PROTOCOL TITLE:

Cocoa to Improve Walking Performance in Peripheral Artery Disease: The COCOA-PAD Study

PRINCIPAL INVESTIGATOR:

Mary McDermott, MD
Department of General Internal Medicine
312-503-6419
mdm608@northwestern.edu

NCT NUMBER:

NCT02876887

VERSION NUMBER:

Version #15.

VERSION DATE:

5.24.18

Table of Contents

1.0 Objectives

1.1 Purpose, Objectives, Specific Aims

Our prior work demonstrates that older people with lower extremity peripheral artery disease (PAD) have greater functional impairment and faster functional decline compared to those without PAD (1-4). PAD-related functional limitations are associated with increased healthcare costs and loss of independence (5-10). Yet few medical therapies have been shown to improve functioning or prevent mobility loss in older people with PAD.

People with PAD have impaired endothelial function, impaired calf muscle perfusion, reduced skeletal muscle mitochondrial activity, and reduced calf muscle mass and strength (11-20). Preliminary pre-clinical and human evidence show that cocoa and its major flavanol component, epicatechin, have therapeutic properties that reverse the pathophysiologic impairments that are present in PAD. Specifically, cocoa and epicatechin improve endothelial function, increase skeletal muscle mitochondrial function, increase skeletal muscle angiogenesis, reduce skeletal muscle levels of myostatin, and increase skeletal muscle levels of follistatin (21-31). Myostatin is a growth differentiation factor that promotes muscle wasting. Follistatin blocks myostatin's action, thereby promoting muscle growth. By reducing myostatin and increasing follistatin, epicatechin-rich cocoa has potential to increase muscle mass and strength (22,25). A small study in humans with type 2 diabetes and heart failure showed that epicatechin-rich cocoa increased skeletal muscle mitochondrial biogenesis, increased mitochondrial cristae abundance, and improved grip strength (25). A small randomized cross-over trial in PAD patients showed that dark chocolate (>85% cocoa), but not milk chocolate (<35% cocoa), acutely increased treadmill walking time, improved endothelial function, and reduced oxidative stress two hours after chocolate ingestion (31). However, no randomized trials have studied whether chronic daily epicatechin-rich cocoa improves walking ability in patients with PAD.

The COCOA-PAD Study is a pilot double-blind, randomized controlled trial to test our hypothesis that epicatechin-rich cocoa improves endothelial function, increases calf skeletal muscle mitochondrial function and biogenesis, and favorably alters skeletal muscle levels of myostatin, follistatin, and mitochondrial transcription factor-alpha (TFAM), thereby improving lower extremity functioning in older people with PAD. If our proposed pilot study supports our hypotheses, results will be used to design a large, definitive randomized clinical trial of epicatechin-rich cocoa in older people with PAD.

Forty-four participants with PAD age 60 and older will be randomized to epicatechin-rich cocoa vs. an epicatechin-free and cocoa-free placebo (matched in appearance and taste) daily for six months. Our primary

outcome is the six-minute walk, a well-validated measure of walking endurance in PAD (32-34). Secondary outcomes are pain-free and maximal treadmill walking performance, brachial artery flow-mediated dilation (FMD), accelerometry-measured physical activity, calf muscle biopsy measures, and MRI-measured calf muscle perfusion. To distinguish between the acute vs. chronic effects of cocoa, the six-minute walk and brachial artery FMD measures will be obtained while off of study beverages for 2.5 hours (acute) and 24 hours (chronic), respectively. . The treadmill stress test will be measured while off of study beverages for 48 hours. Remaining outcomes will be measured while still taking study beverages whenever possible.

Primary Aim. Among 44 participants with PAD age 60 and older, we will determine whether epicatechin-rich cocoa daily for six months improves six-minute walk performance compared to placebo.

Secondary Aims. 1. Among 44 participants with PAD age 60 and older, we will determine whether epicatechin-rich cocoa daily for six months improves maximal and pain-free treadmill walking time, increases brachial artery FMD, and increases accelerometer-measured physical activity compared to placebo. 2. Among 44 participants with PAD age 60 and older, we will determine whether epicatechin-rich cocoa daily for six months favorably improves the following measures obtained with calf skeletal muscle biopsy: activity of Cytochrome C Oxidase (COX) and citrate synthase, calf muscle levels of peroxisome proliferative activated receptor-γ co-activator 1α (PGC-1α), capillary density, myostatin, follistatin, and mitochondrial transcription factor-alpha (TFAM), compared to placebo. 3. Among 44 participants with PAD age 60 and older, we will determine whether epicatechin-rich cocoa daily for six months increases calf skeletal muscle perfusion, measured by magnetic resonance imaging (MRI), compared to placebo. 4. Among participants with PAD age 60 and older, we will determine whether epicatechin-rich cocoa daily for six months increases calf skeletal muscle biopsy measures of calf skeletal muscle regeneration and reduces oxidative stress, compared to placebo. Specific measures of calf skeletal muscle regeneration that we will study consist of satellite cell number and activation state and centrally nucleated and embryonic myosin heavy chain-expressing fibers. Specific measures of calf skeletal muscle oxidative stress will be nitrotyrosine and 4-hydroxynonenal (4-HNE).

Exploratory Aims. Among participants with PAD age 60 and older randomized to epicatechin-rich cocoa, we will determine whether the degree of improvement in six-minute walk performance and treadmill walking performance, respectively, is correlated with the degree of improvement in a) calf skeletal muscle perfusion; b) calf skeletal muscle measures of

regeneration; and c) calf skeletal muscle measures of oxidative stress.

1.2 Hypotheses.

We hypothesize that:
1. PAD participants randomized to epicatechin-rich cocoa will achieve greater increases in six-minute walk performance at six-month follow-up, compared to those randomized to placebo.
2. PAD participants randomized to epicatechin-rich cocoa will have greater increases in treadmill walking time, brachial artery FMD, and physical activity at six-month follow-up, compared to placebo.
3. Epicatechin-rich cocoa will improve each calf skeletal muscle measure listed above, compared to placebo.
4. PAD participants randomized to epicatechin-rich cocoa will achieve greater increases in MRI-measured calf muscle perfusion at 6-month follow-up, compared to those randomized to placebo.
5. PAD participants randomized to epicatechin-rich cocoa will have more favorable changes in each calf muscle biopsy measure, compared to those randomized to placebo.

2.0 Background

2.1 Relevant prior experience and gaps in current knowledge

The COCOA-PAD Study builds on our prior work. We have successfully completed large randomized trials of PAD patients. Our prior work demonstrates that people with PAD have greater functional impairment and more rapid functional decline than those without PAD (1-5). The functional impairment in PAD is associated with loss of independence, increased mortality, and poor quality of life (6-10,35).

Few medical therapies are available to improve functional impairment in PAD. Only two medications, pentoxifylline and cilostazol, are FDA-approved for treating PAD-associated walking impairment. Of these, pentoxifylline is usually ineffective and benefits from cilostazol are modest (36-39). New therapies are urgently needed to improve walking performance in patients with PAD.

2.2 Relevant Preliminary Data.

A. Calf skeletal muscle fibers and mitochondria are damaged in PAD and contribute to functional decline. Endothelial function is impaired in PAD. First, our work shows that people with PAD have smaller calf muscle area, increased calf muscle fat content, and poorer leg strength compared to people without PAD (19,20,40,41). Greater severity of these calf muscle impairments are associated with greater functional impairment and increased mobility loss in PAD (41-43). Second, electron microscopy

demonstrates pathologic changes in myofibrils, mitochondria, and sarcolemma of calf myofibers in patients with PAD (16,44,45). More severe oxidative stress, such as that associated with walking-related ischemia reperfusion of calf muscle, is associated with more severe myofiber damage (44). Third, PAD patients have reduced calf muscle mitochondrial energy production, measured by reduced adenosine triphosphate (ATP) production, compared to people without PAD (44-50). Lower ATP production is associated with greater functional impairment in PAD (18,44). Fourth, people with PAD have impaired endothelial function compared to people without PAD. Greater endothelial function impairment in PAD is associated with lower physical activity and increased cardiovascular events (11,12,51). Epicatechin-rich cocoa targets all of these pathophysiologic changes that are present in PAD.

B. A5. Pre-clinical evidence suggests that cocoa-derived epicatechin improves skeletal muscle characteristics and vascular function. Epicatechin is a flavanol that exists in high concentrations in cocoa. In pre-clinical studies, epicatechin has therapeutic effects on skeletal muscle. First, Hutteman and colleagues studied 21 rats randomized to one of three groups: Group 1: epicatechin for 30 days; Group 2: epicatechin for 30 days followed by 15 days with no therapy; Group 3: Placebo. At 30 day follow-up, Group 1 had increased skeletal muscle mitochondrial biogenesis and mitochondrial function, measured by increases in PGC-1α, TFAM, mitochondrial cristae quantity, and capillary density (21). PGC-1α is a key promoter of mitochondrial biogenesis and mitochondrial function. TFAM increases mitochondrial transcription. Greater mitochondrial cristae provides greater surface area for ATP production. These favorable changes were sustained at 45 day follow-up in Group 2, 15 days after therapy was completed. Second, Gutierrez-Salmean and colleagues administered 1 mg/kg daily of cocoa-derived epicatechin to ten young (6 months old) and ten old (26 months old) mice (22). At two-week follow-up, skeletal muscle levels of myostatin were reduced in the young and older mice by 15% and 21%, respectively, while skeletal muscle levels of follistatin increased by 56% in the older mice (22). Myostatin is a protein and growth differentiation factor that promotes muscle wasting. Follistatin blocks myostatin's action, increasing muscle growth. Thus, cocoa-derived epicatechin achieved favorable changes in myostatin and follistatin levels that increase muscle mass and strength. Third, Nogueira and colleagues randomized 25 mice into one of four groups for 15 days: Group 1: Water alone; Group 2: Water plus exercise; Group 3: cocoa-derived epicatechin alone; Group 4: cocoa-derived epicatechin plus exercise (23). The groups receiving epicatechin had greater increases in skeletal muscle capillary density, TFAM quantity, mitochondrial cristae abundance, treadmill running ability, and mitochondrial activity, compared to mice receiving water alone (23). Groups receiving epicatechin also had less muscle fatigue. In summary, preclinical evidence shows that epicatechin, the major flavanol

component of cocoa, increases mitochondrial biogenesis, mitochondrial function, and capillary density in skeletal muscle. Epicatechin improves muscle strength and running capacity in animals.

C. Preliminary evidence from humans without PAD suggests that epicatechin-rich cocoa improves mitochondrial function, increases muscle mass, and improves strength. Taub and colleagues administered chocolate squares and cocoa beverages daily for three months to five patients with type 2 diabetes and heart failure (age 41 to 71 years) (25). Total daily epicatechin content in the cocoa products was 100 mgs. In thigh muscle biopsies, nitric oxide, PGC-1α, TFAM, and mitochondrial cristae volume all increased following cocoa treatment. In a separate uncontrolled trial, Gutierrez-Salmean administered cocoa-derived epicatechin to 6 healthy young people (mean age 28.5 years) and 6 older healthy people (mean age 62) (22). Plasma levels of myostatin decreased, follistatin increased, and grip strength improved following epicatechin treatment. These preliminary studies in humans support our hypotheses that cocoa and epicatachin improve muscle mitochondrial function and biogenesis, muscle mass, and strength. However, these studies were limited by small sample sizes and lack of control groups. Further evidence is needed.

D. Dark chocolate acutely improves endothelial function and treadmill walking in PAD. Loffredo and colleagues randomized 20 PAD patients (mean age 69 years) to 40 grams of dark chocolate (>85% cocoa) vs. 40 grams of milk chocolate (<35% cocoa) in a single-blind cross-over study (31). Two hours after ingesting dark chocolate, increases were observed in treadmill walking time (124.8 + 60.8 to 142.2+62.0 seconds, $P<0.001$), brachial artery flow-mediated dilation (FMD) (2.3%+2.2% to 6.3%+2.7%, $P<0.001$), and nitric oxide (11.0 umol/L+5.8 to 17.3 umol/L+5.8, $P=0.001$). Milk chocolate ingestion did not affect any of these outcomes. These data demonstrate an acutely beneficial effect of dark chocolate in PAD. However, the effect of chronic daily administered dark chocolate on clinically important outcomes in PAD is unknown.

In summary, pre-clinical evidence and small human studies show that cocoa and the cocoa-derived flavanol, epicatechin, target key pathophysiologic abnormalities in people with PAD that are linked to functional impairment in PAD (21-28,31).

2.3 Scientific background and significance of research based on existing literature.

A. PAD is common and is associated with increased functional impairment and faster rates of functional decline compared to people without PAD. PAD affects 10-15% of community dwelling men and women age 60 and

older (52-54) and will be increasingly prevalent as the U.S. population survives longer with chronic disease.

B. Cocoa is the dried and fermented seed from *theobroma cacao,* from which chocolate is made (see photo to left). Epicatechins are flavanols, a major ingredient of cocoa, that have therapeutic effects on skeletal muscle and the vasculature (21-30). In pre-clinical models, cocoa and/or its major flavanol, epicatechin, promote skeletal muscle mitochondrial biogenesis, increase capillary growth, improve endothelial function, and reduce ischemia/perfusion injury (21-24). Cocoa and/or its major flavanol, epicatechin, also increase muscle mass and strength by favorably altering skeletal muscle levels of follistatin and myostatin (21,23,25). Pathophysiologic impairments in PAD that can be targeted by favorable effects of epicatechin-enriched cocoa are described below.

Innovation of the research.

A. Cocoa therapy has never been studied as a chronic, daily therapy for PAD. Patients with PAD have reduced calf muscle mitochondrial energy production, reduced calf muscle mass and strength, and impaired endothelial function (13-20,40,41). Cocoa and epicatechin improve mitochondrial energy production, increase mitochondrial biogenesis, increase skeletal muscle mass and strength, and improve endothelial function (11-20). Thus, chronic, daily epicatechin-rich cocoa has the potential for benefit in PAD.

B. The COCOA-PAD Study will measure the effects of chronic daily epicatechin-rich cocoa on changes in calf muscle biopsy measures and endothelial function in PAD. Calf muscle biopsies and brachial artery FMD at baseline and follow-up will delineate biologic pathways associated with improved walking performance in PAD. Our calf skeletal muscle measures of TFAM, follistatin, and myostatin have never been studied before in people with PAD.

C. The study of therapeutic properties of a food-derived product is innovative. This study has potential for far-reaching implications, since cocoa is widely accessible and acceptable to people with PAD. If the hypotheses of our proposed pilot trial are correct, a large definitive trial will be warranted.

Justification for proposed dose.

Our intervention is a dark cocoa beverage, naturally rich in epicatechin, manufactured by The Hershey Company. Previous human study suggests that approximately 70 to 100 mgs of epicatechin per day is optimal for improving skeletal muscle mitochondrial biogenesis, mitochondrial function, and strength (22,25). For example, in the study by Taub et al

described in section A16 (25), the cocoa intervention contained 100 mgs of epicatechin. We will prescribe three servings per day of the epicatechin-rich cocoa beverage, providing 75 mg daily of epicatechin. The Hershey's Company also produces a cocoa-free placebo drink that is packaged identically and appears and tastes identically to the cocoa beverage and has nearly identical nutritional content, including calories. Each beverage (cocoa and placebo) is prepared by dissolving the contents of a packet into water. Participants will be asked to drink three beverages per day.

3.0 Inclusion and Exclusion Criteria

3.1 Screening for eligibility

Initial eligibility criteria will be assessed by telephone. Potential participants who remain eligible after the telephone screening will be scheduled for a baseline visit, where they will undergo additional testing to determine their eligibility for randomization.

3.2 Inclusion and Exclusion Criteria.

Inclusion Criteria. All participants will be age 60 and older. All participants will have PAD. PAD will be defined as follows:

1. An ABI $\leq$ 0.90 at baseline is an inclusion criterion for PAD. ABI $\leq$ 0.90 is a well-accepted standard for defining PAD (55-58).

2. Potential participants with an ABI > 0.90 who have vascular lab evidence of PAD or angiographic evidence of PAD will be eligible.

Exclusion Criteria.

1. Above- or below-knee amputation.

2. Critical limb ischemia.

3. Wheelchair-bound or requiring a cane or walker to ambulate.

4. Walking is limited by a symptom other than PAD.

5. Baseline six-minute walk value of < 500 feet or > 1,600 feet.

6. Lower extremity revascularization, major orthopedic surgery, cardiovascular event, or coronary revascularization in the previous three months.

7. Planned revascularization or major surgery during the next six months.

8. Major medical illness including renal disease requiring dialysis, lung disease requiring oxygen, Parkinson's disease, a life-threatening

illness with life expectancy less than six months, or cancer requiring treatment in the previous two years. [NOTE: potential participants may still qualify if they have had treatment for an early stage cancer in the past two years and the prognosis is excellent. Participants who require oxygen only at night may still qualify.]

9. Mini-Mental Status Examination (MMSE) score < 23 or dementia.

10. Unwilling to attend three visits in one week for final outcome measures.

11. Allergy to chocolate.

12. Unwilling or unable to consume products manufactured on the same equipment that processes peanuts, tree nuts, egg, wheat, soy, and milk.

13. Use of cocoa-containing dietary supplements.

14. Unwilling to give up major dietary sources of epicatechin during the study.

15. Symptoms of heart failure or angina that limit walking activity more than ischemic leg symptoms,, increase in angina, or angina at rest (i.e. unstable angina).

16. Participation in or completion of a clinical trial in the previous three months. [NOTE: after completing a stem cell or gene therapy intervention, participants will become eligible after the final study follow-up visit of the stem cell or gene therapy study so long as at least six months have passed since the final intervention administration. After completing a supplement or drug therapy (other than stem cell or gene therapy), participants will be eligible after the final study follow-up visit as long as at least three months have passed since the final intervention of the trial.]

17. Non-English speaking, a visual impairment that limits walking ability.

18. In addition to the above criteria, investigator discretion will be used to determine if the trial is unsafe or not a good fit for the potential participant.

Additional exclusion criteria for the muscle biopsy portion of the study:

Participants who are taking anti-platelet therapy due to any history of TIA, stroke, or coronary stent or due to an acute coronary event in

the past year will not be eligible to discontinue their anti-platelet therapy for muscle biopsy. However, they may still be eligible for the muscle biopsy if Dr. Sufit and the patient agree to carry out the muscle biopsy while the participant remains on anti-platelet therapy.

3.3 Special Populations.

Vulnerable populations (fetuses, pregnant women, children, prisoners, and institutionalized persons) and adults unable to consent will not be included in this study.

4.0 Study-Wide Number of Subjects

NA

5.0 Study-Wide Recruitment Methods

NA

6.0 Multi-Site Research

NA

7.0 Study Timelines

Each participant's part in this study will last approximately six months. We plan to enroll 44 eligible participants over 10 months. The estimated date to complete primary analyses is three years from the study start date.

8.0 Study Endpoints

The primary study endpoint is the six-month change in the six-minute walk data.

Secondary study endpoints include a) six-month change in maximal and pain-free treadmill walking time; b)six-month change in brachial artery FMD; c) six-month change in accelerometer-measured physical activity; and d) six month change in the following calf skeletal muscle measures: activity of Cytochrome C Oxidase (COX) and citrate synthase, calf muscle levels of peroxisome proliferative activated receptor-γ co-activator 1α (PGC-1α), capillary density, myostatin, follistatin, and mitochondrial transcription factor-alpha (TFAM)

Data will be collected at baseline and at the 6-month follow-up visit.

9.0 Procedures Involved

9.1 Study design.

Telephone screening.

Initial eligibility criteria will be assessed by telephone via a telephone call script. The study will be described to the potential participant and the participant will be asked a series of eligibility questions to determine whether they qualify for a baseline study visit. If they qualify for a baseline visit, we will collect their name, address, telephone number(s), e-mail address, date of birth, gender, race, and ethnicity.

In addition, some participants may be asked questions about their interest in supervised treadmill exercise programs, barriers to their participation in supervised exercise programs, and their ability to participate in supervised treadmill exercise programs. These additional questions will be collected as pilot data for a subsequent study and may be administered at any time point during the COCOA-PAD trial.

Baseline testing.
Participants will be asked to come to Northwestern Memorial Hospital at baseline for the purposes of providing informed consent. In addition, at the baseline study visits, we will obtain the ankle brachial index (ABI) measurement, to ensure they are eligible for the study. We will administer study questionnaires to obtain information about medical history, including presence of comorbidities, medication use, smoking status, and other health characteristics. We will perform baseline physical functioning tests including the six-minute walk test, chair stands, balance testing, and the treadmill walking performance test. We will take a blood sample, measure participants' height and weight, and measure the blood pressure in their arm. We will perform a test of brachial artery flow-mediated dilation (FMD). Physical activity levels will be measured using an accelerometer. In addition, participants will be asked to undergo an MRI and a calf muscle biopsy at baseline. However, potential participants not eligible for MRI testing will still be allowed to participate. Participation in the calf muscle biopsy is optional. Baseline study visits will be performed over multiple days. Participants who have a muscle biopsy will be telephoned at approximately seven days after the muscle biopsy. Participants who report any complaints about their biopsy site (such as significant pain or redness) will be scheduled for an evaluation of their biopsy site by a study physician.

Details of baseline visit procedures are described in section 9.2.

Randomization. Participants will be randomized to either epicatechin-rich cocoa intervention or placebo using a SAS computer program. We will use block randomization with block sizes randomly selected from 4 and 6 to ensure balance. Randomization will be stratified according to baseline six-minute walk performance.

Monthly assessments to monitor adherence. Participants will attend monthly study visits to receive their monthly supply of beverages.

Participants will be asked to bring any remaining beverage packets from the previous month. Packets will be counted and the number of packets remaining from the previous month will be recorded. Participants will be asked to keep a log of their study beverage consumption and will be asked to turn in their log at each monthly visit. Participants will be asked to bring their current medications to these monthly meetings and changes in medication from the previous month will be recorded. In addition, weight and blood pressure will be measured at each monthly visit. If participants are having difficulty adhering to the beverages, staff may call participants in between monthly visits to check in on adherence and to remind them to fill out their monthly adherence log.

Monitoring diet and weight gain. Participants will receive a handout about monitoring and controlling their weight during the study. Participants in the intervention and control groups will be asked to weigh themselves approximately daily and record their weight on a data collection form. Participants who do not have access to a scale will be provided with a scale. They will be asked to bring this weight chart to each monthly visit. Participants observed to gain five percent or more of their body weight compared to baseline during the study will be evaluated by the study team for possible causes of weight gain. If the weight gain is determined due to increased caloric intake (rather than fluid weight gain), they will receive dietary counseling in effort to avoid additional weight gain and to advise them on dietary methods to help with weight control. Study beverages will be reduced or discontinued if a participant gains 20 or more pounds during the study.

Six-month follow-up testing. After six months of participation in the study, participants will be asked to return for follow-up testing. All measures and tests that were conducted at baseline will be repeated.

The six-minute walk and brachial artery FMD will be measured while off of study beverages for 2.5 hours and 24 hours, respectively. . The treadmill stress test will be measured while off of the study beverages for 48 hours.The calf muscle biopsy will be performed after other outcome measures are complete and while on study beverages whenever possible. If extenuating circumstances prevent outcome measurement in these defined time frames, the measurement will be obtained as close as possible to the desired time point/length of time after the final cocoa dose.

Some or all study measures may be repeated at baseline or follow-up for data quality (e.g. if a treadmill test must be stopped due to extremely high blood pressure before the patient completed the test is one potential example of why a measure may need to be repeated).

In some cases, participants may be asked to take study beverages for longer than six months. For example, if a participant has an illness that prevents them from returning at six months for follow-up testing or if they are out of town during their six-month follow-up window. In these instances, participants will be asked to continue taking their study beverages for more than six months. Participants may be asked to take up an additional two months of study beverages.

9.2 Research procedures.

Ankle Brachial Index (ABI). After the participant rests supine for five minutes, the right brachial, dorsalis pedis (DP), posterior tibial (PT) and left DP, PT, and brachial artery pressures are measured using a hand-held Doppler probe. Pressures are measured twice. The ABI is calculated for each leg by dividing the average of the DP and PT pressures by the average brachial pressure (59). If a participant qualifies for a heel-rise ABI based on their initial ABI, they will be asked to perform 50 heel-rises at a rate of one per second followed by additional measurements of the right and left brachial, DP, and PT pressures.

Questionnaire Administration. Participants will be administered IRB-approved study questionnaires by a trained and certified study coordinator.

Six-minute walk. Participants will be asked to walk back and forth along a 100-foot hallway for six minutes. They will be instructed that the purpose of the six-minute walk test is to measure the distance they can walk in six-minutes. A script will be read to describe the study procedure. Participants will be asked whether they feel the test is safe to try and whether they have any questions. Participants who cover a distance of less than 500 feet or more than 1600 feet during the baseline six-minute walk will be excluded from the study. In a pilot study to determine whether the six-minute walk distance is longer if a participant uses a 60 foot course instead of a 100 foot course, a subset of participants may be asked to return to the medical center on a separate day in order to perform the six-minute walk test using a 60 foot course. Note that this additional testing will not be performed within a month before the six-minute walk test performed as the primary outcome measure. Only the six-minute walk test performed on the 100 foot course will be used for the study's primary outcome measure.

Treadmill testing. The Gardner graded treadmill exercise test is the standard, accepted treadmill protocol for measuring change in maximal treadmill walking time in response to interventions among PAD participants (34,60,61-63). In the Gardner exercise protocol, speed is maintained at 2.0 miles per hour (mph) and treadmill grade increases by 2.0% every two minutes. If patients cannot walk at 2.0 mph, treadmill speed is started at 0.50 mph and increased by 0.50 mph every 2 minutes until the participant reaches 2.0 mph, after which the treadmill grade is increased every two minutes (34,60,61-63). The treadmill stress test results will be read by the study

cardiologist. If the results of the stress test are abnormal, participants will be referred back to their own physician for follow-up. These potential participants will be evaluated individually to determine whether it is safe and appropriate for them to continue in the study. It is noted that a regular exercise stress test does not have a high specificity for coronary artery disease. Therefore, some participants will be allowed to continue in the COCOA-PAD Study. This will be determined on a case-by-case basis. For example, if the electrocardiographic tracing is considered a 'false positive' based on a more specific stress test result or based on an angiogram result ordered separately by the participant's physician, then the participant will be allowed to continue in the study. If the participant has stable angina or no angina symptoms and his/her physician considers a six-minute walk test safe, then the participant will be allowed to continue in the study, so long as he/she meets other eligibility criteria. These are some examples of when it may be appropriate for a participant with an abnormal baseline exercise stress test to continue in the COCOA-PAD Study.

Brachial Artery FMD. Brachial artery imaging will be performed by a Registered Diagnostic Cardiac Sonographer using established methods (34,51,64). With the participant supine, a blood pressure cuff over the upper arm is inflated for five minutes at a supra-systolic pressure according to protocol. The brachial artery is imaged (B-mode and Doppler) 5 to 9 cm above the antecubital fossa using a linear array vascular ultrasound transducer (Siemens Medical Solutions). FMD is calculated as the percent change in brachial artery diameter 60 seconds after cuff release. Changes in FMD will be read in Dr. James Stein's laboratory by a reader blinded to participant group assignment.

Accelerometry. Physical activity will be measured objectively and continuously in all participants over 7 days at baseline and follow-up, using the well-validated ActiGraph accelerometer (65-67). Participants wear the ActiGraph on their beltline for seven days, removing it only for bathing or sleeping. If the monitor needs to be worn following the biopsy, measurements will not begin until at least seven days have passed after the biopsy. By this time, any discomfort from the muscle biopsy that may limit activity has resolved.

Blood collection and long-term storage. At baseline and six-month follow-up study visits, participants will have approximately 45 mls of blood drawn for processing and long-term storage at -70 degrees Celsius. Approximately 10% of participants selected by chance will have an additional set of blood drawn for quality assurance, for a total of 90 mL at each visit. Blood obtained will be collected in serum, plasma EDTA, and plasma citrate tubes. Stored blood will await later analyses for biomarkers and other emerging blood markers related to peripheral artery disease that may change in response to the intervention. Genetic testing may also be performed on

stored DNA if the participant agrees to this optional study element on the consent document. Results of the genetic testing on the sample will be stored with other data collected. Samples will be labeled with the participant's study identification number and will not be stored with other health or identifying information. Information associated with the sample will be stored a secure database on password protected computers that are secured by Northwestern University firewalls. Access is limited to study staff. If the samples are shared with other researchers not part of the current study, the PI will grant permission to the other researchers to analyze the samples after receiving IRB approval. Samples will be identified with a study identification number and the other researchers will not have access to PHI. Results of testing on the blood samples will not be shared with the study participants.

Calf muscle biopsies. Biopsies will be performed by Robert Sufit, MD, a board-certified neurologist with > 30 years of experience performing muscle biopsies. He completed all biopsies for our pilot study. The muscle biopsy will be obtained from the medial head of the gastrocnemius muscle in the leg with lowest ABI, at the point that is 67% of the distance between the medial malleolus and the medial aspect of the proximal tibia. This site represents greatest calf muscle diameter in >95% of individuals (68). Anesthesia is achieved with subcutaneous lidocaine. Subcutaneous and adipose tissue are dissected. Approximately 300 mgs of muscle tissue is removed. Approximately 50 mgs is mounted and snap frozen in liquid nitrogen-cooled isopentane for immunohistochemistry and the remainder is frozen directly in liquid nitrogen and stored at -70 degrees Celsius for protein isolation. At 6-month follow-up, we will repeat the biopsy, adjacent to the baseline incision site. In the open biopsy, muscle tissue is directly visualized, providing substantial advantages over blind needle biopsy.

Muscle biopsy specimens frozen at -70 degrees C will be shipped to Dr. Leeuwenburgh's laboratory at the University of Florida and Dr. Peterson's laboratory at University of Kentucky for analyses. Enzyme activities will be measured in whole muscle homogenates using established methods (69). COX activity will be determined spectrophotometrically at 30°C as the maximal rate of oxidation of fully reduced Cytochrome C, measured by the change in absorbance at 550 nm. (69). Citrate Synthase activity will be measured using a kit (Sigma-Aldrich; Catalogue # CS0720), per the manufacturer's instructions. Protein expression of PGC-1α, TFAM, myostatin, and follistatin in whole tissue homogenate will be measured with Standard Western Blot (70-71). Capillary density will also be measured using standard methods (72). Satellite cell number will be quantified by counting Pax7+DAPI+ nuclei and activation state by counting MyoD+Pax7+ DAPI+ nuclei in cryosections and expressed per muscle fiber (51). Regenerating fibers will be identified using an antibody against embryonic myosin heavy chain and by central nucleation to measure

changes in response to epicatechin-rich cocoa administration. Immunohistochemistry will be performed using well standardized, validated methods with excellent test re-test reliability (78,79). Nitro-tyrosine and 4-hydroxynonanal will be measured using Standard Western Blot techniques as described previously (80,81).

Other measures related to skeletal muscle quality and function may also be performed at Northwestern or at other institutions as designated by the Principal Investigator.

MRI. We will use arterial spin labeling with cardiovascular magnetic resonance imaging, methods developed by co-investigator Dr. Christopher Kramer and successfully implemented at Northwestern, to measure changes in calf skeletal muscle perfusionat 3 Tesla between PAD participants receiving epicatechin-rich cocoa vs. placebo. A thigh cuff is inflated in the leg with lowest ABI up to 250 mm Hg. After five minutes, the blood pressure cuff is rapidly deflated. Seven control-tagged image pairs are acquired over 60 seconds using PASL pulse sequence with single-shot echo-planar imaging readouts (field of view 200x200 mm, matrix 64x64, repetition time 4000 ms, echo time 32 ms, slice thickness 10 mm). Perfusion is measured and quantified on a Siemens Healthcare workstation by selectively drawing regions of interest of the hyperemic areas on the perfusion maps.

For pilot data collection, we will obtain MR images that allow us to quantify skeletal muscle and fat in calf muscle of participants.

Medications. Participants and their physicians will be asked not to change their medications during the study if possible. Participants will be asked to bring all of their medications to baseline and follow-up visits and to monthly adherence visits. We will systematically record names and doses of medications and inquire about medication changes at each of these visits. If we find differences in the use of specific medications between study groups (such as statins or cilostazol), we will adjust for these differences.

Other measures. Body mass index (BMI) will be assessed at baseline and follow-up by objectively measuring height and weight. Weight will also be measured at monthly adherence visits. Patient report will be used to document comorbidities. A four-meter walk test will be administered at usual and fastest pace at the baseline and follow-up visits. Participants will be asked to perform the usual paced four-meter walk at usual pace and the "fast paced" four meter walk at their fastest pace. Each of these short walks will be performed twice. Participants will be asked to complete a series of standing balance exercises and chair stands. Participants will have the blood pressure in their arm checked.

Dietary counseling and weight monitoring. Because of the potential for weight gain from study interventions, all participants will be weighed and receive dietary counseling at baseline and each monthly follow-up visit. Dietary counseling will consist of isocalorically removing approximately 180 Kcal from other dietary sources to accommodate the beverage. Total calories per day in the cocoa and placebo are comparable to a can of sugar sweetened soda or juice drink. Participants who gain or lose five or more percent of their baseline body weight during the study will receive additional dietary counseling as appropriate. Participants who gain 20 or more pounds will have their study beverages reduced or discontinued. Participants will also be asked to refrain from eating foods high in epicatechin during the study.

Pilot Data Collection. Some participants may be asked to complete a short questionnaire to allow investigators to better understand whether and how people with peripheral artery disease prefer to carry out walking exercise, and to collect data on whether weight loss is an important goal for people with peripheral artery disease.

9.3 Overview of protection against risks.

Prior to beginning data collection, all study coordinators undergo training and are certified by Dr. McDermott using a detailed checklist for each data collection element. Research coordinators are certified in each element of the study visit. Dr. McDermott or a designee re-certifies coordinators approximately every six months to ensure continued adherence to protocol. Those not adhering to all aspects of the protocol undergo additional training followed by re-certification.

All research staff members have completed human subjects training required by Northwestern's institutional review board (IRB). This training includes education about the importance of maintaining confidentiality of personal health information. The study principal investigator or a co-investigator is available to answer questions that arise during the informed consent process as needed.

Participants are asked to sign a study consent form prior to data collection. The research coordinator reviews study procedures, including risks and benefits associated with study participation. The research coordinator answers participants' questions. Dr. McDermott and other study investigators are available to answer participants' questions. Dr. McDermott's pager, direct telephone line, and home telephone number are provided to participants.

Minimizing risks related to the epicatechin-rich cocoa beverage and placebo. Exclusion criteria include a chocolate allergy. Participants will be

asked to return to the medical center once monthly. At these visits, blood pressure and weight will be measured. At each of these monthly follow-up visits, all participants will receive dietary counseling, under the direction of co-investigator Dr. Linda Van Horn. Dietary counseling will consist of helping participants to identify removal of approximately 180 Kcal from other dietary sources to accommodate the calories associated with both the epicatechin-rich cocoa beverage and placebo (i.e. isocaloric). Safety will also be monitored by our DSMB. However, as described above, the epicatechin-rich cocoa is a food product derived from the theobroma cacao plant. Thus we anticipate that adverse events will be very infrequent.

Minimizing risks related to muscle biopsy. The muscle biopsy procedure will be performed by Dr. Robert Sufit who has more than 30 years of experience performing these muscle biopsies, primarily as part of his clinical practice as a Board-Certified Neurologist. As in our pilot study, completed in preparation for this proposal, the muscle biopsy procedures will be performed under sterile conditions using sterile technique. Local anesthesia will be obtained using subcutaneous lidocaine. All participants will be provided with written and verbal instructions about wound care and will be advised to contact Dr. McDermott immediately if any signs of wound infection occur. In addition, participants with a muscle biopsy will be telephoned approximately seven days after the muscle biopsy and will be asked about their muscle biopsy site. Participants who report significant discomfort or redness at the site of the muscle biopsy site during this telephone call will return for a site visit.

Many PAD participants take anti-platelet therapy to prevent cardiac and cerebrovascular events. If potential participants are taking anti-platelet therapy, they will be asked to hold their anti-platelet therapy during the seven days leading up to the muscle biopsy procedure. Participants who are asked to hold their anti-platelet therapy during the week leading up to the muscle biopsy procedure may experience a heart attack or stroke related to the temporary discontinuation of the anti-platelet therapy. Permission from the participant's physician will be required before participants are asked to discontinue anti-platelet therapy. Participants who are taking warfarin or other anti-coagulant therapy will not be eligible for muscle biopsy.

Minimizing risk related to baseline and follow-up testing. All study coordinators undergo baseline training and are certified by Dr. McDermott before beginning data collection (See Appendix A for an example certification checklist). Training and certification involves ensuring that coordinators are trained in methods to help minimize falls. Dr. McDermott re-certifies coordinators every six months to ensure continued adherence to study protocol. Those who are not adhering to protocol undergo additional training followed by re-certification.

Minimizing risk related to loss of confidentiality. The following methods will be employed to maintain confidentiality of participants. First, study recruitment letters will be mailed, using IRB-approved methods, only after receiving permission from the participant's physician. The personal physician of each study participant will have the option of not allowing investigators to contact the potential participant. Lists of potentially eligible participants will be obtained by individuals who normally have access to these lists as part of their daily work requirements. Recruitment letters for potential participants identified from hospital and outpatient lists are prepared by research staff members whose job is to assist study investigators with recruitment. These research staff members have completed training in the ethical conduct of human subject research, including maintaining participant confidentiality. Recruitment letters to potential participants identified from medical center lists are mailed in sealed envelopes and addressed to the potential participant. All potential participants who receive mailed information about the study after the approval from their physician will have the opportunity to call a voice-mail system to ask NOT to be further contacted about this study. Secondly, only study investigators and trained research staff will have access to the study database. Third, participants will be assigned a unique study identifier. Individual names will ultimately be removed from the study database and only the unique study identifier will be used to distinguish participants in the database. Fourth, collected data will be maintained in locked computer files and file cabinets to which only study investigators have access. Collected data will be used only for research purposes. Any published data will not contain any individual identifiers.

Minimizing risks related to MRI. Participants will be carefully questioned to ensure it is safe for them to undergo MRI testing.

10.0 Data and Specimen Banking

10.1 Storage of specimens.

Muscle specimens and blood specimens for long-term storage will be stored in a freezer belonging to Dr. McDermott's research program at Northwestern University, in the freezer farm in the basement of Olson Pavilion. Specimens will be stored for up to 70 years, after which they will be destroyed.

10.2 Data to be stored or associated with each specimen.

Specimens will be coded; meaning that a key will exist that can link the codes back to the direct subject identifiers. Each participant will be assigned a unique study ID number that can be traced back to the study participant. The muscle specimens and the blood samples that are stored will be labeled with this unique identifier and the date and time of the blood collection.

10.3 Procedures to release data or specimens.

Only Dr. McDermott has control over release of study data or specimens. Any investigators seeking to analyze blood or muscle specimens must contact Dr. McDermott for permission. Each request, if it occurs, will be considered on a case-by-case basis. Dr. McDermott will obtain IRB approval prior to releasing any blood or muscle specimens for analysis, other than those tests specifically named in this application.

11.0 Data and Specimen Management

11.1 Data management.

Data is recorded using preprogrammed instruments and an electronic case report form using secure, HIPAA-compliant REDCap database software on servers maintained by Northwestern's Clinical and Translational Sciences Institute. We have substantial experience with REDCap.

*11.2 Power Estimates/Calculations***.**

The COCOA-PAD Study is a pilot study intended to collect preliminary data to estimate an effect size and to assess the feasibility of our proposed methods. Therefore, we calculated the sample size that allows us to identify an effect size estimate. Our power calculations anticipate a < 10% drop-out rate at 6-month follow-up, based on data from our completed GOALS and SILC randomized trials in PAD participants (34,60). For our primary aim, we will compare changes in six-minute walk performance at 6-month follow-up between the epicatechin-rich cocoa group and the placebo group. Comparisons will be made for measures obtained both while off of the study beverages for 2.5 hours and while off of the study beverages for 24 hours, respectively, in order to assess both acute and chronic effects of cocoa on six-minute walk. A total of 40 participants completing the trial will provide a two-sided 95% confidence interval (CI) with a margin of error of 0.64 standard deviation (SD) for the effect size in change of six-minute walk performance between the cocoa (n=20) and the placebo groups (n=20). Using the estimated SD from our SILC trial (34), the margin of error of 0.64 SD represents approximately 33 meters. Prior studies have defined clinically meaningful changes in the six-minute walk as 20 meters (small meaningful difference) and 50 meters (large meaningful difference) (73,74). Similarly, 40 participants completing the trial provides a two-sided 95% CI with a margin of error of 0.64 SD for the effect size in 6-month change of each secondary outcome between the cocoa and placebo groups.

Statistical Analyses. Data will be analyzed according to each participant's originally assigned group, irrespective of whether the participant adheres to his/her assigned group (i.e. intention to treat principle). For our Primary Aim, we will use a t-distribution to obtain the 95% CI for the difference in changes in six-minute walk performance at 6-month follow-up between the

cocoa and placebo groups. Comparisons at six months will be made to measure both the acute effects (2.5 hours after drinking the study beverages) and the chronic effects (24 hours after drinking the study beverages). The characteristics of participants in the intervention and control group will be compared using t or chi-square tests. If there is indication of major imbalance, we will perform analysis of covariance (ANCOVA) with change in six-minute walk distance as the response variable to evaluate differences between the two groups, adjusting for potentially imbalanced covariates. If there is evidence that the normality assumption for the distribution of outcome measures is violated, we will either apply appropriate transformation to the original measures before conducting the aforementioned analyses or obtain a nonparametric estimate and 95% CI for the difference in median between the cocoa and placebo groups (74). These methods will also be used to analyze outcomes in our secondary specific aims.

11.3 *After recruitment was completed, and prior to reviewing results, investigators determined that a one-sided test with a significance level of 0.10 will be used to in the statistical analyses. Correspondingly, the point estimator and one-sided 90% CIs will be obtained for the differences in all outcomes between the cocoa and the placebo groups. Since the trial is a pilot study collecting preliminary evidence on the potential benefit of epicatechin-rich cocoa on change in walking ability and other outcomes in PAD patients, one-sided tests are justified. For the same reason, the decision on whether a larger independent confirmatory study is warranted to prove the hypothesized treatment effect of cocoa will be made based on all relevant evidence in this pilot study collectively, which include but are not restricted to the statistical significance. Steps to secure data to maintain confidentiality during storage, use, and transmission.*

First, all research assistants must complete training in protection of subject privacy and prevention of disclosure of identifying information.

Second, all data collection forms are maintained in a secure office space.

Third, our study databases are maintained in locked computer files or on secure hard-drives that are password protected; to which only authorized staff have access. Dr. McDermott or a study manager must provide permission for programmers and research assistants to access study databases.

Fourth, a study identification number will be assigned to each participant. This identification number will be used to label blood specimens, for example. In addition, most pages of our data collection forms will have only the study identification number listed (and not the participant's name, for example).

11.4 Quality Control.

Health interviewers will be trained by a senior coordinator and certified by Dr. McDermott in each data collection element, using a detailed checklist. Health interviewers are rigorously evaluated for adherence to protocol, delineated in our manuals, prior to beginning data collection. They are re-certified approximately every six months by a project manager or Dr. McDermott. When deficiencies are identified, interviewers undergo additional training and re-assessment. Ten percent of participants are randomly identified for quality control. This subset has their ABI measured twice by independent examiners and may have a split sample of muscle tissue (i.e. two samples from the same participant) sent for analyses. The second muscle sample is designated an arbitrary identification number to which the technician is blinded. Thus, quality control is monitored continuously. A 20% subset of collected MR images will be interpreted by two independent evaluators. For the skeletal muscle measures, a 10% subset of participants will have an additional quality control muscle specimen analyzed. To achieve this, up to 40% of individuals may have up to 400 mgs of tissue removed.

Blinding for data collection. The study design is double-blinded. Participants, study investigators, and the study coordinator collecting outcome data will be unaware of group assignment.

Missing Data. First, missing data may occur if participants are lost to follow-up. However, in our prior randomized trials of PAD participants, drop-out rates were <10% at 6-month follow-up (34,60,75). Thus, drop-out should be small. Second, we recognize that not all participants may return for a second muscle biopsy at follow-up. However, 95% of participants who completed a muscle biopsy in our pilot study indicated that they were willing to return for a second biopsy. Thus, loss to follow-up should be small. Third, some participants may not adhere to their beverage. We will measure adherence and will perform a sensitivity analysis that includes only those who adhered to 80% or more of their assigned beverage. This pilot study will determine likely adherence rates for a larger trial.

12.0 Provisions to Monitor the Data to Ensure the Safety of Subjects

A Data Safety Monitoring Board (DSMB) will monitor safety throughout the study. Please see separate Data Safety Monitoring Board (DSMB) charter. The DSMB charter will be a living document that can be modified during the study.

The DSMB will meet at least every six months during the study. The DSMB will review and approve the protocol prior to beginning data collection. They will decide on stopping criteria for the study. The biostatisticians and data manager will work with the DSMB to perform interim analyses. Adverse events will be monitored continuously

throughout the study and will be reported to the DSMB in a timely manner according to pre-specified requirements. Analyses for each DSMB meeting will be completed according to the requests of the DSMB. Investigators and DSMB members will be blinded to group assignment. Adverse event rates and interim study results will be reviewed and discussed by the DSMB at the DSMB meetings. At least four categories of adverse events will be defined: a) death; b) cardiovascular events (myocardial infarction, stroke, and coronary arrhythmias); c) hospitalizations; and d) injury or illness causing chronic disability. We will report all serious adverse events to the DSMB in a timely fashion. In addition to these serious adverse events, we will monitor participant weight and blood pressure during monthly visits throughout the study. We will use a designated data collection form to record these events. As noted above, however, we anticipate that that epicatechin-rich cocoa beverage will have few significant adverse events.

13.0 Withdrawal of Subjects

13.1 Anticipated circumstances under which subjects will be withdrawn from the research without their consent, including stopping participation for safety reasons. We anticipate that subject withdrawal from the research without their consent will be infrequent. However, a potential example is if a participant develops symptoms during the study and the Principal Investigator feels that the symptoms could make the study unsafe for the participant to continue. In this circumstance, the participant would be advised to follow-up with their physician. If the participant refuses to follow-up with their physician, it may be necessary for the participant to be withdrawn without their consent.

13.2 Procedures when subjects withdraw from the research. Subjects may withdraw from the research at any time. If they decide to leave the research, they should contact the principal investigator, Dr. Mary McDermott. If they stop being in the research, already collected data may not be removed from the study database. They will be asked whether the investigator can collect data from their routine medical care. If the subject agrees, this data will be handled the same as research data.

14.0 Risks to Subjects

The placebo beverage has the same caloric intake and nutritional characteristics as the cocoa beverage except that the placebo drink contains no cocoa or flavanols. In general, older patients with PAD have a high prevalence of comorbid diseases, particularly coronary artery disease, cerebrovascular disease, diabetes mellitus, and pulmonary disease. Thus the patient population is likely to be of generally poorer health than that of older men and women without PAD in the general population.

Risks associated with cocoa therapy. Cocoa is the dried and fermented seed from *theobroma cacao,* from which chocolate is made. Epicatechins are flavanols and a major ingredient of cocoa that has therapeutic effects on skeletal muscle and the vasculature (21-30). Our cocoa-beverage intervention is a food product, manufactured by the Hershey Company. Similar cocoa drinks are widely available for commercial use. The flavanol epicatechin, when administered in therapeutic doses for research purposes, can cause lowering of blood pressure (76). Thus, blood pressure levels will be measured monthly during the study. The epicatechin-rich cocoa drink and the placebo will each deliver approximately 180 calories per day in the doses prescribed (three drinks per day). Weight will be monitored monthly throughout study participation.

Risks associated with the muscle biopsy. The muscle biopsy is associated with several potential risks. These include discomfort during the muscle biopsy procedure and immediately afterward, scarring from the muscle biopsy skin incision, bleeding, and infection. In addition, potential participants who are asked to hold their anti-platelet therapy during the week leading up to the muscle biopsy procedure may experience a cardiovascular event related to the temporary discontinuation of the anti-platelet therapy. First, to minimize risk related to muscle biopsy, all participants undergoing muscle biopsy will receive a written hand-out regarding signs to watch for that may indicate wound infection. They will also be verbally instructed in this. Each participant will be instructed to call Dr. McDermott immediately if any signs of infection occur. Participants will be telephoned approximately seven days after the muscle biopsy and asked for any symptoms that may suggest an infection or delayed healing. Second, permission from the participant's physician will be required before participants are asked to discontinue anti-platelet therapy.

Six-minute walk test, four-meter walks, balance, and chair stands. The physical functioning tests may be associated with muscle fatigue or soreness. These symptoms typically resolve with rest.

These tests may be associated with the risk of falling or coronary ischemia or dyspnea due to heart failure or lung disease. Rarely, falling may result in a fracture. However, the research assistant who will collect these data has been trained to prevent falling. The risk of a fracture secondary to a fall during the testing is less than 1 in 5,000. If a participant experiences chest pain, research assistants are trained to page Dr. McDermott immediately. If the chest discomfort does not immediately resolve with rest, participants are escorted to Northwestern's Emergency Department, which is located in the same building as the location of the tests. Dr. McDermott facilitates follow-up, by contacting participants' physicians, for those who experience new

chest discomfort during testing. In our experience, the risk of chest discomfort is approximately 1 in 750.

Risks associated with ABI measurement. The ankle brachial index measurement consists of measuring systolic blood pressure in each extremity using a hand-held Doppler. The ABI is non-invasive, safe and does not have any known lasting side effects. During the ankle brachial index test, participants may experience discomfort from the inflated blood pressure cuff. However, this discomfort resolves immediately when the cuff is released.

Risks associated with questionnaire administration. Participation includes a risk of loss of confidentiality regarding personal health information. However, all research staff have undergone formal human subjects training. They are trained to protect the privacy of research subject participants.

Risks associated with drawing blood. The potential risks of drawing blood include a bruise at the site of vein puncture, inflammation of the vein, and infection. Participants undergoing a blood draw may experience lightheadedness, dizziness, or fainting.

Risks associated with MRI testing. The MRI scanner makes loud banging noises while doing a measurement. Participants will be provided with earplugs or earphones to protect against the noise. MRI testing can be difficult for people with claustrophobia. Potential participants who are claustrophobic will not be encouraged to participate in MRI testing.

15.0 Potential Benefits to Subjects

Participants who are randomized to the intervention could experience improved functional performance and prevent mobility loss, if our hypotheses are correct.

If the COCOA-PAD Study supports our hypotheses, results will be used to design a large, definitive randomized controlled trial of epicatechin-rich cocoa to improve lower extremity functioning and prevent mobility loss in the large and growing number of older people who are disabled with PAD.

16.0 Vulnerable Populations

NA

17.0 Community-Based Participatory Research

NA

18.0 Sharing of Results with Subjects

Participants will receive results of their ankle brachial index (ABI) test and will be provided with a "result letter" at the end of their baseline visit. ABI and stress test results will be mailed to the participant's physician by request. They will not be provided with other study results routinely. However, participants will be notified of abnormal stress test results or abnormal blood pressure results.

19.0 Setting

Baseline and follow-up data collection will take place at Northwestern Memorial Hospital in the Galter Pavilion (675 N. St. Clair) on floors 11 or 18, 680 N. Lake Shore Drive on the 14th floor, or at 750 N. Lake Shore Drive.

20.0 Resources Available

Our multidisciplinary team includes internationally recognized investigators in PAD (Drs. Criqui, Kibbe, McDermott), functional decline (Drs. Ferrucci, Guralnik, McDermott), skeletal muscle molecular and cellular biology (Drs. Ferrucci, Peterson, Leeuwenburgh, Sufit), cocoa/epicatechin (Drs. Villarreal, Van Horn), and MRI (Dr. Kramer). Dr. James Stein, an internationally recognized expert in endothelial function, and Dr. Linda Van Horn, an internationally recognized diet and nutrition expert, have joined the investigative team. Dr. Van Horn will assist with dietary counseling to prevent study-related weight gain. Dr. Christopher Kramer is internationally recognized for his work developing MRI methods for measuring calf muscle perfusion in patients with PAD. We have successfully completed observational studies of functioning in PAD participants (R01-HL58099, R01-HL64739, and R01-HL71223L) (1-5,7,19,22,32) and randomized clinical trials of exercise or behavioral interventions in PAD participants: (R01-HL073351, R01-HL073912, and R01-HL088589) (34,60,75). We have experience with all outcomes proposed. We are an ideal investigative team to conduct the COCOA-PAD Study.

We have substantial experience with calf muscle biopsies in PAD participants. Since September 1, 2013, we have completed 54 muscle biopsies in a pilot study of people with and without PAD. Biopsies were performed by co-investigator Dr. Sufit, who has more than 30 years of experience performing muscle biopsies. Most of the muscle specimens remain frozen at -70 degrees Celsius, since resources are not available for analyzing all of them. Available resources allowed us to analyze specimens from 17 participants (9 with PAD) in Dr. Leeuwenburgh's laboratory. Our results show that more severe leg ischemia is associated with poorer mitochondrial activity and that poorer mitochondrial activity is associated with poorer six-minute walk. Citrate synthase activity, a measure of mitochondrial function and quantity, and COX activity each correlated with PAD severity, measured by the ankle brachial index (ABI) (correlations=0.492 and 0.769, respectively). Among PAD participants,

citrate synthase and COX activity were correlated with the six-minute walk (0.411 and 0.397, respectively).

Dr. Christiaan Leeuwenburgh is an internationally recognized expert in skeletal muscle biology and mitochondrial function. Dr. Leeuwenburgh's laboratory will measure COX and citrate synthase activity and levels of protein markers of mitochondrial biogenesis (PGC-1α and TFAM) and proteins associated with skeletal muscle mass (follistatin and myostatin) as well as measures of muscle oxidative stress in samples obtained in the COCOA-PAD Study. Dr. Leeuwenburgh's laboratory performed all of the measures from our muscle biopsy pilot study. Dr. Charlotte Peterson's laboratory at University of Kentucky will perform immunohistochemical analyses of muscle regeneration. Dr. Peterson is internationally recognized for her work focusing on elucidation of cellular and molecular mechanisms that control skeletal muscle structure and function. Dr. James Stein of the University of Wisconsin Medical Center, will measure brachial artery FMD from images collected at Northwestern. Dr. McDermott and Dr. Stein have worked together on previous randomized trials of PAD participants, funded by the NIH (34,51,77). Addition co-investigators include Drs. Michael H. Criqui (University of California at San Diego), Jack M. Guralnik (University of Marlyand), Luigi Ferrucci (National Institute on Aging), and Francisco Villarreal (University of California at San Diego). Drs. Criqui, Guralnik, and Ferrucci have worked with Dr. McDermott on PAD studies of functional impairment for over eleven years and bring expertise in functional assessment, PAD, and clinical trials to the study team. Dr. Villarreal is a nationally-recognized expert in cocoa and epicatechin therapy who has conducted pre-clinical studies and a small preliminary human trial of cocoa therapy for patients with diabetes and heart failure (22,23,25-27).

21.0 Prior Approvals

NA

22.0 Recruitment Methods

22.1 When, where, and how potential subjects will be recruited.

PAD participants will be identified from among individuals with PAD who have participated previously in research conducted by Dr. McDermott and/or who have expressed an interest in participating in future studies conducted by Dr. McDermott.

In addition, some PAD participants may be identified from among consecutive patients diagnosed with PAD in the non-invasive vascular laboratory at Northwestern Medical Group (NMG). Dr. Mark Eskandari is medical director of the non-invasive vascular laboratory at NMG and will assist with identifying potential participants from the non-invasive vascular laboratory. As director of the vascular laboratory at NMG, Dr. Eskandari

formally reads many of the non-invasive vascular laboratory tests. He maintains all non-invasive vascular test results in his vascular laboratory. As director of the vascular laboratory, Dr. Eskandari could conceivably contact the patients whose test results are maintained in his laboratory. However, Dr. Eskandari prefers that the contact of potential participants in studies come from the physicians referring him for testing. Lists of patients who have undergone lower extremity arterial testing in the non-invasive vascular laboratory are generated monthly and e-mailed from NMG to Dr. McDermott using an encrypted file. A research assistant, working on behalf of Dr. Eskandari, will contact referring physicians of potential participants identified from the vascular laboratory via fax, phone, page, or electronic message (EPIC or e-mail), to ask for permission to contact their patient about the study. If a reply is not received within three weeks, up to five letters are mailed from Dr. McDermott about the research study. We have substantial experience with our recruitment methods for our previous or ongoing studies.

We also propose to obtain lists of consecutive patients with a diagnosis of lower extremity peripheral arterial disease and individuals at high risk for peripheral artery disease from Northwestern's Enterprise Data Warehouse (EDW). EDW lists will be obtained by an individual who is employed by the Division of General Internal Medicine who has received training and permission to obtain the lists from the EDW.

Similar methods will be used as those described above, in which the patient's physician will be contacted via fax, telephone, page, or electronic message (EPIC or email) to ask for permission to contact their patient about the study. If a reply is not received within three weeks, up to five letters are mailed from Dr. McDermott about the research study.

In the recruitment letters, recipients are asked to call us if they are interested in participation or if they do not want to be contacted further. Potential participants who do not call us within three weeks of the first mailed recruitment letter may be telephoned by study staff and invited to participate.

In addition, we will use newspaper, television, and radio advertising to identify potential participants for this study. We will also use brochures, flyers, or posters that we will post in relevant office practices and public areas. We will use advertising on public transportation or online advertisements.

We will obtain a list of patients who are in an eligible age range for the study and live in the Chicago area from a mass mailing company. Using this, we will send postcards to those individuals on the list. The postcards will instruct people to call a study number if they are interested.

Participants who have participated in previous studies and indicated interest in future studies will be contacted. Participants who we screen for ongoing studies who may have PAD but are ineligible for that study and interested participating in a study may be screened for this study.

We may also use the PCOR-NET for recruitment. PCOR-NET is a PCORI-funded network of institutions in the Chicago area. The purpose of the PCOR-NET is to assist investigators with recruitment for clinical trials. PCOR-NET has its own IRB (University of Illinois at Chicago). PCOR-NET uses ICD-9 codes and the electronic health record to identify potential participants who have PAD. These patients with PAD receive a recruitment letter that describes the COCOA-PAD trial and invites the PAD patients to participate.

22.2 Payment

Participants will receive $25 for completing six-month follow-up testing and $25 per MRI.

If the participant undergoes the optional muscle biopsy portion of the study, they will receive $100 per muscle biopsy. Therefore, participants will receive up to $275 for taking part in this research study.

Participants will be given assistance and/or reimbursement for expenses related to travel such as parking, bus/train fare, taxi fare, and mileage, if requested. A receipt will be required for taxi fare reimbursement. Participants will be provided up to $90 per visit for travel reimbursement. If they require the use of our taxi service, we will estimate the fare on www.taxifarefinder.com. A one-way fare estimate must be less than or equal to $45 (i.e. round trip of $90) in order for the study to provide taxi service.

23.0 Local Number of Subjects

We will identify and randomize 44 eligible participants.

24.0 Confidentiality

NA

25.0 Provisions to Protect the Privacy Interests of Subjects

All research staff undergo training (human subjects training) in the protection of participant confidentiality and privacy. Research staff have access to medical records only for the purpose of conducting research that is approved by the IRB.

Questionnaires and all research procedures will be conducted in an enclosed space by a trained and certified research assistant. Dr. McDermott personally certifies research assistants in data collection to help ensure that participants are treated with the highest level of professionalism.

26.0 Compensation for Research-Related Injury

If the subject needs medical care because of taking part in this research study, they should contact the investigator and medical care will be made available. This care will be billed to the subject, their insurance, or other third party. Northwestern University has no program to pay for medical care for research-related injury.

27.0 Economic Burden to Subjects

NA

28.0 Consent Process

The "SOP: Informed Consent Process for Research (HRP-090)" will be followed. Participants will be consented by a research assistant who has been trained and certified by Dr. McDermott in obtaining informed consent. A research assistant will explain the study to potential participants by telephone prior to their first study visit. When a potential participant arrives to the medical center for study participation, the research assistant will explain the full details of the research study, including risks and benefits. The informed consent process will take place at the initial baseline study visit in a private area on Northwestern's medical campus.

Potential participants will be provided plenty of time to read the consent form. The research assistant will answer questions and Dr. McDermott or another study investigator at Northwestern is also available to answer any questions that participants may have about the research. If the participant would like more time to discuss the research study with their physician or family member before signing the consent document, they will be allowed to do so and the study visit will be rescheduled for a later date.

Potential participants who do not speak English, subjects who are not yet adults, cognitively impaired adults, and adults unable to provide written consent will not be eligible for study participation.

29.0 Process to Document Consent in Writing

The "SOP: Written Documentation of Consent (HRP-091)" will be followed.

30.0 Drugs or Devices

The Hershey Company will send cocoa-epicatechin and placebo beverages in identically appearing packets to Dr. McDermott. Packaging of the cocoa and placebo packets are identical and distinguished only by Lot ID number (D or F). Investigators and study staff and study participants are blinded to which group is 'D' vs. 'F'. REDCap will randomize to either group D or F and participants will be provided with a month's supply of cocoa packets labeled with either D or F according to their randomization assignment.

Participants randomized to the intervention will consume three epicatechin-rich cocoa beverages per day (for instance, participants may consume two in the morning and one in the afternoon/early evening). Participants randomized to placebo will take three identical-appearing placebo beverages per day (for instance, participants may consume two in the morning and one in the afternoon/early evening).

Study beverage packets will be stored in a locked closet located on the 10th floor of 750 N. Lake Shore Drive. Study packets are stored at room temperature, not exceeding 90 degrees F. An electronic thermometer is used within the office space that tracks and records temperature daily and will alarm if the temperature exceeds 90 degrees. Packets will be counted and dispensed in a large handled bag to participants. A monthly supply will include 90 packets (3 packets per day X 30 days). Two individuals on the study team will independently check to ensure that the study randomization group assignment (Group D or F) matches the labeling on the packets before dispensing to the participant. A log will be kept to record the dates and number of packets that are dispensed throughout the study period.

The Food and Drug Administration concluded that an IND is not required.

31.0 References

1. McDermott MM, Greenland P, Liu K, Guralnik JM, Criqui MH, Dolan NC, Chan C, Celic L, Pearce WH, Schneider JR, Sharma L, Clark E, Gibson D, Martin GJ. Leg symptoms in peripheral arterial disease: associated clinical characteristics and functional impairment. *JAMA* 2001;286:1599-606. PMID: 11585483.

2. McDermott MM, Liu K, Greenland P, Guralnik JM, Criqui MH, Chan C, Pearce WH, Schneider JR, Ferrucci L, Celic L, Taylor LM, Vonesh E, Martin GJ, Clark E. Functional decline in peripheral arterial disease: associations with the ankle brachial index and leg symptoms. *JAMA* 2004;292:453-61. PMID: 15280343.

3. McDermott MM, Greenland P, Liu K, Guralnik JM, Celic L, Criqui MH, Chan C, Martin GJ, Schneider J, Pearce WH, Taylor LM, Clark E. The ankle brachial index is associated with leg function and physical activity: the Walking and Leg Circulation Study. *Ann Intern Med* 2002;136:873-83. PMID: 12069561.

4. McDermott MM, Guralnik JM, Tian L, Liu K, Ferrucci L, Liao Y, Sharma L, Criqui MH. Associations of borderline and low normal ankle-brachial index values with functional decline at 5-year follow-up: the WALCS (Walking and Leg Circulation Study). *J Am Coll Cardiol* 2009;53:1056-62. PMID: 19298919.

5. McDermott MM, Guralnik JM, Tian L, Ferrucci L, Liu K, Liao Y, Criqui MH. Baseline functional performance predicts the rate of mobility loss in persons with peripheral arterial disease. *J Am Coll Cardiol* 2007;50:974-82. PMID: 17765125.

6. Fried LP, Guralnik JM. Disability in older adults: evidence regarding significance, etiology, and risk. *J Am Geriatr Soc* 1997;45:92-100. PMID: 8994496.

7. McDermott MM, Tian L, Liu K, Guralnik JM, Ferrucci L, Tan J, Pearce WH, Schneider JR, Criqui MH. Prognostic value of functional performance for mortality in patients with peripheral artery disease. *J Am Coll Cardiol* 2008;51:1482-9. PMID: 18402904.

8. Guralnik JM, Simonsick EM, Ferrucci L, Glynn RJ, Berkman LF, Blazer DG, Scherr PA, Wallace RB. A short physical performance battery assessing lower extremity function: association with self-reported disability and prediction of mortality and nursing home admission. *J Gerontol* 1994;49:M85-94. PMID: 8126356.

9. Guralnik JM, Ferrucci L, Simonsick EM, Salive ME, Wallace RB. Lower-extremity function in persons over the age of 70 years as a predictor of subsequent disability. *N Engl J Med* 1995;332:556-61. PMID: 7838189.

10. Guralnik JM, Ferrucci L, Pieper CF, Leveille SG, Markides KS, Ostir GV, Studenski S, Berkman LF, Wallace RB. Lower extremity function and subsequent disability:

consistency across studies, predictive models, and value of gait speed alone compared with the short physical performance battery. *J Gerontol A Biol Sci Med Sci* 2000;55:M221-31. PMID: 10811152.

11. Heinen Y, Stegemann E, Sansone R, Benedens K, Wagstaff R, Balzer J, Rassaf T, Lauer T, Kelm M, Heiss C. Local association between endothelial dysfunction and intimal hyperplasia: relevance in peripheral artery disease. *J Am Heart Assoc.* 2015;4:e0014672. Doi:10.1161/JAHA.114.001472. PMCID:PMC4345872.

12. Gokce N, Keaney JF Jr., Hunter LM, Watkins MT, Nedeljkovic ZS, Menzoian JO, Vita JA. Predictive value of noninvasively determined endothelial dysfunction for long-term cardiovascular events in patients with peripheral vascular disease. *J Am Coll Cardiol.* 2003;41:1769-1775. PMID: 12767663.

13. Pipinos II, Judge AR, Zhu Z, Selsby JT, Swanson SA, Johanning JM, Baxter BT, Lynch TG, Dodd SL. Mitochondrial defects and oxidative damage in patients with peripheral arterial disease. *Free Radic Biol Med.* 2006;41:262-9. PMID: 16814106.

14. Pipinos II, Sharov VG, Shepard AD, Anagnostopoulos PV, Katsamouris A, Todor A, Filis KA, Sabbah HN. Abnormal mitochondrial respiration in skeletal muscle in patients with peripheral arterial disease. *J Vasc Surg.* 2003;38:827-32. PMID: 14560237.

15. Anderson JD, Epstein FH, Meyer CH, Hagspiel KD, Wang H, Berr SS, Harthun NL, Weltman A, Dimaria JM, West AM, Kramer CM. Multifactorial determinants of functional capacity in peripheral arterial disease: Uncoupling of calf muscle perfusion and metabolism. *J Am Coll Cardiol.* 2009;54:628-35. PMCID: PMC2768062

16. Pipinos II, Judge AR, Selsby JT, Zhu Z, Swanson SA, Nella AA, Dodd SL. The myopathy of peripheral arterial occlusive disease: Part 2. Oxidative stress, neuropathy, and shift in muscle fiber type. *Vasc Endovascular Surg.* 2008;42:101-12. PMID: 18390972.

17. Pipinos II, Swanson SA, Zhu Z, Nella AA, Weiss DJ, Gutti TL, McComb RD, Baxter BT, Lynch TG, Casale GP. Chronically ischemic mouse skeletal muscle exhibits myopathy in association with mitochondrial dysfunction and oxidative damage. *Am J Physiol Regul Integr Comp Physiol.* 2008;295:R290-6. PMCID: PMC2494805

18. Hou XY, Green S, Askew CD, Barker G, Green A, Walker PJ. Skeletal muscle mitochondrial ATP production rate and walking performance in peripheral arterial disease. *Clin Physiol Funct Imaging.* 2002;22:226-32. PMID: 12076351.

19. McDermott MM, Tian L, Ferrucci L, Liu K, Guralnik JM, Liao Y, Pearce WH, Criqui MH. Associations between lower extremity ischemia, upper and lower extremity strength, and functional impairment with peripheral arterial disease. *J Am Geriatr Soc* 2008;56:724-9. PMID: 18284536.

20. McDermott MM, Criqui MH, Greenland P, Guralnik JM, Liu K, Pearce WH, Taylor L, Chan C, Celic L, Woolley C, O'Brien MP, Schneider JR. Leg strength in peripheral arterial disease: Associations with disease severity and lower-extremity performance. *J Vasc Surg* 2004;39:523–30. PMID: 14981443.

21. Hüttemann M, Lee I, Perkins GA, Britton SL, Koch LG, Malek MH. (-)-Epicatechin is associated with increased angiogenic and mitochondrial signaling in the hindlimb of rats selectively bred for innate low running capacity. *Clin Sci (Lond*) 2013;124:663-74. PMID: 23252598.

22. Gutierrez-Salmean G, Ciaraldi TP, Nogueira L, Barboza J, Taub PR, Hogan MC, Henry RR, Meaney E, Villarreal F, Ceballos G, Ramirez-Sanchez I. Effects of (-)-epicatechin on molecular modulators of skeletal muscle growth and differentiation. *J Nutr Biochem* 2014;25:91-4. PMID: 24314870.

23. Nogueira L, Ramirez-Sanchez I, Perkins GA, Murphy A, Taub PR, Ceballos G, Villarreal FJ, Hogan MC, Malek MH. (-)-Epicatechin enhances fatigue resistance and oxidative capacity in mouse muscle. *J Physiol* 2011;589:4615-31. PMID: 21788351.

24. Watanabe N, Inagawa K, Shibata M, Osakabe N. Flavan-3-ol fraction from cocoa powder promotes mitochondrial biogenesis in skeletal muscle in mice. *Lipids Health Dis* 2014;13:64. doi:10.1186/1476-511X-13-64. PMID: 24708519.

25. Taub PR, Ramirez-Sanchez I, Ciaraldi TP, Perkins G, Murphy AN, Naviaux R, Hogan M, Maisel AS, Henry RR, Ceballos G, Villarreal F. Alterations in skeletal muscle indicators of mitochondrial structure and biogenesis in patients with type 2 diabetes and heart failure: effects of epicatechin rich cocoa. *Clin Transl Sci* 2012;5:43-7. PMID: 22376256.

26. Ramirez-Sanchez I, Taub PR, Ciaraldi TP, Nogueira L, Coe T, Perkins G, Hogan M, Maisel AS, Henry RR, Ceballos G, Villarreal F. (-)-Epicatechin rich cocoa mediated modulation of oxidative stress regulators in skeletal muscle of heart failure and type 2 diabetes patients. *Int J Cardiol* 2013;168:3982-90. PMID: 23870648

27. Taub PR, Ramirez-Sanchez I, Ciaraldi TP, Gonzalez-Basurto S, Coral-Vazquez R, Perkins G, Hogan M, Maisel AS, Henry RR, Ceballos G, Villareal F. Perturbations in skeletal muscle sarcomere structure in patients with heart failure and type 2 diabetes: restorative effects of (-)-epicatechin-rich cocoa. *Clin Sci (Lond)* 2013;125:383-9. PMID: 23642227

28. Balzer J, Rassaf T, Heiss C, Kleinbongard, Lauer T, Merx M, Heussen N, Gross HB, Keen CL, Schroeter H, Kelm M. Sustained benefits in vascular function through flavanol-containing cocoa in medicated diabetic patients: A double-masked, randomized, controlled trial. *J Am Coll Cardiol*. 2008;51:2141-2149. PMID: 18510961.

29. Flammer AJ, Hermann F, Sudano I, Speiker L, Hermann M, Cooper KA, Serafini M, Lüscher TF, Ruschitzka F, Noll G, Corti R. Dark chocolate improves coronary vasomotion and reduces platelet reactivity. *Circulation* 2007;116:2376-82. PMID: 17984375.

30. Lee I, Huttemann M, Kruger A, Bollig-Fischer A, Malek MH. (-)-Epicatechin combined with 8 weeks of treadmill exercise is associated with increased angiogenic and mitochondrial signaling in mice. *Front Pharmacol* 2015;6:43. PMID: 25821434.

31. Loffredo L, Perri L, Catasca E, Pignatelli P, Brancorsini M, Nocella C, De Falco E, Bartimoccia S, Frati G, Carnevale R, Violi F. Dark chocolate acutely improves walking autonomy in patients with peripheral artery disease. *J Am Heart Assoc* 2014;3:e001072. doi:10.1161/JAHA.114.001072. PMID: 24990275

32. McDermott MM, Ades PA, Dyer A, Guralnik JM, Kibbe M, Criqui MH. Corridor-based functional performance measures correlate better with physical activity during daily life than treadmill measures in persons with peripheral arterial disease. *J Vasc Surg* 2008;48:1231-7. PMCID: PMC2885131

33. McDermott MM, Guralnik JM, Criqui MH, Liu K, Kibbe MR, Ferrucci L. Six-minute walk is a better outcome measure than treadmill walking tests in therapeutic trials of patients with peripheral artery disease. *Circulation* 2014;130:61-8. PMID: 24982117.

34. McDermott MM, Ades P, Guralnik JM, Dyer A, Ferrucci L, Liu K, Nelson M, Lloyd-Jones D, Van Horn L, Garside D, Kibbe M, Domanchuk K, Stein JH, Liao Y, Tao H, Green D, Pearce WH, Schneider JR, McPherson D, Laing ST, McCarthy WJ, Shroff A, Criqui MH. Treadmill exercise and resistance training in patients with peripheral arterial disease with and without intermittent claudication: a randomized controlled trial. *JAMA* 2009;301:165-74. PMID: 19141764.

35. McDermott MM, Tian L, Liu K, Guralnik JM, Ferrucci L, Tan J, Pearce WH, Schneider JR, Criqui MH. Prognostic value of functional performance for mortality in patients with peripheral artery disease. *J Am Coll Cardiol* 2008;51:1482-9. PMID: 18402904.

36. Girolami B, Bernardi E, Prins MH, Ten Cate JW, Hettiarachchi R, Prandoni P, Girolami A, Buller HR. Treatment of intermittent claudication with physical training, smoking cessation, pentoxifylline, or nafronyl: a meta-analysis. *Arch Intern Med* 1999;159:337-45. PMID: 10030306.

37. Dawson DL, Cutler BS, Meissner MH, Strandness DE Jr. Cilostazol has beneficial effects in treatment of intermittent claudication: Results from a multi-center, randomized, prospective, double-blind trial. *Circulation* 1998;98:678-86. PMID: 9715861.

38. Money SR, Herd JA, Isaacsohn JL, Davidson M, Cutler B, Heckman J, Forbes WP. Effect of cilostazol on walking distances in patients with intermittent claudication caused by peripheral vascular disease. *J Vasc Surg* 1998;27:267-74. PMID: 9510281.

39. Beebe HG, Dawson DL, Cutler BS, Herd JA, Strandness DE Jr, Bortey EB, Forbes WP. A new pharmacological treatment for intermittent claudication: results of a randomized, multi-center trial. *Arch Intern Med* 1999;159:2041-50. PMID: 10510990.

40. McDermott MM, Hoff F, Ferrucci L, Pearce WH, Guralnik JM, Tian L, Liu K, Schneider JR, Sharma L, Tan J, Criqui MH. Lower extremity ischemia, calf skeletal muscle characteristics, and functional impairment in peripheral arterial disease. *J Am Geriatr* Soc 2007;55:400–6. PMID: 17341243.

41. McDermott MM, Ferrucci L, Guralnik J, Tian L, Liu K, Hoff F, Liao Y, Criqui MH. Pathophysiological changes in calf muscle predict mobility loss at 2-year follow-up in men and women with peripheral arterial disease. *Circulation* 2009;120:1048-55. PMID: 19738138.

42. Anderson JD, Epstein FH, Meyer CH, Hagspiel KD, Wang H, Berr SS, Harthun NL, Weltman A, Dimaria JM, West AM, Kramer CM. Multifactorial determinants of functional capacity in peripheral arterial disease: uncoupling of calf muscle perfusion and metabolism. *J Am Coll* Cardiol 2009;54:628-35. PMID: 19660694.

43. Herman SD, Liu K, Tian L, Guralnik JM, Ferrucci L, Criqui MH, Liao Y, McDermott MM. Baseline lower extremity strength and subsequent decline in functional performance at 6-year follow-up in persons with lower extremity peripheral arterial disease. *J Am Geriatr Soc* 2009;57:2246-52. PMID: 19874404.

44. Weiss DJ, Casale GP, Koutakis P, Nella AA, Swanson SA, Zhu Z, Miserlis D, Johanning JM, Pipinos II. Oxidative damage and myofiber degeneration in the gastrocnemius of patients with peripheral arterial disease. *J Transl Med* 2013;11:230. doi: 10.1186/1479-5876-11-230. PMID: 24067235.

45. Pipinos II, Judge AR, Zhu Z, Selsby JT, Swanson SA, Johanning JM, Baxter BT, Lynch TG, Dodd SL. Mitochondrial defects and oxidative damage in patients with peripheral arterial disease. *Free Radic Biol Med* 2006;41:262-9. PMID: 16814106.

46. Pipinos II, Sharov VG, Shepard AD, Anagnostopoulos PV, Katsamouris A, Todor A, Filis KA, Sabbah HN. Abnormal mitochondrial respiration in skeletal muscle in patients with peripheral arterial disease. *J Vasc Surg* 2003;38:827-32. PMID: 14560237.

47. Marbini A, Gmignani F, Scoditti U, Rustichelli P, Bragaglia MM, Govoni E. Abnormal muscle mitochondria in ischemic claudication. *Acta Neurol Belg* 1986;86:304-10. PMID: 3811830.

48. Mäkitie J, Teräväinen H. Histochemical changes in striated muscle in patients with intermittent claudication. *Arch Pathol Lab Med* 1977;101:658-63. PMID: 579309.

49. Makris KI, Nella AA, Zhu Z, Swanson SA, Casale GP, Gutti TL, Judge AR, Pipinos II. Mitochondriopathy of peripheral arterial disease. *Vascular* 2007;15:336-43. PMID: 18053417.

50. Pipinos II, Shepard AD, Anagnostopoulos PV, Katsamouris A, Boska MD. Phosphorus 31 nuclear magnetic resonance spectroscopy suggests a mitochondrial defect in claudicating skeletal muscle. *J Vasc Surg* 2000;31:944-52. PMID: 10805885.

51. Payvandi L, Dyer A, McPherson D, Ades P, Stein J, Liu K, Ferrucci L, Criqui MH, Guralnik JM, Lloyd-Jones D, Kibbe MR, Liang ST, Kane B, Pearce WH, Verta M, McCarthy WJ, Schneider JR, Shroff A, McDermott MM. Physical activity during daily life and brachial artery flow-mediated dilation in peripheral arterial disease. *Vasc Med.* 2009;14:193-201. PMCID: PMC2749502

52. McDermott MM, Fried L, Simonsick E, Ling S, Guralnik JM. Asymptomatic peripheral arterial disease is independently associated with impaired lower extremity functioning: the women's health and aging study. *Circulation* 2000;101:1007-12. PMID: 10704168.

53. Newman AB, Siscovick DS, Manolio TA, Polak J, Fried LP, Borhani NO, Wolfson SK. Ankle-arm index as a marker of atherosclerosis in the Cardiovascular Health Study. Cardiovascular Heart Study (CHS) Collaborative Research Group. *Circulation* 1993;88:837-4. PMID: 8353913.

54. Vogt MT, Cauley JA, Newman AB, Kuller LH, Hulley SB. Decreased ankle/arm blood pressure index and mortality in elderly women. *JAMA* 1993;270:465-9. PMID: 8320785.

55. Lijmer JG, Hunink MG, van den Dungen JJ, Loonstra J, Smit AJ. ROC analysis of noninvasive tests for peripheral arterial disease. *Ultrasound Med Biol* 1996;22:391-8. PMID: 8795165.

56. Bernstein EF, Fronek A. Current status of non-invasive tests in the diagnosis of peripheral arterial disease. *Surg Clin North Am* 1982;62:473-87. PMID: 7089825.

57. Fung YC. Blood flow in arteries: pressure and velocity waves in large arteries and the effects of geometric nonuniformity. In: *Biodynamics: circulation.* New York, NY: Springer-Verlag; 1984:133-136

58. Yao JS. New techniques of objective arterial evaluation. *Arch Surg* 1973;106:600-4. PMID: 4572350.

59. Aboyans V, Criqui MH, Abraham P, Allison MA, Creager MA, Diehm C, Fowkes

FG, Hiatt WR, Jonsson B, Lacroix P, Marin B, McDermott MM, Norgren L, Pande RL, Preux PM, Stoffers HE, Treat-Jacobson D, American Heart Association Council on Peripheral Vascular Disease. Measurement and interpretation of the ankle-brachial index: A scientific statement from the American Heart Association. Circulation 2012;126:2890-2909. PMID: 23159553.

60. McDermott MM, Liu K, Guralnik JM, Criqui MH, Spring B, Tian L, Domanchuk K, Ferrucci L, Lloyd-Jones D, Kibbe M, Tao H, Zhao L, Liao Y, Rejeski WJ. Home-based walking exercise intervention in peripheral artery disease: a randomized clinical trial. *JAMA* 2013;310:57-65. PMID: 23821089.

61. Gardner AW, Skinner JS, Cantwell BW, Smith LK. Progressive vs. single-stage treadmill tests for evaluation of claudication. *Med Sci Sports Exerc* 1991;23:402-8. PMID: 2056896.

62. Gardner AW, Skinner JS, Cantwell BW, Smith LK. Effects of handrail support on claudication and hemodynamic responses to single-stage and progressive treadmill protocols in peripheral vascular occlusive disease. *Am J Cardiol* 1991;68:99-105. PMID: 2058556.

63. Hiatt WR, Regensteiner JG, Hargarten ME, Wolfel EE, Brass EP. Benefit of exercise conditioning for patients with peripheral arterial disease. *Circulation* 1990;81:602-9. PMID: 2404633.

64. Yeboah J, Folsom AR, Burke GL, Johnson C, Polak JF, Post W, Lima JA, Crouse JR, Herrington DM. Predictive value of brachial flow-mediated dilation for incident cardiovascular events in a population-based study: The multi-ethnic study of atherosclerosis. *Circulation.* 2009;120:502-9. PMCID: PMC2740975.

65. Rothney MP, Brychta RJ, Meade NN, Chen KY, Buchowski MS. Validation of the ActiGraph two-regression model for predicting energy expenditure. *Med Sci Sports Exerc.* 2010;42:1785-92. PMCID: PMC2919650.

66. Cooper AR, Page A, Fox KR, Misson J. Physical activity patterns in normal, overweight and obese individuals using minute-by-minute accelerometry. *Eur J Clin Nutr.* 2000;54:887-94. PMID: 11114687.

67. Kelly LA, McMillan DG, Anderson A, Fippinger M, Fillerup G, Rider J. Validity of ActiGraphs uniaxial and triaxial accelerometers for assessment of physical activity in adults in laboratory conditions. *BMC Med Phys.* 2013;13:5. PMID: 24279826.

68. Mitsiopoulos N, Baumgartner RN, Heymsfield SB, Lyons W, Gallagher D, Ross R. Cadaver validation of skeletal muscle measurement by magnetic resonance imaging and computerized tomography. *J Appl Physiol (1985).* 1998;85:115-22. PMID: 9655763

69. Adhihetty PJ, Taivassalo T, Haller RG, Walkinshaw DR, Hood DA. The effect of training on the expression of mitochondrial biogenesis- and apoptosis-related proteins in skeletal muscle of patients with mtDNA defects. *Am J Physiol Endocrinol Metab* 2007 September;293(3):E672-E680. PMID: 17551003.

70. Joseph AM, Adhietty PJ, Buford TW, Wohlgemuth SE, Lees HA, Nguyen LM, Aranda JM, Sandesara BD, Pahor M, Manini T, Marzetti E, Leeuwenburgh C. The impact of aging on mitochondrial function and biogenesis pathways in skeletal muscle of sedentary high- and low-functioning elderly individuals. *Aging Cell* 2012;11:801-809. PMID: 22681576.

71. Wohlgemuth SE1, Lees HA, Marzetti E, Manini TM, Aranda JM, Daniels MJ, Pahor M, Perri MG, Leeuwenburgh C, Anton SD. An exploratory analysis of the effects of a weight loss plus exercise program on cellular quality control mechanisms in older overweight women. *Rejuvenation Res* 2011;14:315-24. PMID: 21631380.

72. Duscha BD, Robbins JL, Jones WS, Kraus WE, Lye RJ, Sanders JM, Allen JD, Regensteiner JG, Hiatt WR, Annex BH. Angiogenesis in skeletal muscle precede improvements in peak oxygen uptake in peripheral artery disease patients. *Arterioscler Thromb Vasc Biol* 2011;31:2742-8. PMCID: PMC3578302

73. Perera S, Mody SH, Woodman RC, Studenski SA. Meaningful change and responsiveness in common physical performance measures in older adults. *J Am Geriatr Soc* 2006;54:743-749. PMID: 16696738.

74. Puhan MA, Mador MJ, Held U, Goldstein R, Guyatt GH, Schunemann HJ. Interpretation of treatment changes in 6-minute walk distance in patients with COPD. *Eur Respir J* 2008;32:637–643. PMID: 18550610.

75. McDermott MM, Reed G, Greenland P, Mazor KM, Pagoto S, Ockene JK, Graff R, Merriam PA, Leung K, Manheim L, Kibbe MR, Olendzki B, Pearce WH, Ockene IS. Activating peripheral arterial disease patients to reduce cholesterol: a randomized trial. *Am J Med* 2011;124:557-65. PMID: 21605733.

76. Decker C. Calculating a nonparametric estimate and confidence interval using SAS software. In SAS Global Forum; 2000; Indianapolis, Indiana. http://www.lexjansen.com/pharmasug/2000/Coders/cc01.pdf. Accessed July 7, 2015.

77. Corti R, Flammer AJ, Hollenberg NK, Lüscher TF. Cocoa and cardiovascular health. *Circulation* 2009;119:1433-41. PMID: 19289648.

78. White S, McDermott MM, Sufit RL, Kosmac K, Bugg AW, Gonzalez-Freire M, Ferrucci L, Tian L, Gao Y, Kibbe MR, Criqui MH, Leeuwenburgh C, Peterson CA. Walking performance is positively correlated to calf muscle fiber size in peripheral artery disease subjects, but fibers show aberrant mitophagy: an observational study. *Journal of Translational Medicine* 2016;14:284. PMID: 27687713.

79. Fry CS, Lee JD, Jackson JR, Kirby TJ, Stasko SA, Liu H, Dupont-Versteegden EE, McCarthy JJ, Peterson CA. Regulation of the muscle fiber microenvironment by activated satellite cells during hypertrophy. FASEB J 2014;28:1654-1656. PMID: 24376025. PMCID: PMC3963024.

80. Joseph AM, Adhihetty PJ, Buford TW, Wohlgemuth SE, Lees HA, Nguyen LM, Aranda JM, Sandesara BD, Pahor M, Manini TM, Marzetti E, Leeuwenburgh C. The impact of aging on mitochondrial function and biogenesis pathways in skeletal muscle of sedentary high- and low-functioning elderly individuals. *Aging Cell.* 2012;11:801-9. PMID: 22681576. PMCID: PMC3444680.

81. Adhihetty PJ, Taivassalo T, Haller RG, Walkinshaw DR, Hood DA. The effect of training on the expression of mitochondrial biogenesis- and apoptosis-related proteins in skeletal muscle of patients with mtDNA defects. *Am J Physiol Endocrinol Metab.* 2007;293:E672-80. PMID: 17551003.