All subjects who have AEs, whether considered associated with the study intervention or not, must be monitored to determine the outcome. The clinical course of the AE will be followed according to accepted standards of medical practice, even after the end of the period of observation, until a satisfactory explanation is found, or Dr. Forman considers it medically justifiable to terminate follow-up.

All untoward medical occurrences observed in subjects receiving the study intervention will be recorded on the study AE log or case report form depending on severity by the study coordinator under the supervision of Dr. Forman. The CRFs will then be reviewed for completeness and internal consistency. Subsequently, the CRFs will be recorded in REDCap, an electronic password-guarded study database. In addition to internal

safeguards built into a computerized system, external safeguards will be put in place to ensure that access to the computerized system and to the data is restricted to authorized personnel. Training conducted by qualified individuals on a continuing basis will be provided to individuals in the specific operations regarding computerized systems that they are to perform during the course of the study.

Adverse events reporting timeline

Any death of a research subject participating in research overseen by the VAPHS IRB determined by the PI/member of the study team to be unexpected and related to the research. The local report must be made immediately to the IRB office. A written notification must also be submitted to the IRB within 1 business day of becoming aware of the death.

Any serious, unexpected, and local (internal) adverse event related to the research must submit a reportable event form in IRBNet within 5 business days.

Both event sections must also be added to the serious adverse event log and submitted at the time of Continuing Review.

Package Uploads (Section 6.0):

- **DSMB Charter, if applicable**

7.0 Privacy and Confidentiality

Standardized case report forms (CRF) will be used for recording all data from each subject. The investigator will ensure that these CRFs are properly completed. Either original de-identified source documents will be used or, in a minimum number of instances, medical data that are extracted from medical records (e.g., recordings of vitals) and CRFs will be audited against the appropriate component of the medical record. The CRFs will be kept current to reflect subject status at each phase during the trial. In all cases, subject will not be identified on CRF by name. Appropriate coded identifications (ID) will be used. Every effort will be made to collect complete data from each study visit. Documents which contain identifiable private health information (PHI) (i.e., informed consent document, contact information, prior medical records) will be separated from the deidentified CRFs at conclusion of subject participation. A participant master list will be maintained to link participant study ID with identifying PHI. The master list will be stored securely on the project-specific VA research drive in a password-protected file with access limited to approved study personnel. Causes of missing data will be fully documented.

We will obtain approximately 100-200mg from the participants vastus lateralis using muscle needling biopsy. The VA (Research) has allocated -80 freezers for banking tissue. The Freezers will be located at a separate room located at the ROB in room GA-221. The tissue will be catalogued using Freezerworks software or in REDCap. Tissue samples will be stored indefinitely and only accessible by the study team for future research

Each Biospecimen will be assigned and labeled with a unique tracking (Inventory Management) code that is distinct from the originating study code/ID and that does not

contain HIPAA identifiers. Freezerworks and/or REDCap has been chosen for this purpose and has been setup at the VA. Freezerworks is hosted on a University of Pittsburgh Server separate from the VA network and servers. These codes will be used for Inventory Management and no additional labeling generated by the contributing investigator will exist on Biospecimen containers. When applicable, tracking (Inventory Management) codes are linked to the contributing investigator's study code/ID in the associated Data Repository. The Contributing Investigator and limited Repository Staff (only those with access to the Data Repository) will be able to link Inventory Management codes to the Contributing Investigator's study code/ID. REDCap will be used to store this data if Freezerwork is not operational by the time we start to store specimen.

The Biorepository Director and Steward will review the Biorepository Inventory at least biennially to identify Biospecimens that may be appropriate for destruction and disposal. Before any Biospecimens may be destroyed, VA's Office of Research and Development must be consulted. Once approved, specimens will be destroyed in accordance with the following guidelines:

- Confirm destruction of specific specimens with the qualified investigator and/or Biorepository Director prior to disposal
- Retrieve specimens from storage facility
- Confirm the identity of specimens in the Inventory System and on the specimen label.
- Remove all labels. For labels that cannot be removed, obliterate any information, including alphanumeric information and barcodes (if possible).
- Dispose of specimens in the appropriate biohazardous waste container, as per institutional procedure
- Record specimen(s) disposal in the Inventory System.

Package Uploads (Section 7.0):

- **VHA Research Protocol Privacy Review Checklist (VA Form 10-250)**
- **Department of Veterans Affairs Enterprise Research Data Security Plan ERDSP in the submission package.**
- **DMAP or Resource Sharing Plan if this research is part of a grant.**
- **VA Data Storage and Retrieval Worksheet if you will be collecting and/or storing VA research data at a non-VA location**
- **Honest Broker Certification, if applicable**

8.0 Collaborative/Multisite Research
N/A

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